RESEARCH ARTICLE

Anti-metastatic Effects on B16F10 Melanoma Cells of Extracts and Two Prenylated Xanthones Isolated from *Maclura amboinensis* Bl. Roots

Pongpun Siripong¹*, Kitiya Rassamee¹, Suratsawadee Piyaviriyakul¹, Jantana Yahuafai¹, Kwanjai Kanokmedhakul²

Abstract

Inhibitory effects of *Maclura amboinensis* Bl, one plant used traditionally for the treatment of cancers, on metastatic potential of highly metastatic B16F10 melanoma cells were investigated in vitro. Cell proliferation was assessed using the MTT colorimetric assay. Details of metastatic capabilities including invasion, migration and adhesion of B16F10 melanoma cells were examined by Boyden Chamber invasion and migration, scratch motility and cell attachment assays, respectively. The results demonstrated that *n*-hexane and chloroform extracts exhibited potent anti-proliferative effects (p<0.01), whereas the methanol and aqueous extracts had less pronounced effects after 24 h exposure. Bioactivity-guided chromatographic fractionation of both active *n*-hexane and chloroform extracts led to the isolation of two main prenylated xanthones and characterization as macluraxanthone and gerontoxanthone-I, respectively, their structures being identified by comparison with the spectral data. Interestingly, both exhibited potent effective effects. At non-toxic effective doses, *n*-hexane and chloroform extracts (10 and 30 µg/ml) as well as macluraxanthone and gerontoxanthone-I (3 and 10 µM) significantly inhibited B16F10 cell invasion, to a greater extent than 10 µM doxorubicin, while reducing migration of cancer cells without cellular cytotoxicity. Moreover, exposure of B16F10 melanoma cells to high concentrations of chloroform (30 µg/ml) and gerontoxanthone-I (20 µM) for 24 h resulted in delayed adhesion and retarded colonization. As insights into mechanisms of action, typical morphological changes of apoptotic cells e.g. membrane blebbing, chromatin condensation, nuclear fragmentation, apoptotic bodies and loss of adhesion as well as cell cycle arrest in the G1 phase with increase of sub-G1 cell proportions, detected by Hoechst 33342 staining and flow cytometry were observed, suggesting DNA damage and subsequent apoptotic cell death. Taken together, our findings indicate for the first time that active *n*-hexane and chloroform extracts as well as macluraxanthone and gerontoxanthone-I isolated from *Maclura amboinensis* Bl. roots affect multistep of cancer metastasis processes including proliferation, adhesion, invasion and migration, possibly through induction of apoptosis of highly metastatic B16F10 melanoma cells. Based on these data, *M. amboinensis* Bl. represents a potential candidate novel chemopreventive and/or chemotherapeutic agent. Additionally, they also support its ethno-medicinal usage for cancer prevention and/or chemotherapy.

Keywords: *Maclura amboinensis* - prenylated xanthones - antimetastasis - anti-proliferative - apoptosis induction

*Asian Pacific J Cancer Prev, 13, 3519-3528*

Introduction

Cancer metastasis is the most important cause of cancer death in patients. During the metastatic cascade, metastasizing tumor cells interact with various host cells, extracellular matrices and basement membrane components. Such adhesive interaction may enhance the survival or invasiveness of tumor cells. Metastasis is a multi-step process which involves a series of the steps, including cellular adhesion to the basement membrane, invasion through the basement membrane, transfer via the circulation, extravasation and proliferation at a distant site (Fidler and Hart, 1980). Therefore, inhibition any of these steps is of great significance of cancer treatment (Nicloson, 1988). Since, most anticancer drugs are not sufficiently tumor selective and sometime cause hematopoietic disorders and resistance to the chemotherapeutic regimen, the drugs which possessed anti-metastatic efficacy and low toxicity on normal tissues are required. Currently, it is well document that natural compounds are one of the most important sources of potential anticancer drugs (Surh, 2003; Gordaliza, 2007).

*Maclura amboinensis* Bl. (family Moraceae), one of Thai medicinal plant which used as traditionally for the treatment of cancers, was selected for further investigated. *Maclura or Cudrania* plant is a desiduous climber

¹Natural Products Research Section, Research Division, National Cancer Institute, Bangkok; ²Department of Chemistry, Faculty of Science, Khon Kaen University, Khon Kaen, Thailand  *For correspondence: siripong_nci@yahoo.com
distributed over Republic of China, Japan, Korea, Australia and also in Thailand. Its aqueous extract has been used to dye cotton yellow. For medical purposes, the decoction of roots and cortex are locally used in the treatment of malaria, fever, diuretic, hepatitis, liver disease and cancers (Lee et al., 2010). This plant is well known as the source of xanthones, flavonoids, terpenoids and stilbenes (Lee et al., 2005). Previously, some bioactive extracts of *Cudrania or Maclura spp.* were found to possess anti-inflammatory (Chang et al., 2008), anti-oxidative (Chang et al., 1994), antitumor (Lee et al., 2005; Lee et al., 2006; Jeong et al., 2009: 2012), hepatoprotective (An et al., 2006), antibacterial (Fukai et al., 2004), antifungal (Wang et al., 2005), antitumor effects and cytotoxicity against various cancer cells (Seo, et al., 2001; Zou et al., 2004; Lee et al., 2005; Wang et al., 2005; Wang et al., 2005: 2010; Kim et al., 2007; Rho et al., 2007; Kuang et al., 2011). More extensive phytochemical and pharmacological studies, several xanthones and flavonoids as the main active components have been identified from this genus and some of them have been reported to possess significant pharmacological properties, including anti-inflammatory (Lin et al., 2012), anti-cancer (Rho et al., 2007; Wang et al., 2010; Kuang et al., 2011), antibacterial (Fukai, et al., 2004) and anti-HIV (Groweiss et al., 2000) activities. For instance, macularaxanthone B and C isolated from *Maclura tinctoria* exhibited anti-HIV activity (Groweiss et al., 2000). Isoalvaxanthone, a prenylxanthone, isolated from *Cudrania cochinchinensis* (Lour.) inhibited colon cancer cell proliferation, migration and invasion through inactivating Rac 1 and AP-1 (Wang et al., 2005). Macularaxanthone and gerontoxanthone-I isolated from *Cratoxylum maingayi* and *C. cochinchinensis* showed anti-malaria effect and cytotoxicity against NCI-H187 cells (Laphookhieo et al., 2009). Treatment with either allanxanthone C or macularaxanthone isolated from *Guttiferae* resulted in a concentration-dependent inhibition cell growth of lymphocytic leukemia (CLL), induced the accumulation in the G_{1}/G_{2} cell cycle phase as well as being capable of *in vivo* antileukemic effects in a xenograft murine model of human lymphocytic leukemia (Menasria et al., 2008). However, to the best of our knowledge, no scientific reports are available on the inhibitory effects of *Maclura amboinensis* Bl. in cancer cells *in vitro* and *in vivo*.

