View metadata, citation and similar papers at core.ac.uk

brought to you by 🗴 CORE

provided by Journal Of Agrobiotechnology (Journal of UniSZA - Un Cytotoxicity and Infinition of UniSZA - Un Damnacanthal and Nordamnacanthal Isolated from Roots of Morinda elliptica

*Noorjahan B. M. A. ¹, A. M. Ali², Yeap S. K. ¹, Subaimi M. ³, N. H. Lajis⁴, Mashitoh A. R. ¹, Ho W. Y. ¹ and Ismail N. H. ⁵

 ¹Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, MALAYSIA. noorjahan@biotech.upm.edu.my
²Faculty Agriculture and Biotechnology, Universiti Darul Iman Malaysia, 21300 Kuala Terengganu, Terengganu Darul Iman, MALAYSIA.
³Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, MALAYSIA.
⁴Institute of Biosciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, MALAYSIA.
⁵Faculty of Applied Sciences, Universiti Teknologi MARA, 40000 Shah Alam, Selangor Darul Ehsan

⁵Faculty of Applied Sciences, Universiti Teknologi MARA, 40000 Shah Alam, Selangor Darul Ehsan, MALAYSIA.

ABSTRACT

Morinda elliptica has been used traditionally as a medicine to treat various diseases in Malaysia and Southeast Asia. In this present study, the cytotoxicity and immunomodulatory effects of damnacanthal and nordamnacanthal isolated from the roots of *Morinda elliptica* were studied. Cytotoxic study was performed on human leukemia cell lines (HL-60) and mouse myelomonocyte leukemia (Wehi-3B) cell line, whereas immunomodulatory effect was evaluated by using lymphocytes proliferation assay on mice thymocytes and human peripheral blood mononuclear cells (PBMC). The production of extracellular human interleukin-2 (IL-2) and human interleukin-12 (IL-12) were also assessed by using the enzyme-linked immunosorbent assay (ELISA). The results indicated that both damnacanthal and nordamnacanthal inhibited the proliferation of HL-60 cells with the IC₅₀ of 4.0 µg/mL and 20 µg/mL, respectively, and were able to activate mice thymocytes and human peripheral blood mononuclear cells (PBMC) at low concentration (0.47 µg/mL). Moreover, the production of extracellular human interleukin-2 (IL-2) and human interleukin-12 (IL-12) from both compounds-activated PBMC were prominently upregulated after 24 hours and the concentration remained almost constant up to 72 hours. Based on the results, both damnacanthal and nordamnacanthal can be cytotoxic and immunomodulatory agents which have the potential in cancer treatment.

Keywords: Morinda elliptica, damnacanthal, nordamnacanthal, immunomodulation, cytotoxicity

ABSTRAK

Morinda elliptica telah digunakan secara tradisional sebagai ubat untuk merawat pelbagai penyakit di Malaysia dan Asia Tenggara. Dalam kajian ini, kesan sitotoksik dan imunopemodulatan oleh damnacanthal dan nordamnacanthal yang dipencilkan dari akar *Morinda elliptica* telah dikaji. Kajian sitotoksik telah dijalankan pada sel jajar leukemia manusia (HL-60) dan sel myelomonosit leukemia mencit (Wehi-3B), manakala kesan pengawalaturan mononuklear darah periferi manusia (PBMC). Penghasilan interleukin-2 (IL-2) dan interleukin-12 (IL-12) ekstrasel manusia telah juga dinilai dengan menggunakan asai penjerapan imun-berangkai-enzim (ELISA). Keputusan menunjukkan bahawa

kedua-dua damnacanthal dan nordamnacanthal menyekat penggandaan sel HL-60 dengan IC_{50} 4.0 µg/mL dan 20 µg/mL masing-masing, dan dapat mengaktifkan timosit mencit dan PBMC pada kepekatan rendah (0.47 µg/mL). Di samping itu, penghasilan IL-2 dan IL-12 ekstrasel manusia dari PBMC yang diaktifkan oleh kedua sebatian telah dengan jelasnya meningkat selepas 24 jam dan kepekatan kekal malar sehingga 72 jam. Berdasarkan kepada keputusan ini, kedua-dua damnacanthal dan nordamnacanthal boleh menjadi agen sitotoksik dan imunopemodulatan yang mempunyai potensi dalam rawatan barah.

Kata kunci: Morinda elliptica, damnacanthal, nordamnacanthal, imunomodulatan, sitotoksisiti

INTRODUCTION

Morinda species are the most popular herbs worldwide. One of these species is *Morinda elliptica* which is widely distributed in Peninsular Malaysia. This herb has been used traditionally as a medicine to treat various diseases such as headache, cholera, diarrhea, gastritis and fever (Ong and Norzalina, 1999). In Malaysia, the pounded leaves of this herb were commonly added to rice for people who have loss of appetite (Jasril *et al.*, 2003). In addition, this herb is also claimed by local people to stimulate the immune system and thus prevent the formation and proliferation of cancers including the malignant ones.

