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Full Length Research Paper

Cytotoxicity evaluation of five selected Malaysian Phyllanthaceae species on various human cancer cell lines

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The present study was undertaken to evaluate the cytotoxic activities of the crude methanol, hexane and ethyl acetate extracts of medicinal plants, *Phyllanthus niruri*, *Phyllanthus pectinatus, Phyllanthus acidus, Phyllanthus roseus* and *Baccaurea motleyana.* Cytotoxic activities were screened by an *in vitro* assay system of growth inhibition against four human cancer cell lines, namely breast cancer cell line (MCF7), epidermal carcinoma of cervix cell line (CaSki), ovarian cancer cell (SKOV3) and colon cancer cell line (HT29). Extracts which exhibited IC_{50} value less than 20.0 µg/ml, were considered active. Crude methanol and crude ethyl acetate extracts of *P. pectinatus* (leaves) were the most active on SKOV3 cell with an IC_{50} value of 4.8 ± 1.04 and 5.8 ± 0.76 µg/ml, respectively. Crude ethyl acetate extract of *P. pectinatus* (fruit) exhibited potent cytotoxicity with an IC_{50} value of 18.1 ± 0.66 and 19.4 ± 0.53 µg/ml, when tested against MCF7 and CaSki cells, respectively. This study revealed that *P. pectinatus* may have a great potential to be exploited for the search of anticancer drug.

Key words: Cytotoxicity, Malaysia, Phyllanthaceae, *Phyllanthus*, *Baccaurea*.

INTRODUCTION

Since the beginning of life, plants have served humankind as source of fuels, foods, clothing, shelter and medicines. Plants contain numerous chemicals as a result of their natural metabolic activities. These chemicals may be essential for plant growth or as by-products of its metabolism; they may also be potentially useful as food or as medicine (Jamia, 2006). Until today, approximately 60% of the world's population still relies almost entirely on plants for medication (Harvey, 2000) and it continue to provide mankind with new remedies.

Plant derived natural products have been holding a great promise in providing invaluable compounds of starting points for the development of new potential drugs. In recent years, there has been a great deal of attention and discoveries in exploiting plant kingdom for pharmaceutical application and the interest in plants as a source of potential therapeutic agents, particularly as anticancer agents continues with higher plants contributing not less than 25% of the total natural product drugs in clinical use in the world (Harvey, 2000; Rocha et al., 2001; Newman et al., 2003; Cragg and Newman, 2005; Fakim, 2006; Agarwal and Shishodia , 2006; Mc Chesney et al., 2007).

There are approximately 300,000 species of higher plants in the terrestrial habitats of the world (Samy et al., 2005; McChesney et al., 2007), and 10,000 of this plants documented to have medicinal uses (McChesney et al., 2007). It is estimated that Southeast Asian tropical rain forests supported some 6,500 medicinal plant species with 1800 plant species found in the Malaysian rain forests and 10% of them reported to have some medicinal value (Samy et al., 2005; Fakim, 2006). As Malaysia is rich in natural resources, plants have played a major role and appreciated for treating illness in Malaysian traditional medicines for many years (Muhamad and Mustafa, 1998). Many modern drugs with plant origin have been discovered following folklore claims of their efficacy combined with the extensive ethnobotanical knowledge of local peoples.

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Phyllanthaceae species are widely distributed in most tropical and subtropical countries consisting about 2000 species with 60 genera (Samuel et al., 2005). Phyllanthaceae species have long been extensively used in folk medicine in most countries for thousand of years including Malaysia, in the treatment of a broad spectrum of diseases, such as disturbances of the kidney and urinary bladder, intestinal infections and diabetes (Kumaran and Karunakaran, 2007), and reported to have pharmacological effects such as antiinflammatory, analgesic, HIV-1 reverse transcriptase inhibitor, antineoplastic activity, antihepatoxic, mutagenic effect in bacterial and antiallergic (Calixto et al., 1998).

Therefore, the present study was carried out to investigate the cytotoxic potential of five medicinal plants of Phyllanthaceae namely, *Phyllanthus niruri, Phyllanthus pectinatus*, *Phyllathus acidus*, *Phyllanthus roseus* and *Baccaurea motleyana* on four different human cancer cell lines.

