Anti-Cancer Activity of *Tectona hamiltoniana*-An Endemic Plant of Myanmar

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Summary: The ethanolic extracts of barks and leaves of *Tectona hamiltoniana* (Verbenaceae) were tested for anti-cancer activity against MCF-7 (Human breast cancer) and NCI-H460 (Lung cancer) cell lines employing sulphorhodamine B (SRB) bioassay. These extracts demonstrated cytotoxicity with GI_{50} values ranging between 24-33 μg/mL against both cell lines. Upon further fractionation, dichloromethane fraction appeared to be most active against the MCF-7 cell line (GI_{50} value of 3.4±0.9 μg/mL) leading to the isolation of lupane type triterpenoids, betulinic acid (**1**), betulin aldehyde (**2**) and betulin (**3**). Compound **2** and **3**, both showed significant cytotoxic effect against both cancerous cell lines (GI_{50} value range 6-11μM).

Key words: Tectona hamiltoniana; Anti-cancer; Lupane type triterpenoid.

Introduction

There are over 7,000 plants species in Myanmar and many of them have been recognized as medicinal plants [1].Traditional medicine in Myanmar is widely used by about 80% of the population as an alternate to modern medicine. It was enriched by the Myanmar traditions and adaptations throughout the centuries [2].

Tectona is a genus of tropical hard wood trees in the family Verbenaceae, native to the South and Southeast Asia, and is commonly found as a component of monsoon forest vegetation. They are deciduous large trees, growing up to 30-40 m. There are three species: *T. grandis* (common teak wood tree) is the most important, with a wide distribution in India and Indo-China, *T. hamiltoniana* (dahat teak) is an endemic species confined to Myanmar occurring in the dry zones (Prome district and upper Myanmar), where it is declared endangered, and *T. philippinensis* (Philippine teak) is endemic to the Philippines, and is also endangered [3]. The wood of *T. hamiltoniana* is used locally for fuel and construction and barks are used for medicinal purp-se. These species has a small area of distribution and is in need of conservation attention [4].

The World Health Organization estimates that approximately 80% of the world's inhabitants rely on traditional medicine for their primary health care [5]. Cancer is a major public health burden in both developed and developing countries. It was estimated that there were 10.9 million new cases, 6.7 million deaths and 24.6 million persons living with cancer around the world in 2002 [6]. Plants have long been used in the treatment of cancer [7]. Among the cancer patients in the USA, the use of complementary and alternative medicine is represented mainly by plants, ranges between 30-75% [8]. Therefore, the present study was aimed to evaluate the *in vitro* anti-cancer activity of *T. hamiltoniana* extracts.

Results and Discussion

Crude ethanolic extracts (leaves and barks) of *Tectona hamiltoniana*, were tested for their anti-cancer activity against MCF-7 (Breast cancer) and NCI-H460 (lung cancer) cell lines by employing sulphorhodamine B (SRB) bioassay. Both the extracts showed significant inhibitory effects on the cell lines tested. The ethanolic extract of *T. hamiltoniana* barks and leaves inhibited MCF-7 and NCI-H460 cell lines with almost similar GI_{50} values of 24 μg/ mL and 32 μg/ mL, respectively (Table-1). Therefore, ethanolic extract of barks of *T. hamiltoniana* was further partitioned with solvents of increasing polarity. The dichloromethane fraction of *T. hamiltoniana* barks was found to be most active against MCF-7 cell line with GI_{50} value of 3.42±0.91 μg/ mL, followed by petroleum ether extract with GI_{50} value of 17.98±2.64 μg/ mL (Table-2).

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Table-1: The cytotoxic effect of ethanolic extracts of *Tectona hamiltoniana*.

Plant		ExtractYield (% W/W)	Cell lines	GI_{50}*	TGI*	LD_{50}*
Tectona hamiltoniana	Barks	28.3	MCF-7	24.67±2.68	68.67±15.9	211±12
			NCI-H460	33.33±3.33	161.67±5.7	>250
	Leaves	15.6	MCF-7	24.75±2.33	76.67±4.17	129.71±12.42
			NCI-H460	32.52±6.37	91.5±12.35	163.49±10.39

SEM = Standard error of mean, where, n = 3 * **µg/mL(Mean±SEM)**

Table-**2**: The cytotoxic effects of fractions and isolated compounds from *Tectona hamiltoniana.*

