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Antiviral and Cytotoxic Activities of Some Plants Used in Malaysian Indigenous Medicine

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ABSTRAK

Ekstrak etanol 61 tumbuhan perubatan yang digunakan di Malaysia telah disaring untuk aktiviti antivirus and sitotoksik. Aktiviti antivirus telah diuji terhadap virus "herpes simplex"-jenis 1 (HSV-1), dan "vesicular stomatitis" (VSV), dan ujian sitotoksik dijalankan menggunakan sel-sel HeLa. Ekstrak Calotropis gigantea, Costus speciosus, Eugenia michelii, Hedyotis auricularia, Mentha arvensis, Orthosiphon aristatus, Polygonum minus *dan* Ricinus communis *menunjukkan aktiviti perencatan terhadap kedua-dua virus (MIC: 0.002-0.1 mg/ml). Aktiviti antivirus khusus terhadap virus HSV-1 telah ditunjukkan oleh ekstrak* Alternanthera sessilis, Blumea chinensis, Eleusine indica, Euphorbia hirta, Freycinetia malaccensis, Leea indica *dan* Solanum americanum *(0.001-0.1 mg/ml). Ekstrak* Acalypha indica, Bertholletia excelsa, Cerbera manghas, Codiaeum variegatum, Plectranthus amboinicus, Centella asiatica, Mirabilis jalapa, Morinda elliptica, Oenanthe javanica, Piper sarmentosum *dan* Premna odorata *menunjukkan aktiviti antivirus khusus terhadap virus VSV (MIC: 0.005-0.1 mg/ml). Aktiviti sitotoksik pula hadir dalam ekstrak* Acalypha indica, Andrographis paniculata, Centella asiatica, Cerbera manghas, Codiaeum variegatum, Cosmos caudatus, Elephantopus scaber, Etlingera elatior, Eugenia michelii, Freycinetia malaccensis, Hibiscus rosa-sinensis, Lecythis ollaria, Mentha arvensis, Mirabilis jalapa, Morinda elliptica, Ocimum tenuiflorum, Piper sarmentosum *dan* Polygonum minus *(CD_{50}: 0.001-0.1 mg/ml). Kedua-dua aktiviti antivirus and sitotoksik ditunjukkan oleh ekstrak* Eugenia michelii, Mentha arvensis *dan* Polygonum minus.

ABSTRACT

Ethanolic extracts of 61 medicinal plants used in Malaysia were screened for antiviral and cytotoxic activities. Antiviral activity was tested against the herpes simplex type-1 (HSV-1) and vesicular stomatitis (VSV) viruses, and cytotoxicity was assayed using the HeLa cell line. Antiviral activity against both viruses was present in the extracts from Calotropis gigantea, Costus speciosus, Eugenia michelii, Hedyotis auricularia, Mentha arvensis, Orthosiphon aristatus, Polygonum minus *and* Ricinus communis *(MIC: 0.002-0.1 mg/ml). The extracts from* Alternanthera sessilis, Blumea chinensis, Eleusine indica, Euphorbia hirta, Freycinetia malaccensis, Leea indica *and* Solanum americanum *were active in selectively inhibiting HSV-1 (0.001-0.1 mg/ml). Selective activity against VSV was shown by the extracts from* Acalypha indica, Bertholletia excelsa, Cerbera manghas, Codiaeum variegatum, Plectranthus amboinicus, Centella asiatica, Mirabilis jalapa, Morinda elliptica, Oenanthe javanica, Piper sarmentosum *and* Premna odorata *(MIC: 0.005-0.1 mg/ml). Cytotoxic activity was present in the extracts from* Acalypha indica, Andrographis paniculata, Cerbera manghas, Codiaeum variegatum, Cosmos caudatus, Elephantopus scaber, Etlingera elatior, E ugenia michelii, Freycinetia malaccensis, Hibiscus rosa-sinensis,

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Centella asiatica, Lecythis ollaria, Mentha arvensis, Mirabilis jalapa, Morinda elliptica, Ocimum tenuiflorum, Piper sarmentosum *and* Polygonum minus *(CD50: 0.001-0.1 mg/ml).Co-existing antiviral and cytotoxic activities were shown by* Eugenia michelii, Mentha arvensis *and* Polygonum minus.