In the present study, the inhibitory effects of four extracts and two major compounds isolated from *M. amboinensis* Bl. roots on cell proliferation as well as cancer metastasis properties such as adhesion, invasion and migration which play a crucial role in the pathogenesis of cancer metastasis on a highly metastatic B16F10 melanoma cells *in vitro* were evaluated. In addition, mechanistic of apoptosis- inducing by these drugs was also explored.

**Materials and Methods**

A reagent for MTT assay: 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyl -tetrazolium bromide, propidium iodide (PI), ribonuclease A, dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Doxorubicin, an anticancer drug, was obtained from Merck. Dulbecco’s modified Eagles medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin, 0.25% trypsin/ ethylenediaminetetraacetic acid (EDTA) solution, trypsin blue and phosphate-buffered saline (FBS) were obtained from Gibco Life Technologies Inc. (Rockville, MA, USA). Annexin V/PI-FITC apoptosis detection kit, fibronectin and Matrigel were from BD Biosciences (San Diego, CA, USA). Hoechst 33342 staining kit was purchased from Invitrogen (Life Technologies Corp., USA). All other reagents and chemicals used were of the highest purity grade available.

**Plant Materials**

Roots and stems of *Maclura amboinensis* Bl. (MA) were collected in Pattani province, Thailand. This plant was authenticated by Dr. Kongkanda Chayamarit, Director of Botanical Garden Organization, Department of National Park, Wildlife and Plant Conservation, Ministry of Natural Resource and Environment of Thailand. A voucher specimen (NCIP No. 0130) was deposited in the Herbarium of the National Products Research Section, Research Division, National Cancer Institute, Bangkok, Thailand.

**Preparation of crude extracts of MA**

Dried coarsely powder of roots (1 kg) of *M. amboinensis* Bl. was extracted exhaustively in a Soxhlet apparatus with n-hexane, followed by chloroform and methanol. Concentration of the extracts under reduce pressure afforded the extracts of n-hexane (40.8 g), chloroform (35.2 g) and methanol (42.7 g), respectively. For the aqueous extract, the powder root (1 kg) was refluxed with distilled water for 2 h. The filtrate was then concentrated *in vacuo* and lyophilized. The residue (377.1 g) was kept in the freezer at -20°C until used. All crude extracts were determined for the antiproliferative activity against various cancer cells in *vitro*, and the n-hexane and chloroform extracts exhibited potent effective effects. All extracts were dissolved in dimethylsulfoxide (DMSO) and added to Dulbecco’s modified Eagle’s medium (DMEM) with a maximum final DMSO concentration of 0.1%.

**Purification and identification of isolated compounds**

The active n-hexane and chloroform extracts were subjected to column chromatography on silica gel and eluted with n-hexane, n-hexane and chloroform, chloroform as well as chloroform and methanol by gradient systems. Fractions of 75 ml were collected and then combined (*t.l.c*) to yield 6 fractions (A-F). Fractions B-E, which showed a significant anti-proliferative activity against cancer cells, were further purified by repeated silica gel column chromatography. After recrystallization with n-hexane, two main isolated compounds (MA-1,128.9 mg and MA-2, 287.3 mg) were obtained as yellow needles from fraction D and E, respectively. Structural identification of these isolated compounds was confirmed by mixed melting point and comparison of the spectral data (UV, IR, 1H and 13C NMR and MS). The chemical structure of two main xanthones is presented in Figure 1.
**Figure 1. Chemical Structures of Two Main Prenylated Xanthones Isolated from Active Extracts of *M. amboinensis* Bl. Roots**

