RESEARCH ARTICLE

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

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## Abstract

**Inhibitory effects of *Maclura amboinenesis* 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 $\mu$g/ml) as well as macluraxanthone and gerontoxanthone-I (3 and 10 $\mu$M) significantly inhibited B16F10 cell invasion, to a greater extent than 10 $\mu$M doxorubicin, while reducing migration of cancer cells without cellular cytotoxicity. Moreover, exposure of B16F10 melanoma cells to high concentrations of chloroform (30 $\mu$g/ml) and geratoxanthone-I (20 $\mu$M) 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

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## 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

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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, triterpenoids 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-lipid peroxidative (Chang et al., 1994), antioxidative (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: 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, macluraxanthone 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). Macluraxanthone 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 macluraxanthone isolated from Guttiferaes resulted in a concentration-dependent inhibition cell growth of lymphocytitic leukemia (CLL), induced the accumulation in the $G_0/G_1$ cell cycle phase as well as were 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 main 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 metastastic 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, trypan 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 Natural 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 (1kg) 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, $^{1}H$ and $^{13}CNMR$ and MS). The chemical structure of two main xanthones is presented in Figure 1.

Macluraxanthone (MA-1): yellow needles from

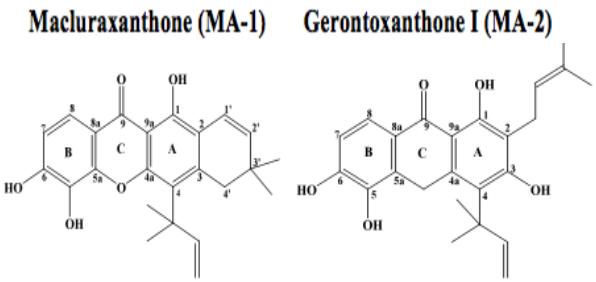


**Figure 1. Chemical Structures of Two Main Prenylated Xanthones Isolated from Active Extracts of *M. amboinensis* Bl. Roots**

*n*-hexane-AcOEt 4:1, m.p. 181-182°C (lit. 181-183°C and 204-206°C); $C_{23}H_{22}O_6$; M.W. 394; EI-MS m/z: 394 ($M^+$, 78), 379 ($(M-Me)^+$, 100), 365(14), 353(22), 351(18), 338(12), 241(8), 182(5); UV$\lambda_{max}$ MeOH (log Σ): 232, 278, 324, 332; IR $\nu_{max}$ (KBr) $cm^{-1}$; 3456, 3280 (free-OH), 1654(C=O), 1635, 1584, 1465, 1417; $^1$H-NMR ($CDCl_3$) δ13.52 (1H, *s,* 1-OH), 7.69 (1H, *d,* J=8.8 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=10.07Hz, H-12), 5.26 (1H, *dd,* J=17.7 and 1.52 Hz, Ha-18), 5.08 (1H, *dd,* J=10.7 and 1.52 Hz, Hb-18) 1.68(6H, *s,* 16-2Me), 1.54(6H, *s,* 13-2Me); $^{13}$CNMR ($CDCl_3$) δ: 158.9 (C-1), 103.0 (C-2), 156.8 (C-3), 113.0(C-4), 156.7 (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), 105.6 (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), 103.3(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); $C_{23}H_{24}O_6$; 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), 156(6); UV$\lambda_{max}$(MeOH)(logΣ) nm: 200, 249, 280, 325; IR $\nu_{max}$(KBr) $cm^{-1}$; 3652, 3408 (free-OH), 3152, 2976, 2928, 1632(C=O), 1584, 1558, 1472, 1449, 1324, 1280 and 967; $^1$H-NMR ($CDCl_3$) δ: 13.63(1H, *s,* 1-OH), 7.74(1H, *d,* J=8.8Hz, 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, Ha-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); 1.81(3H, *s,* Me), 1.71(6H, *s,* 2Me); $^{13}$CNMR ($CDCl_3$) δ: 161.4(C-1), 112.6(C-2), 159.0(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% $CO_2$ 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 ($3x10^3$ 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 $IC_{50}$ 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 2μg/50μl of fibronectin and the upper surface was coated with 10μg/50 μl of Matrigel, whereas the migrative ability was performed on the non-coating filters with Matrigel. In the both assays, B16F10 melanoma cells at the density of $2x10^5$ 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% $CO_2$ 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 using a Microplate Reader (Benchmark 550, Bio-Rad, USA). Each experiment was done in quadruplicate. Three independent experiments were performed. Data are expressed as percentages compared to control.