MATERIALS AND METHODS

Plant materials

The relevant parts of the Phyllanthaceae species were collected from different part of Peninsular Malaysia during the month of October 2007. *P. niruri* (aerial part), *P. acidus* (leaves) and *B. motleyana* (leaves) were collected from a home garden at Port Dickson, *P. pectinatus* (leaves and fruits) was collected from Rimba Ilmu, University of Malaya and *P. roseus* was collected from Gunung Machincang, Langkawi Island. Authentication of *P. niruri (*KLU 42610), P. pectinatus (KLU 47659), *P. acidus* (KLU 47679), *P. roseus* (KLU 47660) and *B. motleyana* (KUL 30038) was carried out in the herbarium of the Rimba Ilmu Botanical Garden, Institute of Biological Sciences, University of Malaya and voucher material for this study was deposited at the same herbarium. A list of related work for the species of plants studied was shown in Table 1.

Preparation of crude extracts from Phyllanthaceae spp.

The leaves, aerial part and fruit of selected Phyllanthaceae spp. were cleaned immediately to remove any extraneous material, sliced and dried in a hot-air oven (Memmert) at 40 to 50°C. The dried materials were ground into a powder and soaked in methanol with ratio 1:10 for 3 days at room temperature with shaking. The solvent-containing extract was then decanted and filtered. The extraction of the ground sample was further repeated (3x) in a methanol with 1:10 ratio each time. The filtrate from each extraction combined and the excess solvent evaporated under reduced pressure using a rotary evaporator (Buchi, Switzerland) to give a dark-greenish extract. The remaining portion of methanol extract was further shaken vigorously with hexane. Hexane soluble obtained were poured into a clean flask and this step was repeated with fresh hexane until the resultant hexane added remains almost colorless after shaking. Remaining hexane insoluble was subjected to solvent-solvent extraction with mixture of ethyl acetate and distilled water (1:1) followed by fairly vigorous mixing. This mixture was then successively fractionated using separating funnel in which two distinct layers were formed. The water residue (bottom layer) was released and discarded while the ethyl acetate phase (top layer) was released into a clean beaker. Resultant filtrate was concentrated under reduced pressure using a rotary evaporator and yielded crude hexane and ethyl acetate extract of each plant. All the crude extracts were weighed and dissolved in dimethylsulfoxide (DMSO) (Sigma, USA), to form stock solutions of 20 mg/ml prior the assay and kept in desiccators.

Cell culture

Human breast cancer cell line (MCF7), ovarian cancer cell line (SKOV3), epidermal carcinoma of cervix cell line (CaSki), colon cancer cell line (HT29), and normal lung fibroblast cell (MRC5) were purchased from American Type Culture Collection (ATCC, USA). MCF7, CaSki and HT29 cells were cultured in RPMI 1640 media (Sigma, USA); SKOV3 in DMEM media (Sigma, USA) and MRC5 in MEM media (Sigma, USA) supplemented with 10% v/v foetal bovine serum (PAA Lab., Austria) as a complete growth media. Cells were maintained in 25 cm^3 flasks with 10 ml of media and were incubated at 37°C in an incubator with 5% CO_2 in a humidified atmosphere (ShelLab.). The culture was subcultured every 2 or 3 days and routinely checked under an inverted microscope (Leica DMI 3000B) for any contamination.

Neutral red cytotoxicity assay

The neutral red cytotoxicity assay is based on the initial protocol described by Borenfreund and Puerner (1984). Cells were detached from the flask with 1.0 ml solution of accutase (iCT, CA) in phosphate buffer solution (PBS) pH 7.4. The cell pellet was obtained by centrifugation at 1000 rpm for 5 min (Kubota 2420, Tokyo) and the density of the viable cells was counted by 0.4% of tryphan blue exclusion method using a haemocytometer. Cells were then plated in 96-well microtiter plate, at a concentration of 30 000 cells/ml. The plate was incubated in a CO_2 incubator at 37°C for 3 h to allow the cells to adhere before addition of the test agents. After 3 h, the crude extracts were then added to the wells at six different concentrations of 1, 10, 25, 50, 75 and 100 µg/ml of each crude extract. The cells were incubated with the extracts for 72 h. Negative control was the well containing untreated cells (without addition of any extract).