Morinda elliptica has long been known to contain a substantial amount of anthraquinones such as damnacanthal and nordamnacanthal. Both damnacanthal and nordamnacanthal have some unique chemical and biological properties. It was reported that damnacanthal isolated from noni roots acted as an inhibitor of *ras* function, which is believed to be associated with the signal transduction in several human cancers such as lungs, colon and leukemia (Hiramatsu *et al.*, 1993). Damnacanthal has been shown to have a potent inhibitory activity towards tyrosine kinase such as *Lck, Scr, Lym* and EGF receptors (Hiwasa *et al.*, 1999). Furthermore, damnacanthal which is solubilized in DMSO also affects intracellular Ca²⁺ mobilization as demonstrated in cultured bovine coronary endothelial cells (Aoki *et al.*, 2000) and showed an intense inhibitory effect against DNA topoisomerase 11 with an IC₅₀ of 20 μ g/mL (Tosa *et al.*, 1998).

It was demonstrated that damnacanthal and nordamnacanthal isolated from *Morinda elliptica* were cytotoxic towards MCF-7 (breast carcinoma) and CEM-SS (T-lymphoblastic leukemia) cell lines. Both compounds were also found to have strong antimicrobial activity while only damnacanthal showed moderate activity against HIV (Ali *et al.*, 2000). More recently, Masakazu *et al.* (2006) discovered that damnacanthal possesses as an inhibitor of viral protein R (Vpr) which is important in the development of anti-HIV therapy.

Undoubtedly, both compounds exhibited various pharmacological activities. However, no specific study has been reported on the immunomodulatory activity of damnacanthal and nordamnacanthal in mice and human immune cells. In this study, the immunomodulatory and cytotoxic activities of both compounds were evaluated.

MATERIALS AND METHODS

Materials

Damnacanthal and nordamnacanthal isolated from the roots of *Morinda elliptica* were kindly supplied by Professor Dr. Md. Nordin Hj. Lajis, from the Laboratory of Natural Products, Institute of Biosciences, Universiti Putra Malaysia. The concentrated compounds were dissolved in dimethylsulphoxide (DMSO) (Sigma, USA) to get a stock solution of 10 mg/mL. The substock solution of 0.06 mg/mL was prepared by diluting 6 μ L of the stock solution into 994 μ L serum-free culture medium, RPMI 1640 (Sigma, USA) (the percentage of DMSO in the experiment should not exceed 0.5). The stock and substock solutions were both stored at 4 °C.

Concanavalin A (Con A) (Sigma, USA), lipopolysaccharide (LPS) (from Salmonella enteritidis) (Sigma, USA) and Pokeweed mitogen (PWM) (1 mg/mL) were used as a positive control. This commercial immunomodulator was prepared by dissolving it with RPMI 1640 (Sigma, USA). The substock stock and solutions were prepared above. as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RPMI 1640, phosphate buffered saline (PBS), Dulbecco's Modified Eagle Medium (DMEM), 2-mercaptoethanol, 6-mercaptopurine and dimethyl sulphoxide (DMSO) were all purchased from Sigma. Foetal bovine serum (FBS) from PAA, IL 2 and IL 12 Instant Enzyme Link Immunosorbent Assay (ELISA) kit was purchased from Bender MedSystems, Austria, and Ficoll-Paque Plus was purchased from Amersham Biosciences.

Preparation of Mice Thymus Cell Suspensions

ICR mice, 5-8 weeks old, were used in all experiments. The animals were purchased from Animal House, Universiti Putra Malaysia. The animals were housed under standard conditions at 25 ± 2 °C and fed with standard pellets and tap water. The mice were protected from stress or specific control. This work has been approved by the Animal Care and Use Committee, Universiti Putra Malaysia (UPM), (Ref: UPM/ FPV/ PS/ 3.2.1.551/ AUP-R2).

The mice were killed by cervical dislocation. The thymus which is located above the heart, was removed and quickly washed with Hank's Balanced Salts Solution (HBSS) in a petri dish. The thymus was minced and pressed through a sterile wire mesh screen with a rubber syringe plunger. The cell suspension was washed with PBS supplemented with 0.1% (w/v) BSA and 0.06% (w/v) sodium citrate (PBS-BSA-SC) and spun down at 200 x g for 10 minutes. The step was repeated until the pellet was clean as shown by absence of any debris or contaminant. The supernatant was discarded, and 4 mL of Dulbecco's Modified Eagle Medium (DMEM) with 10% (v/v) heat inactivated serum was added. The pellet was resuspended and cell counting was performed, to determine the lymphocyte cell number in the suspension. All of the above steps were carried out under sterile conditions in a biological safety cabinet to prevent any contamination.