INTRODUCTION

Plants are an important source of therapeutics from which 25% of the pharmaceuticals in current use have been derived (Farnsworth and Bingel 1977). However, of the estimated 250,000 species of higher plants existing throughout the world, only a fraction have been examined for pharmacological activities (Balick 1990). Phytotherapeutics exhibit a wide range of pharmacological activities, including anticancer and antiviral activities (Farnsworth and Kaas 1981; Hudson 1989). Anticancer drugs, such as the indole alkaloids vincristine and vinblastine, and podophyllotoxin derivatives etoposide and teniposide, are prominent chemotherapeutics of plant origin which were obtained either directly through isolation or derived from lead structures (Arcamone *et al.* 1980). Therefore, the screening of higher plants for antiviral and anticancer agents has been actively pursued on an international scale, especially by the US National Cancer Institute (Farnsworth and Kaas 1981; Hudson 1989). Furthermore, mammalian cell culture systems have greatly aided the routine screening of plant extracts and compounds for anticancer activity using cytotoxicity and antiviral activity, which previously relied upon time-consuming, expensive and cumbersome *in-vivo* models. These screening efforts have resulted in the discovery of several prospective antiviral and anticancer compounds currently undergoing clinical trials. Taxol is the most notable example of these compounds (Wiernik *et al.* 1987).

Although extensive phytochemical surveys have been carried out on the flora of Malaysia (Goh *et al.* 1993 and references cited therein), only a few reports deal with screening for pharmacological activities such as antimicrobial, antitumour, antitumour-promoting and cardiovascular-related activities (Nakanishi *et al.* 1965; Yadav *et al.* 1989; Ali *et al.* 1995; Goh *et al.* 1995). Reports by Teo *et al.* (1990) and references cited therein, Ahmad *et al.* (1992, 1993), Chan *et al.* (1992), Kashman *et al.* (1992), Mahmud *et al.* (1993), Patil *et al.* (1993), Alias *et al.* (1995), Ali *et al.* (1996), and Wong and Tan (1996) are examples of studies confined to the antiviral and cytotoxic activities of extracts and compounds from one or two plant species; consequently, these studies cannot be considered as screening reports.

In the present work we screened 61 local and introduced plant species widely used as anti-infective and anticancer agents in Malaysian indigenous medicine (traditional, ethno- and folk-medicine) for antiviral and cytotoxic activities. We adopted an ethnopharmacological approach to screening because it is more likely to yield a higher number of plants with significant biological activity than screening by random selection (Balick 1990). The ethanolic extracts of these medicinal plants were tested for antiviral activities against both herpes simplex type-1 (DNA type) and vesicular stomatitis (RNA type) viruses using Vero cells, and the cytotoxicity assay was done using the HeLa (human cervical carcinoma) cell line.

MATERIALS AND METHODS

Plants

Plant parts were collected from the Medicinal Plant Garden, Universiti Pertanian Malaysia, and identified by A. Ghani Yunus.

Plant Extracts

Samples (20 mg) of leaves from each plant (and fruits from *Cerbera manghas*) were sliced into small pieces (*ca.* 1 cm × 1 cm) and macerated in 60 ml of 80% (v/v) ethanol. After being left for one week at room temperature, the extracts were filtered using Whatman No. 1 filter paper and then evaporated at 40°C under vacuum. The residues were then stored as stock solutions of 10 mg/ml in 90% (v/v) ethanol at 4°C.

Cultivation of Cells

Vero and HeLa cell lines were obtained from the RIKEN Cell Bank, Tsukuba, Japan and cultured in RPMI-1640 medium supplemented with 5% (v/v) foetal calf serum (FCS), 100 IU/ml penicillin and 100 µg/ml streptomycin as a complete growth medium (CGM). Cells were maintained in 25 cm^2 flask with 10 ml of CGM at 37°C with 5% (v/v) CO_2 until attaining confluence. Confluent cells were removed from the surface of the flask by treatment with 1 ml of 0.025% (w/v) trypsin prepared in phosphate-buffered saline (PBS) solution. CGM was then added to the trypsin-treated cells to achieve a cell concentration of 1-2 × 10^4 cells/ml.

Virus Stocks

Herpes simplex virus type-1 (HSV-1) and vesicular stomatitis virus (VSV), which are DNA and RNA virus type respectively, were obtained from the Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minnesota, USA. Virus stocks were prepared as aliquots of culture medium from Vero cells infected at a multiplicity of infection of 0.1 and cultured for 3 days at 37°C. These aliquots were subsequently stored at -70°C. Working stocks of virus were prepared by serially diluting in culture medium (RPMI-1640) virus stocks to the end-points required for each virus. Serial dilutions of virus stocks in RPMI-1640 medium were assayed to their end-points using Vero monolayers in microtitre plates. These virus working stocks were stored at 4°C until further use.