*Scratch Motility Assay*

To confirm the migration capacity of the MA-treated cells, the scratch motility assay was carried out (Shin et al., 2008). B16F10 melanoma cells ($1x10^5$ cells/well) were seeded into a 24 well culture plate and were allowed to grow overnight to reach confluency. The monolayer was then scratched with a pipette tip, washed with PBS twice to remove floating cells, and treated with tested samples at their respective $IC_{50}$ values. Doxorubicin and 0.1% DMSO in medium were used as positive and negative controls. After each incubation period of 6, 12 and 24 h, the cells migrated into the scratched area was photographed under a phase-contrast inverted microscope (ECLIPSE Ti-U, Nikon, Japan). The distance that cells had migrated into the cell-free space was measured using a microruler. The width of each migrated area was used to calculate the relative proportion wounded at time zero. Each experiment was performed in triplicate.

*Cell Attachment Assay*

Cell attachment assay was examined as described previously and slightly modifications (Xia et al., 2005). Briefly, B16F10 melanoma cells ($1x10^5$ cells/well) were treated with tested drugs; *n*-hexane, chloroform and methanol extracts (3, 10 and 30 $\mu$g/ml); aqueous extract (10, 30 and 100 $\mu$g/ml) as well as two main xanthones; MA-1 and MA-2 (3, 10 and 20 $\mu$M), respectively and incubated at 37°C. Doxorubicin (3 and 10 $\mu$M) and 0.1% DMSO in medium were used as positive and negative controls. After 24 h exposure, all treated and untreated cells were detached using 0.25% trypsin-EDTA and plated them back at the same density on a new 24 well-culture plate. At the end of each incubation period for 6, 12 and 24 h., the cells attachment status and morphology was observed and photographs were captured under a phase-contrast inverted microscope (ECLIPSE Ti-U, Nikon, Japan). Subsequently, cell viability was analyzed using the MTT assay. Each experiment was performed in triplicate.

*Cell Morphology Observation*

Morphological changes of apoptotic cancer cells were evaluated by Hoechst 33342 staining (Li et al., 2005). Briefly, B16F10 melanoma cells ($1x10^4$ cells/well) were grown on 8-well chamber slide (Nalge Nunc Int., USA) and treated with four extracts; *n*-hexane, chloroform and methanol (3, 10 and 30 $\mu$g/ml) and aqueous extract (10, 30 and 100 $\mu$g/ml) as well as two main xanthones; MA-1 and MA-2 (3, 10 and 20 $\mu$M) for 24 h, respectively. Doxorubicin (3 and 10 $\mu$M) and 0.1% DMSO in medium were used as positive and negative controls. At the end of incubation, cells were harvested and fixed with 4% paraformaldehyde for 10 min and then washed three times with PBS. Nuclear DNA was denatured with pre-cooled ethanol/acetic acid (2:1) at -20°C for 5 min. After washing with PBS twice, the fixed cells were stained with 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% $CO_2$ for 24 h. Cells were then treated with four extracts; *n*-hexane, chloroform, methanol (3, 10 and 30 $\mu$g/ml) and aqueous extract (250, 500 and 750 $\mu$g/ml) as well as two main xanthones; MA-1 and MA-2 (3, 10 and 20 $\mu$M) for 24 h. Doxorubicin (1, 3 and 10 $\mu$M) 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 $\mu$g/ml ribonuclease A at 37°C for 20 min. Cellular DNA was labeled with 250 $\mu$l PI (100 $\mu$g/ml) in PBS at least 30 min in the dark at room temperature and then filtered through a 40 $\mu$m 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 quantify drugs-induced apoptotic cell death, flow cytometry was examined after staining with fluorecein-conjugated Annexin V-FITC and PI using an Annexin V-FITC/PI apoptosis detection kit, according to the manufacture's instruction. Briefly, at the end of treatment, both adherent and floating cells were harvested, washed twice with ice-cold PBS, and then double-labeled with 5 $\mu$l annexin V-fluorescein and 5 $\mu$l PI in 100 $\mu$l of binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). After 15 min incubation at room temperature, 400 $\mu$l of binding buffer was added in each sample and cells were then analyzed by a FACSCalibur Flow Cytometer of 20,000 cells in each group. Annexin-V binds to those cells that express phosphatidylserine on the outer layer of cell membrane. This allows for the discrimination of living cells (unstained with either fluorochrome) from apoptotic cells (stained only with annexin-V) and necrotic cells (stained with both of annexin-V and PI). Data analysis was performed with the FACSDiva Version 6.1.3 Software. All experiments were performed in duplicate