Isolation of Peripheral Blood Mononuclear Cells (PBMC)

Venous blood (20-25 mL) was collected aseptically from 20 healthy donors in preservative free heparin tubes. The blood was diluted with phosphate buffered saline (PBS) pH 7.4, and layered onto Ficoll plus at the ratio of diluted blood with Ficoll 2:1 (Amersham). After centrifugation at 400 x g for 50 minutes, the lymphocytes were collected at the interface and washed three times with the PBS. The cells were resuspended in DMEM with 10% (v/v) foetal bovine serum and antibiotics.

Cell Line

The cell lines used were human promyelocytic leukemia (HL-60) and mouse embryo fibroblast (3T3). HL-60 was obtained from RIKEN Cell Bank, Tsukuba, Japan and 3T3 was obtained from the American Type Culture Collection ATCC (Rockville, MD). The cells were grown and maintained in RPMI 1640 (Sigma, USA) medium; supplemented with 10% (v/v) fetal bovine serum (Flow Laboratory, Australia), 100 IU/mL penicillin (Flow Laboratory, Australia) and 100 g/mL streptomycin (Flow Laboratory, Australia) at 37 °C, 5% CO, and 90% humidity.

MTT Cell Viability Assay

The effects of both compounds on cell viability of mice thymocytes, human PBMC, HL-60, Wehi-3B and NIH 3T3 were first determined by using a colorimetric technique, which is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Mosmann, 1983). Briefly, $100 \,\mu\text{L}$ of DMEM/RPMI 1640 media with 10% (v/v) of FBS was added into all the wells except row A in the 96-well plate (TPP, Switzerland). Then, 100 µL of diluted compounds at 60 µg/mL was added into row A and row B. A series of twofold dilution of compound was carried out down from row B until row G. The row H was left untouched and the excess solution (100 μ L) was discarded, then 100 µL of cells (mice thymocytes, human PBMC, HL-60, Wehi-3B or NIH 3T3) with cell concentration at 4×10^{5} cells/mL was added into all wells in the 96-well plate and incubated at 37 °C, 5% CO, and 90% humidity for 72 h. After the incubation period, 20 μ L of MTT (Sigma, USA) at 5 mg/mL was added into each well in the 96-well plate and incubated for 4 h at 37 °C, 5% CO₂ and 90% humidity. One hundred and seventy microlitres of medium with MTT was removed from every well and 100 µL DMSO (Sigma, USA) was added to each well to compound and solubilize the formazan crystal by incubating for 20 min at 37 °C, 5% CO, incubator. Finally, the plate was read at 570 nm and reference wavelength at 630 nm by using µ Quant ELISA Reader (Bio-Tek Instruments, USA). The results of the compounds were compared with the result of ConA (1 μ g/mL) and LPS (1 μ g/mL) for immunomodulation, while Doxorubicin (Sigma, USA) for cytotoxicity, and without mitogens/drug or compounds by using the same method. Each compound and control was assayed in triplicate or three times. The cytotoxic dose that kills cells by 50% (IC₅₀) was determined from absorbance (OD) versus concentration curve, and the percentage of proliferation was calculated by the following formula:

Percentage (%) of cell proliferation = $\frac{\text{OD sample X }100}{\text{OD control}}$

Cytokine Production of Human Peripheral Blood Mononuclear Cells

The expressions of extracellular IL-2 and IL-12 were performed by using Cytokine Instant Enzyme Link Immunosorbent Assay (ELISA) kit (Bender MedSystems, Austria). Briefly, the extracted human PBMC with cell concentration at 5 x 10⁵ cells/mL was treated with the same volume of compounds at 30 μ g/mL in 6 well culture plates (TPP, Switzerland). Control cultures without compound and positive control with Pokeweed Mitogen (50 μ g/mL) were prepared simultaneously. The culture was then incubated for respective times (24 hours, 48 hours, and 72 hours). After the corresponding period, the samples were harvested and pelleted. 50 μ L of the supernatant was added onto the strip on the plate of the kit and incubated for 3 hours with 200 rpm shaking. Then, the samples were washed, followed by immediate addition of 3,3',5,5"-tetramethylbenzidine (TMB) peroxidase substrate in the dark. Finally, 1M phosphoric acid stop solution was added and the plate was read at 450 nm, and 620 nm as a reference wavelength, by using μ Quant ELISA Reader (Bio-Tek Instruments, USA) at the Animal Tissue Culture Laboratory, Faculty of Biotechnology and Biomolecular Sciences, UPM. The result was compared to the control strip in the kit. Each compound and control was assayed three

times. Data were expressed as pg/mL.

Statistical Analysis

All experiments were performed in triplicate and the results were expressed as mean \pm standard error.