Fractions/ Compounds	Cell lines	GI_{50}	TGI	LC_{50}
		µg/ mL (Mean±SEM)		
Petroleum ether	MCF-7	19.09±3.39	71±11.93	>100
	NCI-H 460	28.93±3.36	67.67±11.26	>100
Dichloromethahne	MCF-7	3.25±0.16	9.33±1.86	42.5±4.5
	NCI-H-460	6.35±0.61	16±0.5	28±4.5
Ethyl acetate	MCF-7	39.34±0.88	72.83±3.72	96.25±1.25
	NCI-H460	81.307±7.69	>100	>100
n-Butanol	MCF-7	>100	>100	>100
	NCI- H460	>100	>100	>100
Aqueous residue	MCF-7	>100	>100	>100
	NCI-H460	>100	>100	>100
			µM (Mean±SEM)	
betulinic acid	MCF-7	50.26±1.96	>100	>100
	NCI -H460	>100	>100	>100
Betulinaldehyde	MCF-7	6.04±2.04	13.03±5.01	40.01±9.98
	NCI -H460	10.86±2.82	19.5±1.32	38.86±0.87
Betulin	MCF-7	6.63±1.70	20.12±1.50	39.36±1.63
	NCI -H460	11.17±2.66	30.52±5.58	44.17±1.01

SEM = Standard error of mean, where, n = 3

These results suggested that the main anti-cancer constituents are predominantly residing in dichloromethane fraction. Bioassay-guided isolation of dichloromethane fraction of *T. hamiltoniana* barks led to isolation of three known lupane-type triterpenoids *i.e*, betulinic acid (**1**) [9], betulin aldehyde (**2**) [9] and betulin (**3**) [10]. The structures of these compounds were elucidated by combination of various spectroscopic techniques and by comparing their spectral data with the reported values [9-12]. These results showed that the constituents responsible for cytotoxic activity of crude extracts were hydrophobic in nature [Fig. 1].

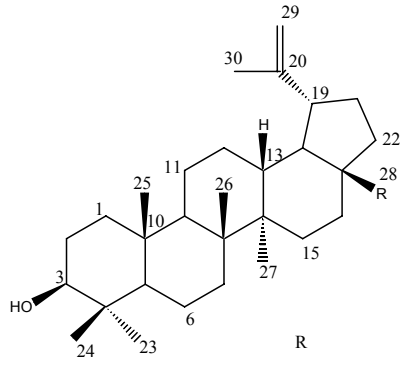

Fig. 1: Isolated lupane-type triterpenes from *T. hamiltoniana.*

It was reported that betulin aldeyde together with lupeol, betulin and betulinic acid have been isolated from the stem bark of other species, *Tectona grandis* [13]. Moreover, 5-hydroxylapachol and lapachol were isolated from the root barks of the *T. grandis*, which were cytotoxic to *Artemia salina* (brine shrimp) showing an IC_{50} of 5 ppm [14]. These findings pointed out that the different species of same genus may have similar phytochemical constituents.

The compounds purified and identified in this study are the first time report from *T. hamiltoniana.* Their cytotoxicity against MCF- 7 and NCI-H460 is shown in Table-2. Many researchers have reported selective anti-tumor activity of betulinic acid against the human melanoma.[15]. Moreover, it also suppresses the growth and apoptosis of other cancer cells, including brain cancer cells, neuroblastomas, ovarian carcinomas and leukemias [16] where as the normal cells such as dermal fibroblasts and pheripheral blood lymphocyts are much less sensitive to growth inhibition by this betulinic acid [17]. In this study, betulinic acid (**1**), demonstrated weak anticancer activity against MCF-7 cell line with GI_{50} value of 50.26±1.96 µM. It failed to show any significant activity towards NCI-H460 cell line. The anti-tumor activity of lupane-derived triterpenoid compounds were first discovered over 20 years ago, when extracts from the stem barks of various plants were tested for cytostatic activity using different *in vivo* cancer model systems.[18, 19] In our study, betulin aldehyde (**2**) was more potent than betulinic acid (**1**) and betulin (**3**) against the MCF-7

and NCI-H460 cell lines with GI_{50} values of 6.047 μM and 10.863 μM respectively. These results indicated that the anti-cancer effects of the isolated compounds were less potent than that of dichloromethane fraction of ethanolic extract of barks. Thus it was favoring the synergistic effect of compounds residing in dichloromethane fraction. Moreover this is the first report of the isolation of these triterpene from *T. hamiltoniana* and further analysis may result in the isolation of more active anticancer compounds. It was concluded the possible potential of *T. hamiltoniana* as anti-cancer agent of plant origin.

Experimental

Reagents and Chemicals

RPMI-1640, fetal calf serum, trypsin-EDTA, glutamine-penicillin-streptomycin solution (GPSS), sulphorhodamine-B, gentamycin sulphate, amphotericin B and doxorubicin were purchased from Sigma chemicals, USA. The solvents and other chemicals used were of analytical grade.