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, $^{1}$H- and $^{13}$C-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 $\mu$g/ml) and aqueous extract (0.1-500 $\mu$g/ml) for 24 h, resulted in a significant reduction of cell viability at the $IC_{50}$ 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 $IC_{50}$ 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).

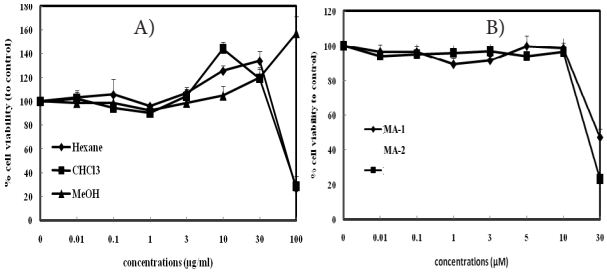


**Figure 2. Effect of MA Extracts and Two Xanthones on B16F10 Melanoma Cell Proliferation.** Cell proliferation was assessed by the MTT colorimetric assay. B16F10 melanoma cells ($3x10^3$ cells/well) were treated with either vehicle (0.1% DMSO) or various concentrations of the extracts; (A) *n*-hexane, chloroform, methanol (0.01-100 $\mu$g/ml) and (B) two prenylated xanthones; macluraxanthone (MA-1) and gerontoxanthone I (MA-2) (0.01-30 $\mu$M) for 24 h. The data are expressed as percentage cell viability and represent as the mean±S.D. of three independent experiments where each treatment was performed in six wells.