RESULTS

Cytotoxicity Effect of Damnacanthal and Nordamnacanthal on Leukemia Cell Lines and Normal Cell

Damnacanthal was found to inhibit both leukemia cell lines tested, HL-60 and Wehi-3B, respectively, with the IC_{50} value less than 5 µg/mL after 72 h of treatment (Table 1). Moreover, it did not inhibit normal cell 3 T3. In contrast, nordamnacanthal only showed a weak toxicity effect towards HL-60 with the IC_{50} value of 23 µg/mL, and it had no effect towards Wehi-3B and 3T3. Doxorubicin exhibited stronger inhibition towards both leukemia cell lines with the IC_{50} value less than 3 µg/mL, but also exhibited a toxicity effect towards normal cell 3T3 with the IC_{50} value of 3 µg/mL.

Table 1. Effects of damnacanthal, nordamnacanthal and doxorubicin towards HL-60,	Wehi-3B a	nd
NIH 3T3 cell lines after 72 hours of treatment.		

Cell	IC 50 value (µg/mL)		
lines	HL-60	Wehi-3B	3T3
Compounds			
Damnacanthal	4.2 ± 0.1	3.3 ± 0.5	> 30
Nordamnacanthal	23.3 ± 0.7	> 30	> 30
Doxorubicin	1.2 ± 0.1	1.0 ± 0.9	3.0 ± 0.8

Mitogenic Activity of Damnacanthal and Nordamnacanthal on Mice Thymocytes

Both compounds were found to significantly stimulate the proliferation of mice thymocytes in a doseand time-dependent fashion (Figures 1 and 2). Damnacanthal exhibited significantly higher proliferation of mice thymocytes as compared to the control group after 24 h, but the proliferation reduced drastically after 48 h and 72 h of incubation. Maximal growth was found after 24 h of incubation with $30 \mu g/mL$ of the compound, achieving a 2.1 fold higher than the positive control, Con A. Con A is a lectin protein originally extracted from jack bean (*Canavalia ensiformis*), which was used as a positive control in evaluating mice thymocytes since this mitogen was known to induce T cells (Ho *et al.*, 2004). It was used at its optimal concentration (2.5 µg/mL), and was found to prominently promote the lymphocyte growth in a time-dependent fashion with maximal growth after 24 h treatment. However, this proliferation decreased significantly after 48 h and 72 h with values of 66.12% and 28.86%, respectively. In addition, nordamnacanthal also stimulated proliferation of mice thymocytes after 24 h treatment (Figure 2). It significantly stimulated higher proliferation of mice thymocytes up to 132.02% at a concentration of 30 μ g/mL after 24 h treatment, achieving 1.5-fold higher as compared to Con A. The proliferation rate of mice thymocytes treated with nordamnacanthal was drastically decreased after 48 h and slightly increased after 72 h treatment, at all concentrations tested. The pattern of proliferation of mice thymocytes by both compounds seemed to be quite similar. Both compounds were not toxic to mice thymocytes even at a higher concentration of 30.0 μ g/mL. Furthermore, it clearly showed that damnacanthal exhibited better proliferation of mice thymocytes than nordamnacanthal and Con A.

Mitogenic Activity of Damnacanthal and Nordamnacanthal on Human Peripheral Blood Mononuclear Cells (PBMC)

Both damnacanthal and nordamnacanthal were found to stimulate the proliferation of PBMC in a dose- and time-dependent manner (Figures 3 and 4). Damnacanthal induced the highest proliferation of PBMC at 24 h treatment with a value of 46.6% at a concentration of $30.0 \,\mu\text{g/mL}$. At this incubation time, it significantly showed better proliferation of PBMC compared to PWM.



Fig. 1. Mitogenic activity of damnacanthal on mice thymocytes using MTT assay. Mice thymocytes were isolated and incubated with increasing concentrations (0.47-30.0 μ g/mL) of damnacanthal or Con A (2.5 μ g/mL, as positive control) in culture medium for 24 h, 48 h and 72 h (P < 0.05). Results were expressed as mean % ratio of MTT absorbance in damnacanthal treated and control well ± standard error of three independent experiments with three wells each.



Fig. 2. Mitogenic activity of nordamnacanthal on mice thymocytes using MTT assay. Mice thymocytes were isolated and incubated with increasing concentrations (0.47-30.0 μg/mL) of nordamnacanthal or Con A (2.5 μg/mL, as positive control) in culture medium for 24 h, 48 h and 72 h (P < 0.05). Results were expressed as mean % ratio of MTT absorbance in nordamnacanthal treated and control well ± standard error of three independent experiments with three wells each.



Compound concentration (µg/ml). Fig. 3. Mitogenic acitivity of damnacanthal on human PBMC using MTT assay. PBMC was isolated and incubated with increasing concentrations (0.47-30.0 µg/mL) of damnacanthal or PWM (50.0 µg/mL, as positive control) in culture medium for 24 h, 48 h and 72 h (P < 0.05). Results were expressed as mean % ratio of MTT absorbance in nordamnacanthal treated and control well ± standard error of three independent experiments with three wells each.