Antiviral Assay

The antiviral test was performed according to the simplified plaque reduction assay (Abou-Karam and Shier 1990). Microtitre plates with confluent monolayer cultures of Vero cells were inverted to remove spent medium. In triplicate, each well was filled with 100 µl of plant extract serially diluted in RPMI-1640 medium. This was followed by the addition of 100 µl of medium containing *ca.* 30 plaque forming units (pfu) of HSV-1 or 10 pfu of VSV, per well of confluent Vero cells. In each plate, wells in the last row were used for controls, which consisted of two treatments: (1) cells not treated with plant extracts and virus, and (2) cells treated only with virus. The plates were incubated for 66 h (HSV-1) and 36 h (VSV) at 37°C, with care taken not to disturb the culture during incubation. Antiviral activity was then scored using an inverted microscope (low power) as the non-cytotoxic minimum inhibitory concentration (MIC, mg/ml) which totally prevented cytopathic effects (CPE).

Cytotoxicity Assay

The assay used was the microtitration cytotoxicity assay (Shier 1983). Varying concentrations of the plant extracts were prepared from the stock solutions by serial dilution in RPMI-1640 medium to give a total volume of 100 µl in each well. Each well was filled with 100 µl of HeLa cell suspension in CGM at 1-2 × 10^4 cells/ml. Controls containing only HeLa cells were included for each sample. The assay for each concentration of plant extract was performed in triplicate and the culture plates were kept at 37°C with 5% (v/v)

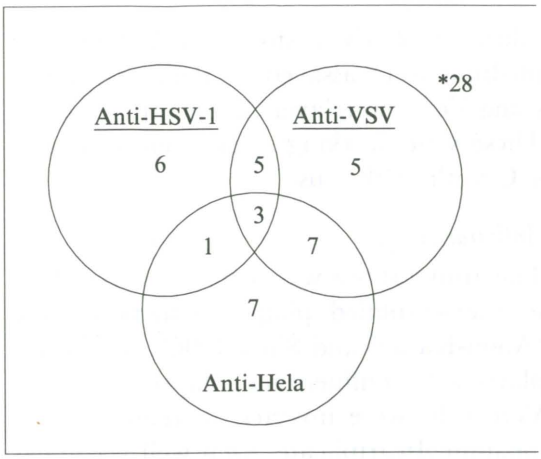

*Fig. 1. The number of plant species showing antiviral and cytotoxic activities are in the circles. Overlapping circles indicate co-existing activities. (*Species that did not show any activity)*

CO_2 for 4 days. Using an inverted microscope (low power), cytotoxicity was determined as the concentration of plant extract which reduced cell number by *ca.* 50% with reference to the control (CD_{50}, mg/ml).

RESULTS AND DISCUSSION

The overall results of the 61 plants from 33 families screened for antiviral and cytotoxic activities are summarized in Fig. 1. Table 1 lists the 28 species (46%) that gave negative results for all three tests. Table 2 lists the 26 species (43%) that exhibited antiviral activity and the 18 species (30%) which showed cytotoxicity.

Eight species (13%) (*Calotropis gigantea, Costus speciosus, Eugenia michelii, Hedyotis auricularia, Mentha arvensis, Orthosiphon aristatus, Polygonum minus* and *Ricinus communis*) showed antiviral activity against both HSV-1 and VSV. The extracts of *Calotropis gigantea, Eugenia michelii* and *Ricinus communis* showed a similar MIC value of 0.01 mg/ml against both viruses, but only *Eugenia michelii* demonstrated cytotoxicity (CD_{50}: 0.05 mg/ml). In the case of *Polygonum minus*, a similar MIC value against HSV-1 was obtained but lesser activity was shown against VSV (MIC: 0.02 mg/ml) and HeLa cells (CD_{50}: 0.1 mg/ml). Very strong anti HSV-1 but weak anti-VSV and cytotoxic activities were observed in the *Mentha arvensis* extract (MIC: 0.002 mg/ml & 0.1 mg/ml; and CD_{50}: 0.1 mg/ml, respectively). On the other hand, strong anti-VSV but weak anti HSV-1 activities were displayed by the extracts of *Costus specious* and *Hedyotis auricularia*, while weak antiviral activity against both viruses was observed in the extract of *Orthosiphon aristatus* (MIC: 0.1 mg/ml).