*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

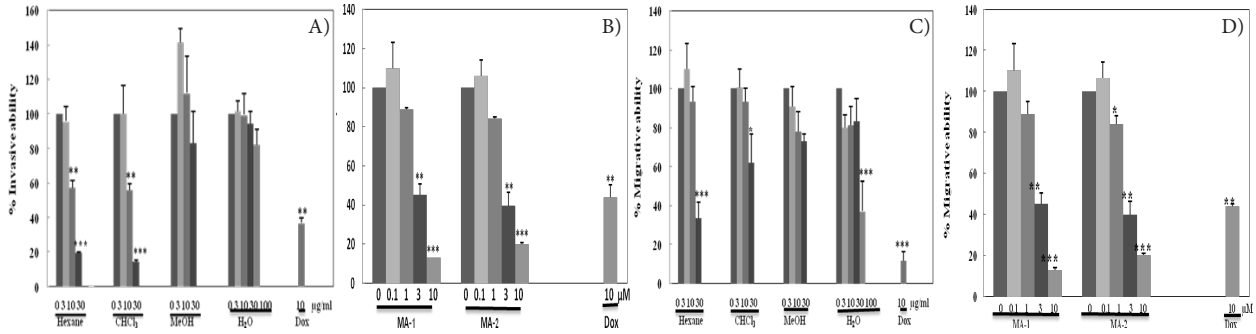


**Figure 3. Effect of MA Extracts and Two Xanthones on B16F10 Melanoma Cell. A-B) Transwell invasion chamber assay was performed to assess the effect on cell invasion.** B16F10 cells ($2x10^5$ cells/chamber) containing with MA extracts and two xanthones were added to Matrigel-coated upper compartment of Boyden Chambers and allowed to invade toward lower compartment filled with 0.6 ml of the same medium. Cells were incubated for 24 h and stained with 0.5% crystal violet. Invasive ability was analyzed with cells on the lower side by dissolving the cell bound crystal violet in 30% acetic acid and subsequent spectrophotometric analysis at 590 nm. **C-D) Transwell migration chamber assay was performed to assess the effect on cell migration.** B16F10 cells ($2x10^5$ cells/chamber) containing with MA extracts and two xanthones were added to non-Matrigel-coated upper compartment of Boyden Chambers and allowed to invade toward lower compartment filled with 0.6 ml of the same medium. Cells were incubated for 24 h and stained with 0.5% crystal violet. Migrative ability was analyzed with cells on the lower side by dissolving the cell bound crystal violet in 30% acetic acid and subsequent spectrophotometric analysis at 590 nm. All data were represented as the mean±S.D. of the results of three independent experiments. *p<0.05, **p<0.01 and ***p<0.001 versus non-treatment control group.

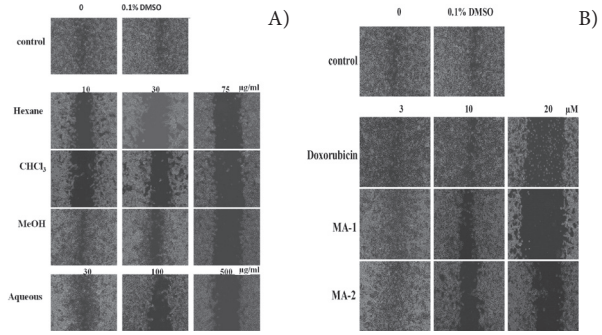


**Figure 4. Effect of MA Extracts and Two Xanthones on B16F10 Melanoma Cell Migration.** Scratch motility or wound healing assay was performed to assess cell migration. Cells were treated with vehicle (0.1% DMSO), four extracts; *n*-hexane, chloroform and methanol extracts (10, 30 and 75 $\mu$g/ml) and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

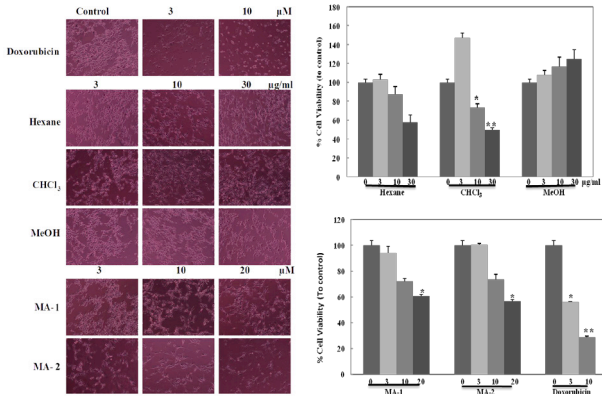


**Figure 5. Effect of MA Extracts and Two Xanthones on B16F10 Melanoma Cell Adhesion.** Cell attachment assay was performed to assess the effect on cell adhesion. B16F0 cells (1x10$^5$ cells/well) treated with four extracts, two main xanthones and Doxorubicin for 24 h. were detached using 0.25% trypsin-EDTA and plated them back with same density on a new culture plate. After each incubation periods 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 was analyzed using the MTT assay after 24 h incubation (Figure 5B and 5C). The data were represented as the mean±S.D. of the results of three independent experiments. *p<0.05 and **p<0.01 versus non-treatment control group.

using a Transwell® Boyden Chamber Assay. The results demonstrated that the *n*-hexane and chloroform extracts at both concentrations of 10 and 30 $\mu$g/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 $\mu$g/ml, 88.2%). Macluraxanthone and gerontoxanthone-I at the concentrations of 3 and 10 $\mu$M 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 $\mu$M 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 $\mu$g/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 $\mu$g/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 $\mu$M 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 $\mu$M doxorubicin (55.9%, Figure 3D).