n-hexane-AcOEt 4:1, m.p. 181-182°C (lit. 181-183°C and 204-206°C); C₇₀H₇₀O₂; M.W. 394; EI-MS m/z: 394 (M⁺, 78), 379 (M-Me)², 100, 365(14), 353(22), 351(18), 338(12), 241(8), 182(5); UVλmax MeOH (log ɛ): 232, 278, 324, 332; IR νmax (free-OH): 3456, 3280 (free-OH), 1654(C=O), 1635, 1584, 1465, 1417; H-NMR (CDCl₃): δ13.52 (1H, s, 1-OH), 7.69 (1H, d, J=8.07 Hz, H-8), 6.95 (1H, d, J=8.8 Hz, H-7), 6.77 (1H, d, J=10.07 Hz, H-11), 6.74 (1H, s, OH 6 or 5), 5.65 (1H, d, J=10.07 Hz, H-12), 5.26 (1H, dd, J=17.7 and 15.2 Hz, H-18), 5.08 (1H, dd, J=10.7 and 1.52 Hz, Hb-18), 1.68 (6H, s, 13-2Me); ²CNMR (CDCl₃): δ: 138.9(C-1), 130.3(C-2), 156.8(C-3), 113.0(C-4), 136.7(C-4a), 144.5(C-4b), 131.0 (C-5), 154.1(C-6'), 113.7(C-7), 117.5(C-8'), 112.7(C-8a), 180.8(C-9), 106.5(C-9a), 116.1(C-11), 127.1 (C-12), 78.2(C-13), 27.9(C-14), 28.2(C-15), 41.4(C-16), 29.7(C-17), 29.7(C-18), 149.0(C-19), 103.5(C-20) (Laphookhieo et al., 2009).

Gerontoxanthone-I (MA-2); pale yellow needles from n-hexane-AcOEt 2:1, m.p. 176-177°C (lit. 178-180°C); C₇₀H₇₀O₂; M.W. 396; EI-MS m/z: 396(M⁺, 20), 381 (M⁺-Me, 10), 340(38), 353[M-Me-CO]⁺, 20), 325(M⁺-C₅H₁₁, 100), 285(58), 258(16), 256(16), 236(16), 154(15), 158, 155, 147, 142, 149, 1324, 1280 and 967; H-NMR (CDCl₃): δ: 13.63 (1H, s, 1-OH), 7.74 (1H, d, J=8.8 Hz, H-8), 6.98 (1H, d, J=8.8 Hz, H-7), 6.73 (1H, dd, J=17.7 and 10.68 Hz, H-17), 6.09(2H, brs, 2-OH 6 or 5), 5.35 (1H, dd, J=10.68 and 1.22 Hz, Hb-18), 3.53(2H, dd, J=17.7 and 1.52 Hz, H-18), 5.08(1H, dd, J=10.7 and 1.52 Hz, Hb-18), 1.68(6H, dt, J=7.02 and 1.84 Hz, 2H-11), 1.89(3H, s, Me); ¹³CNMR (CDCl₃): δ: 161.4(C-1), 112.6(C-2), 115.9(C-3), 111.2(C-4), 155.0(C-4a), 144.7(C-4b), 136.2(C-5), 153.2(C-6'), 113.9(C-7), 117.7(C-8), 110.0(C-8a), 180.8(C-9), 105.9(C-9a), 21.6(C-11), 121.1(C-12), 130.9(C-13), 17.9(C-14), 25.9(C-15), 41.6(C-16), 148.9(C-17), 103.0(C-18), 28.0(C-19), 28.0(C-20) (Laphookhieo et al., 2009).

**Cell culture**

A highly metastatic B16F10 murine melanoma cells (B16F10; ATCC CRL-6475) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were grown as monolayer cultures in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin sulphate. They were incubated in a humidified atmosphere with 5% CO₂ at 37°C. Cells in the exponential phase were used for all experiments.

**Cell proliferation assay**

Cells proliferation was assessed using the MTT colorimetric assay, as described previously (Siripong et al., 2006). Briefly, log phase of B16F10 melanoma cells (3x10⁵ cells/ml) suspended in 100 µl of DMEM medium containing 10% fetal bovine serum, 1% antibiotic, were seeded onto a 96-well culture plate (Costar, Cambridge, MA, U.S.A.). After 24 h pre-incubation, the cells were exposed with serial concentrations of four extracts; n-hexane, chloroform, methanol (0.01-100 µg/ml) and aqueous extract (0.1-500 µg/ml) as well as two main xanthones; macluraxanthone (MA-1) and gerontoxanthone-I (MA-2) (0.01-100 µM) for the indicated times (24, 48 and 72 h). Doxorubicin (0.01-30 µM) and 0.1% DMSO in medium were used as positive and negative controls. At the end of each incubation period, 20 µl MTT solution (5 mg/ml in PBS) was added to each well and further incubated at 37°C for 3 h. After centrifugation at 1,400 rpm for 5 min at 4°C, the medium was aspirated and the formazan product in each well was solubilized with 100 µl DMSO. The absorption at 550 nm wavelength was recorded on a Microplate reader (Benchmark 550, Bio-Rad, USA). Each concentration of drug was performed in six wells for three independent experiments. The IC₅₀ value was calculated by plotting of the percentage of cell viability versus drug concentrations.