Plant Materials

The plants materials (leaves and barks) were collected from the Mandalay division, Myanmar, during September 2007. The voucher specimen (M-62-07) was deposited at the Herbarium of Myanmar Science and Technological Research Department, Ministry of Science and Technology, Yangon, Myanmar. Air-dried and powdered plant parts (300 gm) were extracted with ethanol by percolation process (500 mL X 3 times) and filtered. The filtrate was concentrated and dried using rotary evaporator and stored at -20 °C till further use.

Preparation of Test Sample

The weighed quantities of samples were dissolved in dimethyl sulphoxide (DMSO) to obtain desired concentration of ethanolic extracts (40 mg/ mL), fractions (20 mg/ mL) and isolated compounds (20 mM) and stored at -20 °C till further use. Prior to experiments respective dilutions were prepared in RPMI.

In vitro C*ytotoxicity Assay*

Two human cancer cell lines, MCF-7 (Human breast cancer cell line) and NCI-H460 (Lung cancer cell line), were grown in RPMI-1640 medium, supplemented with fetal calf serum (10%) and L-glutamine (2 mM). The culture flasks were kept in CO_2 (5%) incubator at 37 °C and 100% relative humidity. The adherent cells were obtained using Trypsin–EDTA solution, followed by addition of washing medium (RPMI supplemented with 1% FCS and 1% GPSS). The cell suspension was centrifuged for 10 minutes at 1000 rpm and the cell pellet was re-suspended in 1 mL of washing medium. The cells are counted by using trypan blue (0.4%) stain in haemocytometer and density of live cells were determined. Sulporhodamine B bioassay was employed for screening of anticancer activity of the plant extracts [20-21]. The MCF-7 or NCI-H460 (100 μL) showing viability above 80% were added into 96-well plates at densities of 75,000-100,000 cells/ well, respectively. The plates were incubated in CO_2 incubator for 24 h prior to addition of tested samples. Aliquots of (100 μL) of different dilutions of test extract and compounds were added to the appropriate wells and incubated for 48 h. Then cells were fixed *in situ* by the gentle addition of 50 μL of cold 50% (w/ v) trichloroacetic acid (TCA) (final concentration, 10%) and incubated for 30 minutes at 25 ^{o}C. The supernatant was discarded, and the plates were washed five times with tap water and air dried overnight. Sulporhodamine B (SRB) solution (100 μL) at 0.4% (w/ v) in 1% acetic acid was added into each well, and plates were incubated for 30 minutes at room temperature. After staining, unbound dye was removed by washing three times with 1% acetic acid and the plates were left to air dry. After 24 hours of drying the bound stain was subsequently solubilized 10 mM trizma base, and the absorbance was noted in microroplate reader (Tecan Sunrise Standard Instrument) at 545 nm. All assays were run in triplicate wells and repeated at least three times. Each concentration was prepared in triplicate and doxorubicin was used as positive control. Using the seven absorbance measurements [time zero, (Tz), control growth (C) and test growth in the presence of drug at five concentration levels (Ti)], the percent cell growth was calculated at each drug concentration. The graph was plotted between drug concentrations and percent cell growth to obtain GI_{50} or IC_{50}, TGI and LC_{50}values.

Bioassay-Guided Fractionation and Isolation of Active Compounds

Crude cytotoxic ethanolic extract of barks of *T. hamiltoniana* (82 gm) (GI_{50}= 24.67±2.68 μg/ mL) was suspended in water and subsequently partitioned with petroleum ether, dichloromethane (DCM), ethyl acetate, *n*-butanol and water. The final portion after all partitioning was the aqueous residue. The

petroleum ether (2.36 gm) (GI_{50}= 19.09±3.39 µg/ mL) and the dichloromethane fraction (8.86 gm) (GI_{50}= 3.25 ±0.16 µg/ mL) displayed the better activity against both of the cell lines tested. Therefore DCM fraction was subjected to repeated column chromatography on silica gel (E. Merck, type 60, 70-230 mesh). The first elution of the column was made with a combination of petroleum ether and DCM (4:1) and then with increasing amount of DCM in petroleum ether and finally, with methanol. One hundred and seventy eight fractions were obtained and the fractions with similar composition were combined to give 15 major fractions monitored by TLC analysis. Fractions 3 and 8 obtained from dichloromethane- petroleum ether gave the betulinaldehyde **2**, (221.2 mg) and betulin **3**, (24.9 mg), receptively. The fraction 15 eluted with DCM-petroleum ether (90:10) was purified by recrystalization with methanol to give betulinic acid (**1**) (5.5 gm).