At the end of the incubation period, the media was replaced with medium containing 50 µg/ml neutral red. The plates were incubated for another 3 h to allow for uptake of the vital dye into the lysosomes of viable and uninjured cells. After the incubation period, the media was removed and cells were washed with the neutral red washing solution. The dye was eluted from the cells by adding 200 µl of neutral red resorb solution and incubated them for 30 min at room temperature with rapid agitation on a microtiter plate shaker (LT BioMax 500). The optical density (OD) was measured at 540 nm using microplate reader (Emax, Molecular Devices). Three replicate plates were used to determine the cytotoxicity activity of each extract. The percentage of inhibition of each of the test samples was calculated according to the following equation: % of inhibition = $(OD_{control} - OD_{sample}) / (OD_{control}) \times 100\%$. The IC_{50} is the concentration of extract that causes 50% inhibition or cell death (Chian get al., 2003).

Statistical analysis

Data are presented as mean ± S.D. IC_{50} for each extract was extrapolated from the graphs plotted using the OD values obtained.

RESULTS AND DISCUSSION

In our search for plant derived natural products with cytotoxic activity, we prepared crude methanol, hexane

Table 1. A list of related work for the species of plants studied.

Botanical name	Local name	Cited medicinal uses or related scientific studies
P. niruri L. Ridley	Dukong anak, dukong-dukong anak, Amin buah, Rami buah, Turi hutan and Meniran (Burkill, 1966).	Poultices for skin complaints, including caterpillar itch. Diarrhoea, kidney trouble, gonorrhea, and syphilis; and after a miscarriage and childbirth; and for coughs (Burkill, 1966). Lower hypertension and purify the blood. Wounds healing and scurf. To cure jaundice (Muhamad and Mustafa, 1998). General tonic after childbirth (Ong and Norzalina, 1999). Lipid lowering activity (Khanna et al., 2002). *In vitro* antiplasmodial activity (Sholikhah et al., 2004). Treatment of hepatitis. Inhibition of membrane lipid peroxidation, scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and inhibition of reactive oxygen species (ROS) *in vitro* (Harish and Shivanandappa, 2006). *In vitro* antioxidant and *in vivo* hepatoprotective activity (Sabir and Rocha, 2008). Antihyperuricemic effect (Murugaiyah and Chan, 2009).
P. pectinatus Hooker	Pokok Melaka, Pokok laka (Ani and Faizal, 2003).	Astringent (Salleh, 1997). [Locals treated *P. pectinatus* as a synonym of *P. emblica* (both locally known as Pokok Melaka), which scientifically proven incorrect. A lot of studies also had been carried out in Malaysia on pharmaceutical aspect, but their studies are often on the wrong tree, published under the wrong name (Ng, 2000)].This is the first report on the cytotoxicity of *P. pectinatus*.
P. acidus L. Skeels	Cermai (Engel and Phummai, 2000).	Relief fever with skin manifestations (Engel and Phummai, 2000). Tonic to relief body ache (Kamarudin and Latiff, 2002). Cystic fibrosis treatment (Sousa et al*.,* 2007). Antimicrobial inhibitory activity (Jagesar et al., 2008). Hypotensive activity (Leeya et al., 2010).
P. roseus (Craib and Hutch.) Beille	Labu Kuncing (Khazanah Endau Rompin Herba, 2007).	*In vitro* antiherpes simplex virus type 1 (HSV-1) (Akanitapichat et al., 2005). Increase the male sexual drive (Khazanah Endau Rompin Herba, 2007).
B. motleyana Muell. Arg.	Rambai or rambeh (Burkill, 1966).	Fruits are mainly for home consumption (Chin, 1986). Antimicrobial activity (Suhaila et al., 1994). Treatment of sore eye (Werner, 2002).

and ethyl acetate extracts from five selected Phyllanthaceae species grown in Malaysia. Phyllanthaceae species used for extract preparation are *P. niruri, P.hyllanthus pectinatus* (leaves and fruits), *P. acidus, P. roseus* and *B. motleyana*. Several reasons contribute to the selection of these plants in our studies, such as: (1) their greater distribution in many tropical and subtropical countries; (2) the great number of species in this genus; (3) their

Broad medicine; and (4) the greater diversity of secondary metabolites present in these plants (Kumaran and Karunakaran, 2007).

Complete dose-response curves were generated and IC_{50} values were calculated for these crude extracts (Table 2) against four human cancer cell lines and one human normal cell line. The IC_{50} values shown in Table 2 clearly indicated that the cytotoxic potency of the Phyllanthaceae species was as follows: *P. pectinatus* (leaves) > *P. niruri* > *P. roseus* > *P. pectinatus* (fruits) > *P. acidus* > *B. motleyana*. The cytotoxic effect of the Phyllanthaceae crude extract was more pronounced towards MCF7 cells followed by CaSki, SKOV3 and HT29 cells. This may be due to the different in molecular characteristic of the cells (Verma et al., 2008).