Similar to damnacanthal, nordamnacanthal has significantly exhibited the highest proliferation of PBMC at 24 h treatment; up to 43.1% at a concentration of $30.0 \ \mu\text{g/mL}$. However, the proliferation response induced by nordamnacanthal was less than that of PWM after 48 h and 72 h treatments. Obviously, damnacanthal and nordamnacanthal seemed to stimulate effective PBMC at early incubation hours (24 h) whereas PWM seemed to stimulate effective PBMC at longer incubation hours.



Fig. 4. Mitogenic acitivity of nordamnacanthal on PBMC using MTT assay. PBMC was isolated and incubated with increasing concentrations (0.47-30.0 μ g/mL) of nordamnacanthal or PWM (50 μ g/mL, as positive control) in culture medium for 24 h, 48 h and 72 h (P < 0.05). Results were expressed as mean % ratio of MTT absorbance in nordamnacanthal treated and control well ± standard error of three independent experiments with three wells each.

The Production Extracellular Human IL-2 and Human IL-12

As shown in Figure 5, damnacanthal and nordamnacanthal had significantly induced the production of human extracellular IL-2 in a time-dependent manner. Interestingly, both compounds, damnacanthal and nordamnacanthal, have promoted a higher induction of human IL-2 after 24 h of incubation with values of 151.7 pg/mL and 267.0 pg/mL, respectively; compared to the negative control with a value of 20 pg/mL. However, the concentration of IL-2 decreased significantly after 48 h and 72 h of damnacanthal treatment, with the reduction to only 6.8 and 1.8 fold higher than the negative control, respectively. Nordamnacanthal has significantly induced higher production of human IL-2 after 24 h treatment with 13.3-fold higher compared to the negative control. Similar to damnacanthal, it also reduced the production of human IL-2 after 48 h and 72 h treatments with 2.7-fold and 1.5-fold of negative control, respectively. In contrast, the production of human IL-2 after 48 h of incubation, 25.3-fold higher compared to the negative control. In addition, it also demonstrated a steady induction of human IL-2 throughout the treatment period.



Fig. 5. The production of human IL-2 in culture supernatants upon stimulation of PBMC by damnacanthal, nordamnacanthal and PWM. PBMC was isolated and incubated at 24 h, 48 h and 72 h with active concentrations (damnacanthal and nordamnacanthal at 30.0 μ g/mL) and (PWM at 50.0 μ g/mL), and IL-2 induction was specifically determined by ELISA. Values were the means ± standard error of three experiments (P < 0.05).

As shown in Figure 6, the production of human IL-12 by damnacanthal and nordamnacanthal was higher than that of the negative control, which produced 12 pg/mL of IL-12. There was no significant difference in the concentration of extracellular IL-12 induced by the treatment of damnacanthal on PBMC after 24 h and 72 h of treatment where the values were 61.3 pg/mL and 59.0 pg/mL, respectively (P > 0.05). Results indicated that it had significantly induced the production of human IL-12 at 24 h and 72 h treatments with 5.1 and 4.9-fold higher compared to the negative control, respectively. Damnacanthal obviously exhibited a sharp reduction in the production of human IL-12 after 48 h of treatment with a value of only 1.1-fold higher compared to the control. Interestingly, nordamnacanthal also induced a rather similar amount of human IL-12 after 24 h and 72 h, with the values of 48.0 and 52.0 pg/mL, respectively. Moreover, both compounds exhibited a sharp reduction in the production of human IL-12 after 48 h treatment with 13.3 pg/mL by damnacanthal, and 13.0 pg/mL by nordamnacanthal, respectively, or 1.1 and 1-fold higher compared to the control. This might be due to the fact that both compounds were derived from the same group of anthraquinones.



Fig. 6. The production of human IL-12 in culture supernatants upon stimulation of PBMC by damnacanthal and nordamnacanthal. PBMC was isolated and incubated at 24 h, 48 h and 72 h with active concentrations of damnacanthal and nordamnacanthal at 30 μ g/mL and IL-2 induction was specifically determined by ELISA. Values were the means ± standard error of three experiments (P < 0.05).

DISCUSSION

Damnacanthal and nordamnacanthal are anthraquinones that are produced mainly by plants of the Rubiaceae family. Both compounds were commonly isolated from *Morinda citrifolia*, *Morinda elliptica*, *Morinda lucida*, *Prismatomeris fragrans* and others (Ismail *et al.*, 1997; Tosa *et al.*, 1998; Kwanjai *et al.*, 2004). In this present study, both compounds were shown to possess cytotoxic effects towards several leukemia cell lines with IC_{50} value of less than 10 µg/mL after 72 hours of treatment.