Betulinic acid (**1**)[9] : White crystals, mp 317-318 ^{o}C, EI MS: *m/z* 456; ^{1}H NMR (300 MHz, $CDCl_3$): 0.61 (1H, d, *J* = 9.7 Hz, H-5), 0.67 (3H, s, H-26), 0.74 (3H, s, H-25), 0.85 (3H, s, H-24), 0.87 (3H, s, H-23), 0.89 (3H, s, H-27), 1.60 (3H, s, H-30), 0.8-2.18 (23H), 2.93 (1H, m, H-19), 3.31 (1H, bs, H-3), 4.51 (1H, br s, H-29a), 4.64 (1H, br s, H-29b); ^{13}C-NMR (75 MHz, $CDCl_3$): 178.9 (C-28), 150.5 (C-20), 109.2 (C-29), 78.5 (C-3), 56.0 (C-17), 55.1 (C-5), 50.3 (C-9), 48.9 (C-19), 46.7 (C-18), 42.2 (C-14), 40.4 (C-8), 38.5 (C-1), 38.1 (C-13), 38.0 (C-4), 37.3 (C-10), 36.9 (C-22), 34.1 (C-7), 32.0 (C-16), 30.3 (C-15), 29.4 (C-21), 27.6 (C-23), 26.7 (C-2), 25.3 (C-12), 20.6 (C-11), 18.9 (C-30), 18.0 (C-6), 15.8 (C-26), 15.6 (C-25), 15.0 (C-24), 14.3 (C-27).

Betulin aldehyde (**2**)[9] : White crystals, mp 188-190 ^{o}C, EI MS: *m/z* 440; ^{1}H NMR ($CDCl_3$, 300 MHz): 0.66 (1H, d, *J* = 9.0 Hz, H-5), 0.72 (3H, s, H-25), 0.79 (3H, s, H-26), 0.94 (3H, s, H-24), 0.95 (3H, s, H-23), 1.23 (3H, s, H-27), 1.67 (3H, s, H-30), 0.8-2.0 (23H), 2.81 (1H, m, H-19), 3.16 (1H, dd, *J* = 11.0 Hz, 5.2 Hz, H-3), 4.60 (1H, br s, H-29a), 4.73 (1H, br s, H-29b) and 9.6 (1H, s, H-28); ^{13}C-NMR ($CDCl_3$, 75 MHz): 206.7 (C-28), 149.7 (C-20), 110.1 (C-29), 78.9 (C-3), 59.3 (C-17), 55.2 (C-5), 50.4 (C-9), 48.0 (C-18), 47.5 (C-19), 42.5 (C-14), 40.8 (C-8), 38.8 (C-4), 38.7 (C-13), 38.6 (C-1), 37.1 (C-10), 34.3 (C-7), 37.2 (C-22), 29.8 (C-21), 29.2 (C-15), 32.5 (C-16), 28.7 (C-2), 27.9 (C-23), 25.5 (C-12), 20.7 (C-11), 18.9 (C-30), 18.2 (C-6), 16.1 (C-26), 15.8 (C-25), 15.3 (C-24), 14.2 (C-27).

Betulin (**3**)[10] : White amorphous powder, mp 251-252 ^{o}C, EI MS: *m/z* 442; ^{1}H-NMR ($CDCl_3$, 300 MHz): 0.66 (1H, d, *J* = 9.3 Hz, H-5), 0.74 (3H, s, H-24), 0.80 (3H, s, H-25), 0.95 (3H, s, H-23), 0.96 (3H, s, H-27), 1.00 (3H, s, H-26), 1.66 (3H, s, H-30), 0.85-1.9 (23H), 2.36 (1H, m, H-19), 3.16 (1H, dd, *J* = 10.8, 5.1 Hz, H-3), 3.32 (1H, d, *J* = 10.8 Hz, H-28a), 3.79 (1H, d, *J* = 10.8 Hz, H-28b),4.56 (1H, br s, H-29a), 4.66 (1H, br s, H-29b); ^{13}C-NMR ($CDCl_3$, 75 MHz): 150.4 (C-20), 109.7 (C-29), 78.9 (C-3), 60.5 (C-28), 55.2 (C-5), 50.4 (C-9), 48.0 (C-18), 47.8 (C-19), 47.8 (C-17), 42.7 (C-14), 40.9 (C-8), 38.8 (C-4), 38.7 (C-1), 37.3 (C-13), 37.1 (C-10), 34.2 (C-7), 33.9 (C-22), 29.7 (C-21), 29.1 (C-16), 27.9 (C-23), 27.3 (C-15), 27.0 (C-2), 25.2 (C-12), 20.8 (C-11), 19.0 (C-30), 18.3 (C-6), 16.1 (C-26), 15.8 (C-25), 15.3 (C-24), 14.7 (C-27).

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