According to Geran et al. (1972), a crude extract having an IC50 value equal to or less than 20 µg/ml is considered active. Crude ethyl acetate extract of *P. pectinatus* (fruit) exhibited a potent cytotoxicity with IC50 values of 18.1±0.66 µg/ml, when tested against MCF7 cells. *P. roseus* crude extracts in methanol, hexane and ethyl acetate possessed mild cytotoxic activities with IC50 values of 40.0 ± 5.22, 54.3 ± 8.25 and 24.7 ± 5.58 µg/ml on MCF7 cells. Crude methanol and ethyl acetate extract of *P. pectinatus* (leaves) exhibited the strongest cytotoxicity on SKOV3 cells with IC50 values of 4.8 ± 1.04 and 5.8 ± 0.76 µg/ml, respectively. On CaSki cells, crude ethyl acetate extract of *P. pectinatus* (fruit) exhibited relatively higher cytotoxicity with IC50 value of 19.4 ± 0.53 µg/ml. Crude ethyl acetate extracts of *P. niruri, P. pectinatus* (leaves) and *P. acidus*; and crude hexane extract of *P. roseus* showed a mild cytotoxic activity on CaSki cells with IC50 values range from 43.8 ± 3.21 to 59.2 ± 12.29 µg/ml. On HT29 cells, crude methanol and ethyl acetate extracts of P. pectinatus (leaves) were the most active with IC50 values of 39.1±8.75 and 28.2±3.21 µg/ml, respectively. Crude extracts of *B. motleyana* did not show significant cytotoxic activity in the screening and considered not active.

Overall, the crude extract of Phyllanthaceae species studied exhibited IC50 value more than 100.0 µg/ml on normal MRC5 cells, indicating that the crude extract is not deleterious to non-cancerous cells. These results support the use of these medicinal plant in treating kidney and urinary bladder disturbances, sexual diseases, skin manifestation, wound healing, inflammation, diabetes and hepatitis B (Burkill, 1966; Calixto et al., 1998; Khatoon et al., 2006; Kumaran and Karunakaran, 2007). Inflammation (Coussens and Werb, 2002); hepatitis B (Perz et al., 2006), skin manifestation (Braverman, 2002); gonorrhoea and syphilis (Hayes et al., 2000) have been reported to be associated with several cancer and this clearly indicate the chemoprevention potential of *Phyllanthus* species. There have been a number of reports on the potential of the extracts and compounds derived from *Phyllanthus* in suppressing experimental carcinogenesis in various organs and cells such as liver cells (Jeena et al., 1999), lung carcinoma (Huang et al., 2006), macrophages (Kiemer et al., 2003), uterine carcinoma and gastric adenocarcinoma (Zhang et al., 2004), breast, colon and liver cancer cell lines (Rajkapoor et al., 2007); and ovarian cells (Sanchez-Lamar et al., 1999).

The ethyl acetate extract tend to be more active than the methanol and hexane extract. Different classes of organic compounds of medicinal interest have been isolated and characterized from *Phyllanthus*, including alkaloids, flavonoids, lactones, steroids, terpenoids, lignans and tannins (Liu and McIntosh, 2001; Calixto et al., 1998). These compounds may be responsible for the cytotoxicity actions reported in relation to these plants extract (Liu and McIntosh, 2001).

In the present work, the strongest cytotoxic activities were detected from the crude extract of *P. pectinatus*. Phytochemical report revealed the presence of monoterpene, triterpene, lignan and flavonone in *P. pectinatus* (Ong et al., 2009). A number of dietary monoterpenes have been reported to possess antitumor activity, exhibiting not only the ability to prevent the formation or progression of cancer, but the ability to regress existing malignant tumours (Crowell and Gould, 1994). Triterpenoids have been shown to possess anti inflammatory and anti carcinogenic properties (Manez et al., 1997). Several types of triterpenoids were isolated from different part of *Phyllanthus* species such as lupane and oleanane from the bark of *Phyllanthus flexuosus* (Wada et al., 2001) and dichapetalin from the aerial parts of *Phyllanthus acutissima* (Tuchinda et al., 2008) which showed remarkable cytotoxic activity.