**Transwell invasion and migration assays**

Cell invasion and migration abilities were determined using a Modified Boyden Chamber assay as described previously (Ogasawara et al., 2002; Siripong, et al., 2002; Roy and Maity, 2007). For analyzing the invasive ability, transwell® chambers (Costar 3422, Corning, NY, U.S.A.) were set up with 8 µm pore size of polyvinylpyrrolidone-free carbonate filters (Nucleopore, Pleasanton, USA). The lower surface of the filters was then coated with 2 µg/50µl of fibronectin and the upper surface was coated with 10µg/50 µl of Matrigel, whereas the migratory ability was performed on the non-coating filters with Matrigel. In the both assays, B16F10 melanoma cells at the density of 2x10⁵ cells/chamber, were suspended in DMEM containing 1% BSA in the absence or presence of various concentrations of the drugs (n-hexane, chloroform, methanol extracts (3, 10 and 30 µg/ml); aqueous extract (3, 10, 30 and 100 µg/ml) as well as two main xanthones; MA-1 and MA-2 (1, 3, 5 and 10µM)). Doxorubicin (1 and 10µM) and 0.1% DMSO in medium were used as positive and negative controls. The cell suspension (100µl/chamber) was then applied to the upper compartment of the chambers and incubated in a 24 well culture plate containing 600µl of the same medium at 37°C, 5% CO₂ for 24 h. At the end of incubation, all filters were finally fixed with 30% methanol and then stained with 0.5% crystal violet for 5 min. After gentle rinsing, the cells on the upper surface of filters were wiped off with a cotton swab. Cells that had invaded through the Matrigel and filters were extracted with 30% acetic acid and then measured their absorbances at 590 nm.
5 mg/ml of Hoechst 33342 solution in PBS and incubated at room temperature for 15 min. Finally, all specimens were mounted with Perma Fluor aqueous mounting medium and were then observed under a Phase-Contrast and Fluorescence Inverted Microscope (ECLIPSE Ti-U, Nikon, Japan). Images were captured using CCD camera at a magnification of 400x and calculated with NIS-Elements D 3.0 Software at three fields per slide. Apoptotic cells were identified as cells with condensed and fragmented nuclei.

**DNA Cell Cycle Analysis**

Cell cycle phase distribution was analyzed by flow cytometry with propidium iodide (PI) staining, as described previously (Siripong et al., 2006 & 2009). In brief, B16F10 melanoma cells (1x10^6 cells/dish) were seeded on 60 mm dishes (Corning Incorporation, MA, USA) and incubated at 37°C, 5% CO₂ for 24 h. Cells were then treated with four extracts; n-hexane, chloroform, methanol (3, 10 and 30 µg/ml) and aqueous extract (250, 500 and 750 µg/ml) as well as two main xanthones; MA-1 and MA-2 (3, 10 and 20 µM) for 24 h. Doxorubicin (1, 3 and 10 µM) and 0.1% DMSO in medium were used as positive and negative controls. At the end of incubation, both detached and adherent cells were collected, washed twice with PBS and then fixed overnight in ice-cold 70% ethanol at -20°C. Cell pellets were washed with PBS and suspended in PBS containing 100 µg/ml ribonuclease A at 37°C for 20 min. Cellular DNA was labeled with 250 µl PI (100 µg/ml) in PBS at least 30 min in the dark at room temperature and then filtered through a 40 µm nylon filter. The cell cycle distribution was analyzed for 20,000 events by a FACS Calibur Flow Cytometer (BD Bioscience, San Jose, CA, USA). Cells with a lower DNA content than that of the G1 phase of the cell cycle were considered as hypodiploid cells (sub-G1 phase). The percentage of apoptotic cells was detected using FACSDiva Version 6.1.3 Software (BD Bioscience, San Jose, CA, USA). All experiments were performed in duplicate and reproducibility was checked in three independent experiments.

**Quantification of Apoptotic Cells**

To quantitatively assess the number of apoptotic cells, the Cell Death Detection ELISA kit (Roche) was used. The procedure was performed according to the manufacturer’s instructions. B16F10 melanoma cells (1x10^6 cells/dish) were plated on 6-well plates and incubated for 24 h. Cells were then treated with one of the tested drugs or left untreated. After 24 h, the cells were harvested and subjected to DNA fragmentation analysis. The amount of DNA in each sample was measured using a microplate reader (Benchmark 550, Bio-Rad, USA). Each experiment was performed in triplicate.
and reproducibility was checked in three independent experiments (Siripong et al., 2006: 2009).

**Statistical Analysis**

Data were expressed as the mean values±S.D and were obtained from experiments repeated at least three times. Statistically analysis was performed by one-way analysis of variance (ANOVA) following by Student’s t-test. P-values less than 0.05 were considered significant.

**Results**

**Inhibitory Effect of MA extracts and two main xanthones on cell viability of B16F10 cells**

Figure 1 illustrates the chemical structures of two main isolated compounds; macluraxanthone (MA-1) and gerontoxanthone-I (MA-2) derived from the active *n*-hexane and chloroform extracts of the roots of *Maclura amboinensis* Bl. Their structures are characterized by UV, IR, ¹H- and ¹³C-NMR and MS spectroscopy. The basic structure is a prenylated xanthone.

We firstly screened the inhibitory effects of MA extracts and two main xanthones on the proliferation of a highly metastatic B16F10 melanoma cells using the MTT colorimetric assay. As shown in Figure 2, the exposure of B16F10 melanoma cells to various concentrations of four extracts; *n*-hexane, chloroform, methanol extracts (0.01-100 µg/ml) and aqueous extract (0.1-500 µg/ml) for 24 h, resulted in a significant reduction of cell viability at the IC₅₀ values of 75.2, 76.81, >100, and 374.32 µg/ml, respectively, compared to that of untreated cells (p<0.01). Among them, the *n*-hexane and chloroform extracts exhibited greater antiproliferative effects on the metastatic cells than that of the methanol and aqueous extracts at the indicated concentrations. In addition, macluraxanthone and gerontoxanthone-I-treated B16F10 cells (0.01-30 µM/L) also showed the inhibitory effects on B16F10 melanoma cells at the IC₅₀ values of 19.88 and 27.96 µM/L, respectively. The results suggest that MA reduced the proliferation of B16F10 melanoma cells in a concentration-dependent manner. Similarly, the longer exposures of the MA–treated cells for 48 and 72 h., time-dependent was also obtained (data not shown).