Subsequently, the anti-migratory effect of MA extracts and two xanthones on B16F10 melanoma cells was confirmed by the scratch motility assay. As shown in Figure 4, the untreated B16F10 cells exhibited a complete wound closure activity with in 24 h. incubation. In contrary, the MA-treated cells showed only a limited closure of wound at the end of their respective incubation times (6, 12 and 24 h). As the incubation time increased for 24 h, the migration inhibition rate of the *n*-hexane and chloroform extracts as well as macluraxanthone and gerontoxanthone-I showed markedly suppressed in a dose-dependent manner (Figure 4A and 4B).

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 main 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 numbers of viable treated cells in each group and further incubated at 37°C, 5%$CO_2$ for 6, 12 and 24 h. The rounded cells represent the unattached cells. The higher number of rounded (unattached) cell at a given time point

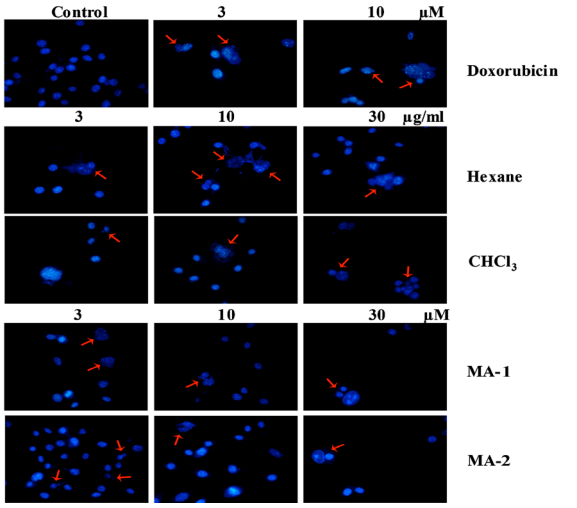


**Figure 6. Morphological Changes of B16F10 Melanoma Cells Treated with MA Extracts and Two Xanthones.** B16F10 melanoma cells (1x10$^4$ cells/well) were seeded on 8 well-chamber slide and allowed to attach for overnight. Cells were treated with either vehicle (0.1% DMSO) or various concentrations of the extracts; *n*-hexane, chloroform, methanol (3, 10 and 30 $\mu$g/ml) and aqueous extracts (10, 30 and 100 $\mu$g/ml) as well as two xanthones; macluraxanthone and gerontoxanthone I (1, 3 and 10 $\mu$M) for 24 h. Doxorubicin (1 and 10 $\mu$M) 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.

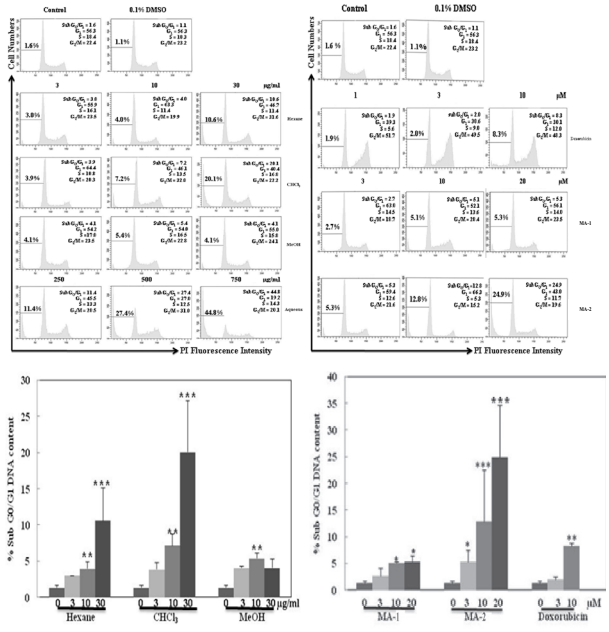


**Figure 7. Effect of MA Extracts and Two Xanthones on Cell Cycle Progression of B16F10 Melanoma Cells.** B16F10 melanoma cells (1x10$^6$ cells) were treated with either vehicle (0.1% DMSO) or various concentrations of the extracts; *n*-hexane, chloroform, methanol (3, 10 and 30 $\mu$g/ml) and aqueous extracts (10, 30 and 100 $\mu$g/ml) as well as two xanthones; macluraxanthone and gerontoxanthone I (1, 3 and 10 $\mu$M) 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 cyotmetry. Doxorubicin was used as positive control and 0.1% DMSO was used as negative control. The data were represented as the mean± S.D. of the results of three independent experiments. *p<0.05; **p<0.01 and ***p<0.001 versus non-treatment control group.