Previous phytochemical studies of *P. niruri* extracts revealed the presence of acyclic triterpene (Singh et al., 1989); phenolic compounds (De Souza et al., 2002); flavonoids, glycosides and tannins (Rajeshkumar et al., 2002); and lignans (phyllanthin, hypophylanthin, phyltetralin and niranthin) (Murugaiyah and Chan, 2007). Previous study showed that 7'-hydroxy-3',4',5,9,9'-pentamethoxy-3,4-methylene dioxy lignan from *P. urinaria* was capable of inhibiting telomerase activity and also could inhibit bcl2 and activate caspase 3 and caspase 8 whose significance in the induction of apoptosis (Giridharan et al., 2002) and lignans-rich fraction and the lignans nirtetralin, niranthin or phyllanthin isolated from *Phyllanthus amarus* exerted cytotoxic effects on K-562 leukaemia cells (Leite et al., 2006); Leeya et al. (2010) isolated adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic acid and kaempferol from the n-butanol extract from leaves of *P. acidus*. Phyllanthusol A and B glycosides, isolated from the root of *P. acidus* have been reported to exhibit cytotoxic activity on KB nasopharyngeal cell lines (Vongvanich et al., 2000). The phytochemistry and cytotoxic activity of *P. roseus* are completely unexplored. *B. motleyana* has not been previously investigated for cytotoxicity but only weak activity was observed in this study.

Table 2. IC_{50} values of crude extracts of selected Phyllanthaceae species on human cell lines.

Plant species	Part of plant tested	Extraction solvent	IC_{50} values[a] (µg/ml) in different cell lines[b]				
			MCF7	SKOV3	CaSki	HT29	MRC5
P. niruri	Aerial part	MeOH	61.7 ± 3.94	40.8 ± 8.29	84.0 ± 4.77	95.5 ± 2.65	98.8 ± 3.33
		Hexane	75.0 ± 1.73	50.5 ± 5.63	>100	98.2 ± 17.96	>100
		EtOAc	31.1 ± 2.72	33.3 ± 1.61	52.8 ± 0.58	79.7 ± 3.75	>100
P. pectinatus	Leaves	MeOH	60.2 ± 1.76	4.8 ± 1.04	95.0 ± 2.00	39.1 ± 8.75	>100
		Hexane	>100	52.3 ± 8.84	>100	>100	>100
		EtOAc	50.5 ± 4.09	5.8 ± 0.76	43.8 ± 3.21	28.2 ± 3.21	>100
P. pectinatus	Fruits	MeOH	51.0 ± 2.65	>100	73.3 ± 2.84	>100	>100
		Hexane	>100	>100	>100	>100	90.5 ± 13.26
		EtOAc	18.1 ± 0.66	69.0 ± 15.76	19.4 ± 0.53	61.5 ± 5.77	>100
P. acidus	Leaves	MeOH	>100	88.0 ± 5.97	>100	>100	>100
		Hexane	96.8 ± 7.11	80.8 ± 8.33	83.3 ± 8.25	>100	>100
		EtOAc	44.2 ± 7.82	67.8 ± 3.55	55.5 ± 14.76	66.5 ± 7.05	>100
P. roseus	Leaves	MeOH	40.0 ± 5.22	>100	>100	60.0 ± 6.54	>100
		Hexane	54.3 ± 8.25	>100	59.2 ± 12.29	58.7 ± 8.39	63.0 ± 4.36
		EtOAc	24.7 ± 5.58	>100	83.0 ± 11.26	89.0 ± 10.15	>100
B. motleyana	Leaves	MeOH	60.6 ± 2.80	>100	>100	60.3 ± 5.20	>100
		Hexane	93.5 ± 5.27	>100	>100	>100	>100
		EtOAc	89.0 ± 3.12	>100	>100	>100	>100

[a] Crude extract with IC_{50} value ≤ 20 µg/ml considered active (Geran et al, 1972).[b] MCF7 (breast cancer); SKOV3 (ovarian cancer); CaSki (cervical cancer); HT29 (colon cancer) and MRC5 (normal lung fibroblast).

Conclusion

Cytotoxicity screening models provide important preliminary data to help to select medicinal plant with potential anticancer properties for future work. Isolation, identification, structural analysis and activity verification of the extracts of Phyllanthus species by bioassay-guided protocol is necessary in order to isolate bioactive secondary metabolites with cytotoxic properties. Further study on the detailed mechanisms of extracts on human cancer cell lines studied may lead to the development of a traditional anti cancer herb.

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