**Effect of the MA extracts and two main xanthones on B16F10 cell invasion**

Cells invasion, migration and adhesion of cancer cells are the critical processes in tumor metastasis (Nicloson, 1988). In order to further evaluate the effects of MA extracts and two xanthones on metastatic capabilities of B16F10 melanoma cells, a non-toxic effective dose of the drug-treated cells for 24 h exposure was chosen for subsequent experiments. Anti-invasive ability of the MA extracts; *n*-hexane, chloroform, methanol extracts (3, 10, 30 µg/ml) and aqueous extracts (3, 10, 30 and 100 µg/ml) as well as two xanthones; macluraxanthone and gerontoxanthone-I (0.1, 1, 3 and 10 µM) was analyzed by...
versus non-treatment control group. The results suggest that the effect of MA extracts and two xanthones on B16F10 melanoma cell adhesion was analyzed using the MTT assay after 24 h incubation (Figure 5A). Cell viability of treated cells and photographs were captured under a phase-contrast inverted microscope (Figure 5A for 24 h). Cell viability of treated cells and photographs were captured under a phase-contrast inverted microscope (Figure 5A for 24 h). Cell viability of treated cells and photographs were captured under a phase-contrast inverted microscope (Figure 5A for 24 h). Cell viability of treated cells and photographs were captured under a phase-contrast inverted microscope (Figure 5A for 24 h). Cell viability of treated cells and photographs were captured under a phase-contrast inverted microscope (Figure 5A for 24 h). Cell viability of treated cells and photographs were captured under a phase-contrast inverted microscope (Figure 5A for 24 h).

Subsequently, the anti-migratory effect of MA extracts and two xanthones on B16F10 melanoma cells confirmed by the scratch motility assay. As shown in Figure 4, the untreated B16F10 cells exhibited a complete wound closure activity within 24 h incubation. In contrast, the MA-treated cells showed only a limited wound closure activity with in 24 h incubation (Figure 4A). The migration inhibition rate of the untreated B16F10 cells was evaluated by the MTT assay and scratch motility assay. As shown in Figure 4, the untreated B16F10 cells exhibited a complete wound closure activity within 24 h incubation. In contrast, the MA-treated cells showed only a limited wound closure activity with in 24 h incubation. The migration inhibition rate of the untreated B16F10 cells was evaluated by the MTT assay and scratch motility assay. As shown in Figure 4, the untreated B16F10 cells exhibited a complete wound closure activity within 24 h incubation. In contrast, the MA-treated cells showed only a limited wound closure activity with in 24 h incubation.

To rule out the possibility that the anti-migratory effect of MA is due to its cytotoxicity, the cell viability of MA-treated B16F10 cells was evaluated by the MTT assay and trypan blue staining. No significant effect on cell viability was observed at low doses, indicating that cell migration ability was suppressed without any cytotoxicity (data not shown).

Based on our findings above, these results revealed that the n-hexane and chloroform extracts as well as macluraxanthone and gerontoxanthone-I significantly inhibit the migration ability of B16F10 melanoma cells without cellular cytotoxicity at non-toxic concentrations.

Effect of MA extracts and two xanthones on B16F10 cell adhesion

Since the adhesion of tumor cells to the extracellular matrix is considered to be important step in the invasive process of metastatic cancer cells, the effect of MA extracts and two xanthones on adhesion was examined by the cell attachment assay. We detached the MA-treated cells from culture plate with 0.25% trypsin-EDTA, plated them back onto a new 24-well culture plate with same density on a new culture plate. After each incubation period of 6, 12 and 24 h, the cells attachment status and morphology was observed and photographs were captured under a phase-contrast inverted microscope (Figure 5A for 24 h). Cell viability of treated cells and photographs were captured under a phase-contrast inverted microscope (Figure 5A for 24 h). Cell viability of treated cells and photographs were captured under a phase-contrast inverted microscope (Figure 5A for 24 h). Cell viability of treated cells and photographs were captured under a phase-contrast inverted microscope (Figure 5A for 24 h). Cell viability of treated cells and photographs were captured under a phase-contrast inverted microscope (Figure 5A for 24 h).

Figure 4. Effect of MA Extracts and Two Xanthones on B16F10 Melanoma Cell Migration. Scratch motility assay or wound healing assay was performed to assess cell migration. Cells were treated with vehicle (0.1% DMSO), four extracts; n-hexane, chloroform and methanol extracts (10, 30 and 75 µg/ml) and aqueous extract (30, 100 and 500 µg/ml) as well as two xanthones; macluraxanthone and gerontoxanthone-I (3, 10 and 20 µM) for 24 h. Doxorubicin (3, 10 and µM) was used as a positive control. Representative photographs of treated and untreated cells are presented (x40 magnification).

Figure 5. Effect of MA Extracts and Two Xanthones on B16F10 Melanoma Cell Adhesion. Cell attachment assay was performed to assess the effect on cell adhesion. B16F10 cells (1x10⁴ cells/well) treated with four extracts, two main xanthones and Doxorubicin for 24 h. were detached using 0.25% trypsin-EDTA and plated them back with same density on a new culture plate. After each incubation period of 6, 12 and 24 h, the cells attachment status and morphology was observed and photographs were captured under a phase-contrast inverted microscope (Figure 5A for 24 h). Cell viability of treated cells and photographs were captured under a phase-contrast inverted microscope (Figure 5A for 24 h). Cell viability of treated cells and photographs were captured under a phase-contrast inverted microscope (Figure 5A for 24 h). Cell viability of treated cells and photographs were captured under a phase-contrast inverted microscope (Figure 5A for 24 h). Cell viability of treated cells and photographs were captured under a phase-contrast inverted microscope (Figure 5A for 24 h).