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 $\mu$g/ml) and gerontoxanthone-I (20 $\mu$M) 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 $\mu$g/ml) and xanthones (10 and 20 $\mu$M) were 42.5% and 50.2% as well as 39.1% and 43.2%, respectively, compared to that of 10 $\mu$M 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 $\mu$g/ml) and aqueous extract (10, 30 and 100 $\mu$g/ml) as well as two xanthones; macluraxanthone and gerontoxanthone-I (3, 10 and 30 $\mu$M) on the morphological changes of B16F10 melanoma cells for 24 h. and detecting under a phase contrast and fluorescence microscope after staining with Hoescht 33342 kit. As shown in Figure 6, typical morphological changes as the characteristic of apoptotic cells e.g. membrane blebbing, cell shrinkage, chromatin condensation, nuclear fragmentation and apoptotic bodies (Li et al., 2005) were observed after exposure the cells with these drugs for 24 h., whereas the untreated cells did not show the evident apoptotic morphological changes. Treatment the B16F10 cells by chloroform and *n*-hexane extracts as well as gerontoxanthone-I and macluraxanthone exhibited high apoptotic cells in all indicated concentrations. However, methanol and aqueous extracts-treated cells had slightly effects (data not shown). The results indicated the active *n*-hexane and chloroform extracts as well as macluraxanthone and gerontoxanthone-I induced cell death by apoptosis action at the indicated treatments.

Several studies have shown that apoptosis might be induced to cell cycle arrest. Therefore, inhibition of the cell cycle has been appreciated as target for the treatment of cancer (Kerr et al., 1994). In the next step, we evaluated

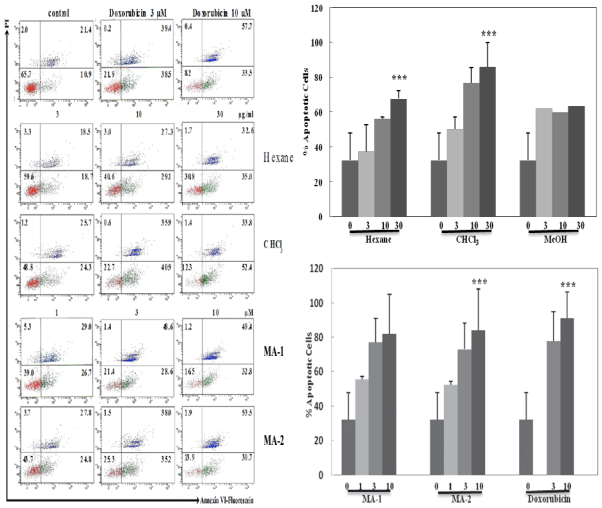


**Figure 8. Induction of Apoptosis in B16F10 Melanoma Cells Treated with MA Extracts and Two Xanthones.** B16F10 melanoma cells (1x10$^6$ cells/dish) were treated with either vehicle (0.1% DMSO) or various concentrations of the extracts; *n*-hexane, chloroform, methanol (3, 10 and 30 $\mu$g/ml) and aqueous extracts (10, 30 and 100 $\mu$g/ml) as well as two xanthones; macluraxanthone and gerontoxanthone I (1, 3 and 10 $\mu$M) 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 xantnones in the same period, the percentages of sub-G1 populations (hypodiploid 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* Bl. 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 (Nicloson, 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 reconstituented 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 $\mu$g/ml) and geratoxanthone-I (20 $\mu$M) 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. amboinensis* 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 amboinensis* Bl. roots remarkably suppressed the multistep 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 isoalvaxanthone isolated from *Cudrania cochinchinensis* (Lour) and cudratricusxanthone G, isolated from *Cudrania tricuspidata* exerted anti-metastatic 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. amboinensis* 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 $G_0/G_1$ 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. amboinensis* 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 amboinensis* Bl. affects multistep 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. amboinensis* 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.

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