Apparently, both compounds showed a toxicity effect towards leukemia cell lines which are categorized as anchorage-independent cell lines. Previously, Ali *et al.* (2000) reported that both damnacanthal and nordamnacanthal compounds exhibited more sensitivity towards the anchorage-independent cell lines. This is due to the fact that the anchorage-dependent cell lines require attachment to a solid matrix in order to proliferate, thus making them interact with each other through cell to cell contact and generate transmembrane signals that not only affected the cell proliferation, migration and differentiation, but also the survival of the cell that prevents it from undergoing apoptosis. Therefore, damnacanthal and nordamnacanthal have been shown to be toxic towards the anchorage-independent cells, particularly on leukemia cell lines groups that are grown in suspension culture.

Even though the chemical structure between damnacanthal and nordamnacanthal is closely related (Figure 7), there was a significant difference on the cytotoxicity effect towards several cell lines due to the existence of methoxyl group (-OCH₃) of damnacanthal at position C-1 whilst nordamnacanthal has hydroxyl group (-OH) at the same position. The existence of this different group was predicted to be the contributing factor to the variable effects of damnacanthal and nordamnacanthal towards cancerous cell lines. According to Ali *et al.* (2000), the presence of hydroxyl group at C-1 and C-3 and/or a formyl group at C-2 in the anthraquinone skeleton, such as nordamnacanthal and rubiacin may exert their cytotoxicity effects towards several cancerous cell lines. Nevertheless, Kamei *et al.* (1998) reported that anthraquinones with more hydroxyl groups were more effective than those with less or without the existence of the group. Konoshima *et al.* (1989) discovered that not only the number but the position of hydroxyl groups would also influence the activity of an

anthraquinone.



Fig. 7. The chemical structures of damnacanthal and nordamnacanthal.

Since damnacanthal and nordamnacanthal were known to possess some unique chemical and biological properties, the cytotoxicity effects of both compounds towards several cell lines appeared to be extremely variable. Recently, Kwanjai *et al.* (2005) demonstrated that damnacanthal and nordamnacanthal isolated from *Prismatomeris fragrans* were cytotoxic towards human small cell lung cancer (NCI-H187) and human breast cancer (BC) cell lines. This result is similar with the previous results obtained by Ali *et al.* (2000) that showed damnacanthal was cytotoxic towards breast carcinoma cells (MCF-7). Besides, Jasril *et al.*, (2003) reported that nordamnacanthal exhibited strong antitumor promoting activity towards Epstein Barr Virus activation in Raji cells. According to Shier *et al.* (1991), purified compounds that require concentration of less than 10 µg/mL to exhibit their cytotoxicity are considered as promising cancer chemotherapy agents. Hence, it seems likely that both damnacanthal and nordamnacanthal have good potential to be developed into new cancer chemotherapy agents.

Immunomodulatory studies revealed that both compounds significantly enhanced the mice thymocytes and PBMC proliferation, as compared to the control group, without causing inhibition at all concentrations tested. This result was in agreement with the results published by Wang *et al.* (2002), where the thymus in animals treated with the juice extract isolated from *Morinda citrifolia* was enlarged. The chemical constituents contained in the *Morinda citrifolia* including damnacanthal and nordamnacanthal may have enhanced immune function by stimulating thymus growth and thus exhibited anti-ageing and anti-cancer activities. Previously, Hirazumi and co-workers (1996) reported that the juice extracted from *Morinda citrifolia* was found to inhibit Lewis lung tumors in mice (LLC) which was associated with the stimulation of T cell immune response and thymocytes proliferation. Besides, mice with injected LLC ingested a daily dose of 15.0 mg of noni juice had a significant increase (119%) in life span (Hirazumi *et al.*, 1996; Hirazumi and Furusawa, 1999). In addition, the ingestion of noni combined with conventional chemotherapy in the treatment of mice with cancer, proved to increase life span (Hirazumi *et al.*, 1994). Hence, those finding did support the capability of both damnacanthal and nordamnacanthal compounds in enhancing the immune system through modulation of thymus proliferation and regeneration.

Moreover, damnacanthal and nordamnacanthal are also included in the group of phenolic compounds. Phenolic compounds have been known to possess broad biological activities, including as an immune enhancer (Maria *et al.*, 2006). According to Zhao *et al.* (2007), previous studies have indicated that many phenolic compounds could stimulate the proliferation of splenocytes. The most common phenolic compound known to have immunomodulator activities is scopoletin (Wang and Su, 2001; Maria *et al.*, 2006). The phenolic compounds could stimulate or suppress the immune system due to the hydroxyl groups in their structure. The hydroxyl groups can affect the enzyme or electron-transferring system, thus resulting in immunomodulating property, particularly in proliferation of lymphocytes and phagocytosis (Manosroi *et al.*, 2003).