Using a Transwell® Boyden Chamber Assay. The results demonstrated that the n-hexane and chloroform extracts at both concentrations of 10 and 30 µg/ml significantly inhibited B16F10 cell invasion by approximately 42.5% and 80.4% as well as 43.9% and 85.3%, respectively (p<0.01 and p<0.001, Figure 3A), compared to that of untreated cells and Doxorubicin (10 µg/ml, 88.2%). Macluraxanthone and gerontoxanthone-I at the concentrations of 3 and 10 µM markedly suppressed the B16F10 cell invasion in the percentages of 54.8 and 89.91 as well as 43.9 and 85.26, respectively, which superior than that of 10 µM doxorubicin (55.9%, Figure 3B). These results suggest that the n-hexane and chloroform extracts as well as macluraxanthone and gerontoxanthone-I are highly effective in preventing B16F10 cell invasion ability in a dose-dependent manners.

Effect of MA extracts and two main xanthones on B16F10 cell migration

To determine whether MA extracts and two xanthone affected metastatic cancer migration, transwell cell migration and scratch motility assays were carried out. In the transwell cell migration assay, n-hexane and chloroform extracts at the concentration of 30 µg/ml significantly suppressed the migration ability of B16F10 melanoma cells (p<0.01 and p<0.05, respectively), whereas aqueous extract had markedly affected at a highest concentration of 100 µg/ml (62.6%, p<0.001). Interestingly, the n-hexane extract had more potent effective effect than that of chloroform extract (66.3% and 37.9%, Figure 3C). Moreover, macluraxanthone and gerontoxanthone-I at the concentrations of 3 and 10 µM also showed reduction in migration of B16F10 melanoma cells in a dose-dependently. Inhibition rates of migration ability were 54.9% and 89.9% as well as 60.3% and 79.9%, respectively, which superior than that of 10 µM doxorubicin (55.9%, Figure 3D).

Based on our findings above, these results revealed that the n-hexane and chloroform extracts as well as macluraxanthone and gerontoxanthone-I significantly inhibit the migration ability of B16F10 melanoma cells without cellular cytotoxicity at non-toxic concentrations.

Anti-metastatic Effects of Maclura amboinensis Bl. on B16F10 Melanoma Cells

DOI:http://dx.doi.org/10.7314/APJCP.2012.13.7.3519

As compared to the untreated cells indicates a delay or defect in their attachment. As shown in Figure 5, most of the untreated cells are begun to adhere to the plate after 6 h of incubation. Cells even form a monolayer after 24 h incubation. In contrast, treatment the B16F10 cells by chloroform extract (30 µg/ml) and gerontoxanthone-I (20 µM) remained in their suspension form after 6 h incubation (data not shown). After extending incubation for 12 and 24 h, some of the treated cells were still unattached. Similar results were obtained with doxorubicin under the same treatment conditions. At the end of each incubation period of 12 and 24 h, cell viability of the MA-treated cells was measured by the MTT assay. These results demonstrated that cell viability of the treated cells by chloroform and n-hexane extracts as well as macluraxanthone and gerontoxanthone-I was markedly decreased in a dose-dependently (p<0.01 and 0.05), suggesting that the adhesion capability of MA-treated cells was retarded (as shown in Figures 5B and 5C). Reduction rates of B16F10 cell viability by their active extracts (10 and 30 µg/ml) and xanthones (10 and 20 µM) were 42.5% and 50.2% as well as 39.1% and 43.2%, respectively, compared to that of 10 µM doxorubicin (71.3%, Figure 5C). These results were consistent with migration and invasion capabilities.

Induction of apoptosis by MA extracts and two main xanthones in B16F10 cells

To clarify whether the inhibitory effect of MA extracts and two xanthones on the growth of B16F10 cells is associated with apoptosis, we confirmed the apoptotic characterizations by several approaches e.g. morphological changes, DNA fragmentation and cell cycle arrest detecting by Hoechst 33342 staining and FACScan flow cytometry, respectively.

We firstly assessed the effects of MA extracts; n-hexane, chloroform and methanol (3, 10 and 30 µg/ml) and aqueous extract (10, 30 and 100 µg/ml) as well as two xanthones; macluraxanthone and gerontoxanthone I (1, 3 and 10 µM) for 24 h. Doxorubicin (1 and 10 µM) was used as a positive control. Hoechst 33342 staining was performed. Cell morphology was observed under a fluorescence microscope. Arrows indicate the cells with DNA fragmentation.

Figure 6. Morphological Changes of B16F10 Melanoma Cells Treated with MA Extracts and Two Xanthones. B16F10 melanoma cells (1x10^6 cells/well) were seeded on 8 well-chamber slide and allowed to attach for overnight. Cells were treated with either vehicle (0.1% DMSO) or various concentrations of the extracts; n-hexane, chloroform, methanol (3, 10 and 30 µg/ml) and aqueous extracts (10, 30 and 100 µg/ml) as well as two xanthones; macluraxanthone and gerontoxanthone I (1, 3 and 10 µM) for 24 h. Doxorubicin (1 and 10 µM) was used as a positive control. Hoechst 33342 staining was performed.

Figure 7. Effect of MA Extracts and Two Xanthones on Cell Cycle Progression of B16F10 Melanoma Cells. B16F10 melanoma cells (1x10^6 cells) were treated with either vehicle (0.1% DMSO) or various concentrations of the extracts; n-hexane, chloroform, methanol (3, 10 and 30 µg/ml) and aqueous extracts (10, 30 and 100 µg/ml) as well as two xanthones; macluraxanthone and gerontoxanthone I (1, 3 and 10 µM) for 24 h. After the end of treatment, cells were harvested, fixed with 70% ethanol and digested with RNase A. Cellular DNA was stained with propidium iodide and DNA content was analyzed by flow cytometry. Doxorubicin was used as positive control and 0.1% DMSO was used as negative control. The data were represented as the means ± S.D. of the results of three independent experiments. *p<0.05; **p<0.01 and ***p<0.001 versus non-treatment control group.
Figure 8. Induction of Apoptosis in B16F10 Melanoma Cells Treated with MA Extracts and Two Xanthones. B16F10 melanoma cells (1x10^6 cells/dish) were treated with either vehicle (0.1% DMSO) or various concentrations of the extracts; n-hexane, chloroform, methanol (3, 10 and 30 µg/ml) and aqueous extracts (10, 30 and 100 µg/ml) as well as two xanthones; macluraxanthone and gerontoxanthone I (1, 3 and 10 µM) for 24 h. Doxorubicin was used as positive control and 0.1% DMSO was used as negative control. After the end of treatment, cells were harvested, stained with Annexin V-FITC and propidium iodide and then analyzed by FACScan flow cytometry. Three dependent experiments were done and gave similar results. The lower right (LR) quadrant of the FACS histogram (A), indicated the percentage of early apoptotic cells (Annexin V-FITC stained cells) and upper right (UR) quadrant indicates the percentage of late apoptotic cells (Annexin V-FITC and PI-stained cells). The population of total apoptotic cells are shown in (B). **p<0.01 and ***p<0.001 versus non-treatment control group.

whether treatment with MA caused in apoptosis induction by cell cycle arrest. Cell cycle distribution was analyzed by flow cytometry after staining the treated cells with propidium iodide (PI). The results demonstrated that n-hexane (10 µg/ml) and chloroform extracts (3 µg/ml) as well as gerontoxanthone-I (3 and 10 µM) and macluraxanthone (3 µM) for 24 h. exposure arrested the cell cycle of B16F10 melanoma cells in G1 phase at the low concentrations, whereas doxorubicin-treated cells exhibited its effect at G2/M phase in all indicated concentrations (1, 3 and 10 µM; Figure 7A and 7B). Consistently, after treatment B16F10 cells with various concentrations the MA extracts and two xanthones in the same period, the percentages of sub-G1 populations (hypoploid cells) subsequently increased which represented the cells undergoing apoptosis (Figures 7C and 7D). Our observations revealed that one of the mechanisms by which *M. amboinensis* BI. inhibited cell proliferation was by the induction of apoptosis.

To quantify apoptotic cells by the treatment of B16F10 cells with the MA extracts and two xanthones, a biparametric analysis was performed using Annexin V and PI fluorescein staining kit, which stained phosphatidylserine residue and DNA, respectively and then analyzing by flow cytometry. As shown in Figure 8, the percentages of early apoptotic cells (annexin V-fluorescein positive and PI negative, inversely located phosphatidylserine with loss of the membrane integrity) and late apoptotic cells were increased dose-dependently after treatment of these drugs at the indicated concentrations. Taken together, these findings clearly confirmed that *M. amboinensis* Bl. inhibited B16F10 melanoma cell proliferation causing by arresting cell cycle progression at G1 phase and leading to apoptosis in later stage.

Discussion

Although *Cudrania* or *Maclura* spp. have been possess significant pharmacological actions, including anti-oxidative, anti-inflammatory, hepatoprotective, anti-HIV, anti-proliferative and antitumor activities etc. but, in our knowledge, no bioactivity of *Maclura amboinensis* Bl. (MA) has been reported up to now. In this present study, for the first time, we found that its root extracts could suppress various steps of tumor metastasis including proliferation, adhesion, invasion and migration capabilities of a highly metastatic B16F10 melanoma cells in *vitro*.

We firstly screened the effects of different extracts from *M. amboinensis* Bl. roots on cell growth of B16F10 melanoma cells in *vitro*. The results demonstrated that its n-hexane and chloroform extracts significantly inhibited cell viability on this cancer metastatic cells when compared with untreated cells. Bioactivity-guided chromatographic fractionation of these active n-hexane and chloroform extracts led to the isolation of two main isolated compounds and characterization as macluraxanthone and gerontoxanthone-I (as shown in Figure 1). Their structures were identified by comparison with the spectral data. The basic chemical structure is a prenylated xanthone. Interestingly, macluraxanthone and gerontoxanthone-I had also potent effective effects. These findings are consistent with the previous reports that macluraxanthone isolated from *Cudrania spp.* showed potent antiproliferative effects on various cancer cells (Lee et al, 2005) as well as macluraxanthone and gerontoxanthone-I isolated from *Cratoxylum maingayi* and *C. cochinchinense* exhibit strong inhibitory effect against a NCI-H187 cancer cells (Laphookhieo et al., 2009). Based on the structure-activity relationship of 1,3,5,6-oxygenated xanthones the presences of two hydroxyl groups at C-5 and C-6 (Jabit et al., 2007) in both compounds as well as one hydroxyl and isoprenyl groups at C-1 and C-2 in gerontoxanthone-I may be important for enhancing the cytotoxicity against cancer cells (Laphookhieo et al., 2009).