IL-2 and IL-12 play an important role in regulating the proliferation, differentiation and cytolytic activity of lymphocytes. Thus, the modulatory effects of damnacanthal and nordamnacanthal on the production of these cytokines were studied. Damnacanthal and nordamnacanthal were found to induce higher production of human IL-2 than that of human IL-12 in a time-dependent manner. PWM was used as a positive control since it had been reported previously to induce IL-2 and IFN- γ production (Stanilova *et al.*, 2005). The present result showed that the induction of human IL-2 by damnacanthal and nordamnacanthal were less than that of PWM throughout the treatment periods, but was higher if compared to their capability to induce the production of human IL-12. IL-2 expression is dependent on T cell activation (Adam *et al.*, 2003). Thus, this finding suggests that both compounds tend to induce T cell compared to B cell, since IL-2 is known as a central cytokine in the regulation of T cell responses (Meena and Donna, 2004). In addition, the population of T and B lymphocytes, in human peripheral blood lymphocytes, in which 90% is T cell while only 10% is B cell (Cerqueira *et al.*, 2004), may contribute to a higher production of human IL-2 in culture supernatant treated with damnacanthal and nordamnacanthal.

Subsequently, it is interesting that damnacanthal and nordamnacanthal were shown to modulate normal lymphocytes (peripheral blood mononuclear cells), induced human IL-2 production and at the same time it was cytotoxic towards CEM-SS cells which originated from T-lymphoblastic cells. The capability of damnacanthal and nordamnacanthal in stimulating the proliferation of T cells and inducing the production of human IL-2 could play a very important role in the killing and suppression of cancer cells. The induction of IL-2 could play an essential role in triggering NK cell population, a heterogenous group of granular lymphocytes that appear very effective in lysing target cancer cells through activation of lymphokine activated killer cells (LAK) (Maria *et al.*, 2006). Moreover, tumor cells of various types possess unique sets of tumor markers that can be recognized by NK cells (Farag and Caliguiri, 2006). In addition, the lytic process by NK cells involved the release of cytotoxic factors for which there seem to be more receptors on the surface of target cancer cells than on malignant self cells. Therefore, this finding suggests that damnacanthal and nordamnacanthal could be developed as a new anticancer chemotherapy agent particularly in treating leukemic cells originated from T-lymphocytes cells, without causing the cytotoxic effect towards normal cells.

CONCLUSION

This study suggests that damnacanthal and nordamnacanthal have a huge potential in combating cancer cells without or with less toxicity effect towards healthy cells, and at the same time might enhance the immune system. However, further studies are needed to elucidate the exact mechanism of action involved in both compounds.

ACKNOWLEDGEMENTS

The authors thank the Ministry of Science, Technology and Innovation of Malaysia for funding the project under the IRPA grant.

REFERENCES

- Adam, J. K., Odhav, B. & Bhoola, K. D. 2003. Immune responses in cancer. *Pharmacology and Therapeutics* **99**: 113-132.
- Ali, A. M., Ismail, N., Mackeen, M., Yazan, L., Mohamed, S., Ho, A. & Lajis, N. 2000. Antiviral, cytotoxic and antimicrobial activities of antharaquinones isolated from the roots of *Morinda elliptica*. *Pharmaceutical Biology* **38**: 3791-3801.
- Aoki, K., Parent, A. & Zhang, J. 2000. Mechanism of damnacanthal-induced [Ca(2+)] elevation in human dermal fibroblasts. *European Journal of Pharmacology* 387: 119-124.
- Cerqueira, F., Cordeiro-Da-Silva, A., Gaspar-Marques, C., Simoes, F., Pinto, M. M. M. & Nascimento, M. S. J. 2004. Effect of abietane diterpenes from *Plectranthus grandidentatus* on T- and B-lymphocytes proliferation. *Bioorganic and Medicinal Chemistry* 12: 217-223.
- Farag, S. S. & Caliguiri, M. A. 2006. Human natural killer cells development and biology. *Blood Reviews* 20: 123-137.
- Hiramatsu, T., Imato, M., Koyono, T. & Umezawa, K. 1993. Induction of normal phenotypes in Ras transformed cells by damnacanthal from *Morinda citrifolia*. *Cancer Letters* **73**: 161-166.
- Hirazumi, A., Furusawa, E., Chou, S. C. & Hokama, Y. 1994. Anti cancer activity of Morinda citrifolia on intraperitoneally implanted Lewis lung carcinoma in syngenic mice. Proceeding of the Western Pharmacological Society 37: 145-146.
- Hirazumi, A., Furusawa, E., Chou, S. C. & Hokama, Y. 1996. Immunomodulation contributes to the anticancer activity of *Morinda citrifolia* (Noni) fruit juice. *Proceeding of the Western Pharmacological Society* 39: 7-9.
- Hirazumi, A., Furusawa, E., Chou, S. C. & Hokama, Y. 1999. Immunomodulatory polysaccharide-rich substances from the fruit juice of *Morinda citrifolia* (Noni) with anti tumor activity. *Phytotherapic Research* 13: 380-387.
- Hiwasa, T., Arase, Y., Chen, Z., Kita, K., Umezawa, K., Ito, H. & Suzuki, N. 1999. Stimulation of ultraviolet-induced apoptosis of human fibroblast UV-1 cells by tyrosine kinase inhibitors. *FEBS Letters* 444: 173-176.
- Ho, C. Y., Clara, B. S. L., Kim, C. F., Leung, K. N., Fung, K. P., Tse, T. F., Helen, H. L. C. & Moses, S. S. C. 2004. Differential effect of *Coriolus versicolor* (Yunzhi) extract on cytokine production by murine lymphocytes *in vitro*. *International Immunopharmacology* 4: 1549-1557.
- Ismail, N. H., Ali, A. M., Aimi, N., Kitajima, M., Takayama, H. & Lajis, N. H. 1997. Anthraquinones from Morinda elliptica. Phytochemistry 45: 1723-1725.
- Jasril, Nordin, H. L., Lim, Y. M., Abdullah, M. A., Sukari, M. A. & Ali, A. M. 2003. Antitumor promoting and antioxidant activities of anthraquinones isolated from the cell suspension culture of *Morinda elliptica*. *Asia Pacific Journal of Molecular Biology and Biotechnology* **11**: 3-7.
- Kamei, H., Koide, T., Kojima, T., Hashimoto, Y. & Hasegawa, M. 1998. Inhibition of cell growth in culture by quinones. *Cancer Biotherapy and Radiopharmaceuticals* 13: 185-188.
- Konoshima, T., Kozuka, M., Koyama, J., Okatani, T. & Tagahara, K. 1989. Studies on inhibitors of skin tumor promotion, VI. Inhibitory effects of quinines of Epstein-Barr virus activation. *Journal* of Natural Products 52: 987-995.
- Kwanjai, K., Somdej, K. & Ruchanee, P. 2005. Biological activity of Anthraquinones and Triterpenoids from *Prismatomeris fragrans. Journal of Ethnopharmacology* **100**: 184-288.
- Manasroi, A., Saraphanchotiwitthaya, A. & Manosroi, J. 2003. Immunomodulatory activities of Clausena excavata Burm. f. wood extracts. Journal of Ethnopharmacology 89: 155-160