Cell invasion, migration and adhesion behaviors are important characteristics of cancer metastasis. Many studies have demonstrated that inhibition of these steps results in the prevention of metastasis and they are targets of anticancer agent development (Niclolson, 1988). In subsequent experiments, inhibitory effect of MA-treated B16F10 cells of cancer metastasis process was explored using the Transwell Boyden Chamber, scratch motility and cell attachment assays, respectively. Our data obtained that the active n-hexane and chloroform extracts as well as macluraxanthone and gerontoxanthone-I at the non-toxic effective doses, significantly inhibited both
B16F10 cell invasion and migration of reconstituted based membrane Matrigel/fibronectin on Transwell chamber in dose-dependent manner. Correspondingly, both active MA extracts and two main xanthones were effective in inhibiting cell migration in the cancer metastatic cells, detecting by Scratch motility assay at the concentrations which did not cause cell death during the assay. Cell adhesion assay revealed that exposures the B16F10 melanoma cells with highest concentrations of chloroform (30 μg/ml) and geratoxanthone-I (20 μM) for 24 h displayed a delay adhesion and retarded colonization, compared to that of untreated and doxorubicin-treated cells. These data imply that the inhibitory effect of M. amboiensis Bl. may be mediated by a direct effect on the metastasis process associated with tumor growth of B16F10 melanoma cells.

It is well document that Cudrania or Maclura plants are a rich sources of xanthones, flavonoids, tritepenoids and stilbenes (Lee et al., 2005). Among them, xanthones and flavonoids which act as active components, are responsible for many pharmacological actions including anti-oxidative, anti-inflammatory and especially antiproliferative and antitumor activities. In this current study, we also found that two main xanthones; macluraxanthone and gerontoxanthone-I isolated from the active n-hexane and chloroform extracts of Maclura amboiensis Bl. roots remarkably suppressed the multitstep of metastatic process of B16F10 cells proliferation, adhesion, invasion and migration at the non-toxic effective doses, indicating that the presence of these active xanthones may be in part responsible for their effects on cell proliferation and metastasis capability of this metastatic cells. Our observations correlate with the earlier reports that two main xanthones of isovalaxanthone isolated from Cudrania cochinchinesis (Lour) and cudraticuxanthone G, isolated from Cudrania tricuspidata exerted anti-metastic action in human colorectal carcinoma (SW620) cells by targeting MMP-2 through regulating the activities of Rac 1, Cdc 42, and their downstream transcriptional factor AP-1 (Wang et al., 2010; Kuang et al., 2011). Further studies have to find out the exact molecular mechanism of actions of the active xanthones and other isolated compounds in inhibiting the cascade of events of metastasis. Identify and purify the other active compounds occurring in the active extracts are in progress.

Apoptosis is fundamental process for maintenance of homeostasis and elimination of damaged or unwanted cells. There are many chemopreventive agents that involved in cancer cell death by induction of apoptosis (Hu and Kavanagh, 2003). Recent evidence suggests that apoptosis of cells is closely related to occurrence, progress and metastasis of tumors (Hung et al., 2008). Thus, promoting apoptosis is regarded as the preferred mechanism of managing cancer cells. Cell cycle arrest, damage to DNA or stress to the cytoplasm or cell membrane may be cause of apoptosis. We hypothesized that M. amboiensis Bl. may exert its cytotoxicity on cancer cells by inducing apoptosis. In view of these findings, typical morphological changes as the apoptotic cells e.g. membrane blebbing, cell shrinkage, chromatin condensation, nuclear fragmentation, apoptotic bodies and loss of adhesion (Kerr et al., 1994) were observed after exposure the cells with these drugs for 24 h. More importantly, we found that chloroform and n-hexane extracts as well as macluraxanthone and gerontoxanthone-I induced the accumulation of B16F10 cells in G1 phase at low doses after 24 h incubation. They may be related with the induction DNA synthesis which plays a crucial role in cell cycle progression (Kerr et al., 1994). As the treatment dose increased, the percentage of cells in the sub-G1 phase (hypodiploid or apoptotic cells) increased accordingly (Figure 8). Correspondingly, using Annexin V/PI fluorescein staining and detecting by flow cytometry allowed the distributions of early apoptosis and necrosis cells from viable cells. Early (annexin V positive and PI negative) and late apoptotic (annexin V and PI positives) of MA-treated B16F10 cells were subsequent increased in dose-dependently. In agreement with these observations, treatment with either allanxanthone C or macluraxanthone purified from Guttiferae tree resulted in a concentration dependent inhibition of the growth of lymphocytic leukemia cells, induction of apoptosis by triggering the mitochondrial pathway (Menasria et al., 2008), an accumulation in the G0/G1 phase of cell cycle progression as well as capable of in vivo antileukemic effect in xenograft SCID CB-17 mice of human lymphocytic leukemia (Loisel et al., 2010). These data are supported the hypothesis that M. amboiensis Bl. could suppress B16F10 melanoma cell proliferation via cell cycle blockage and subsequently induced cell death which may be specific to apoptotic cell death rather than necrosis. More detail experiments are required to confirm this point.

In conclusion, our observations indicate for the first time that Maclura amboiensis Bl. affects multitstep in the complex process of cancer metastasis, including proliferation, adhesion, invasion and migration, possibly through induction of apoptosis of the highly metastatic B16F10 melanoma cells. As evidence from these above results, M. amboiensis Bl. represents a potential candidate of a novel chemopreventive and/or chemotherapeutic agent for cancer metastasis. In addition, they also support the ethno-medicinal usage of this medicinal plant for cancer prevention and chemotherapy. A better understanding on the exact mechanisms on its anti-metastatic activity is needed to further investigation.

Acknowledgements

We gratefully acknowledge financial support in the grant of the National Cancer Institute, Ministry of Public Health, Bangkok, Thailand.

References