- Maria, G. M., Graciela, F., Maria, L. B. A., Paula, L. & Claudia, A. 2006. Comparative immunomodulatory effect of scopoletin on tumoral and normal lymphocytes. *Life Sciences* 79: 2043-2048.
- Masakazu, K., Raymond, P. W., Dong, S. A., Jonathan, P. S., Robert, D., Micheal, E. P., Jing, H. & Irvin, S. Y. C. 2006. Cell-based chemical genetic screen identifies damnacanthal inhibitor of HIV-Vpr induced cell death. *Biochemical and Biophysical Research Communication* 384: 1101-1106.
- Meena, R. C. & Donna, L. F. 2004. Signalling control of memory T cell generation and function. *Seminar in Immunology* **16**: 285-293.
- Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxic assay. *Journal of Immunology Methods* **65**: 55-63.
- Ong, H. C. & Norzalina, J. 1999. Malay herbal medicine in Gemencheh, Negeri Sembilan, Malaysia. *Fitoterapia* 70: 10-14.
- Shier, W. Thomas. 1991. Mammalian Cell Culture on a day: A lab manual of low cost methods. Nat. Inst. of Biotech. and Appl. Micro. (BIOTECH), University of the Philipines at Los Banos College, Laguna. p. 64-71.
- Stanilova, S. A., Dobreva, Z. G., Slavov, E. S. & Mitera, L. D. 2005. C3 binding glycoprotein from *Cuscuta europea* induced different cytokine profile from human PBMC compared to other plant and bacterial immunomodulators. *International Immunopharmacology* 5: 723-734.
- Tosa, H., Ilnuma, M., Asai, F., Tanaka, T., Nozaki, H. & Fujimori, S. 1998. Anthraquinones from Neonaucle calycina and their inhibitory activity against DNA topoisomerase II. Biological and Pharmaceutical Bulletin 21: 641-642.
- Wang, M. Y. & Su, C. 2001. Cancer preventive effect of Morinda citrofolia (Noni). Annals of the New York Academy of Sciences 952: 161-168.
- Wang, M. Y., West, B., Jensen, C. J., Nowicki, D. S. C., Palu, A. K. & Anderson, G. 2002. Morinda citrifolia (Noni): A literature review and recent advances in Noni research. Acta Pharmacologica Sinica 23: 1127-1141.
- Zhao, M., Yang, B., Wang, J., Liu, Y., Yu, L. & Jiang, Y. 2007. Immunomodulatory and anticancer activities of flavonoids extracted from litchi (*Litchi chinensis* Sonn) pericarp. *International Immunopharmacology* 7: 162-177.