Selective antiviral activity towards only

TABLE 1
Species failing to show either antiviral or cytotoxic activity

Family	Species
Amaranthaceae	*Aerva lanata* (L.) Juss.
Anacardiaceae	*Spondias cytherea* Sonnerat
Apocynaceae	*Hunteria zeylanica* (Retz.) Gardn. & Thw.
	Plumeria rubra L.
Bixaceae	*Bixa orellana* L.
Bombaceae	*Ceiba pentandra* Gaertn.
Commelinaceae	*Tradescantia spathacea* Sw.
Euphorbiaceae	*Euphorbia neriifolia* L.
Gramineae	*Cymbopogon citratus* (DC.) Stapf
Labiatae	*Plectranthus scutellaroides* (L.) R. Br.
Leguminosae	*Caesalpinia pulcherrima* (L.) Sw.
	Cassia alata L.
	Cassia fistula L.
Liliaceae	*Allium schoenoprasum* L.
Lythraceae	*Lawsonia inermis* L.
Menispermaceae	*Tinospora crispa* (L.) Hook. *f.* & Th.
Myrtaceae	*Eugenia polyantha* Wight
Oxalidaceae	*Averrhoa carambola* L.
Piperaceae	*Peperomia pellucida* Kunth
	Piper nigrum L.
Plantaginaceae	*Plantago major* L. s.l.
Rubiaceae	*Gardenia augusta* (L.) Merr.
Sapotaceae	*Mimusops elengi* L.
Scrophulariaceae	*Picria fel-terrae* Lour.
Solanaceae	*Datura metel* L
	Datura inoxia Mill.
Zingiberaceae	*Alpinia galanga* (L.) Sw.
	Curcuma mangga Val. & Van Zyp,

TABLE 2
The effect of plant extracts on cells as shown by minimum inhibitory concentration values against herpes simplex virus-type 1 and vesicular stomatitis virus, and CD_{50} values towards HeLa cells

Plant	HSV-1	VSV	Cytotoxicity
	MIC[a] (mg/ml)		CD_{50}[b] (mg/ml)
Acanthaceae			
Andrographis paniculata Nees	-ve	-ve	0.1
Amaranthaceae			
Alternanthera sessilis (L.) DC.	0.001	-ve	-ve
Apocynaceae			
Cerbera manghas L.			
Fruits	-ve	0.05	0.001
Leaves	-ve	0.1	0.02
Asclepiadaceae			
Calotropis gigantea R. Br.	0.01	0.01	-ve
Compositae			
Blumea chinensis DC.	0.005	-ve	-ve
Cosmos caudatus Kunth	-ve	-ve	0.1
Elephantopus scaber L.	-ve	-ve	0.05
Euphorbiaceae			
Acalypha indica L.	-ve	0.01	0.01
Codiaeum variegatum (L.) Bl.	-ve	0.1	0.1
Euphorbia hirta L.	0.1	-ve	-ve
Ricinus communis L.	0.01	0.01	-ve
Gramineae			
Eleusine indica (L.) Gaertn.	0.1	-ve	-ve
Labiatae			
Plectranthus amboinicus (Lour.) Spreng.	-ve	0.1	-ve
Mentha arvensis L.	0.002	0.1	0.1
Ocimum tenuiflorum L.	-ve	-ve	0.1
Orthosiphon aristatus (Bl.) Miq.	0.1	0.1	-ve
Leeaceae			
Leea indica (Burm. f.) Merr.	0.05	-ve	-ve
Malvaceae			
Hibiscus rosa-sinensis L.	-ve	-ve	0.1
Myrtaceae			
Eugenia michelii Lamk.	0.01	0.01	0.05
Lecythidaceae			
Bertholletia excelsa Hump. & Bonpl.	-ve	0.005	-ve
Lecythis ollaria L.	-ve	-ve	0.1

Nyctaginaceae			
Mirabilis jalapa L.	-ve	0.05	0.1
Pandanaceae			
Freycinetia malaccensis Ridl.	0.05	-ve	0.1
Piperaceae			
Piper sarmentosum Roxb.	-ve	0.02	0.1
Polygonaceae			
Polygonum minus Huds.	0.01	0.02	0.1
Rubiaceae			
Hedyotis auricularia L.	0.1	0.05	-ve
Morinda elliptica Ridl.	-ve	0.1	0.003
Solanaceae			
Solanum americanum Mill.	0.1	-ve	-ve
Umbelliferae			
Centella asiatica (L.) Urb.	-ve	0.1	0.1
Oenanthe javanica DC.	-ve	0.02	-ve
Verbenaceae			
Premna odorata Blanco	-ve	0.05	-ve
Zingiberaceae			
Costus speciosus (Koenig) Smith	0.1	0.02	-ve
Etlingera elatior (Jack) R. M. Smith	-ve	-ve	0.1

[a]MIC = minimum inhibitory concentration, i.e. the lowest concentration of plant extract which completely inhibited virus replication.

[B]CD_{50} = cytotoxic dose at 50%, i.e. the concentration of plant extract which reduced the number of HeLa cells by 50%.

HSV-1 was seen in extracts from 7 plants (11%) (in order of decreasing activity, *Alternanthera sessilis, Blumea chinensis, Freycinetia malaccensis, Leea indica, Euphorbia hirta, Eleusine indica*, and *Solanum americanum*) with MIC values within the range of 0.001-0.1 mg/ml. Conversely, 11 plant (18%) extracts possessed selective antiviral activity against VSV (in order of decreasing activity, *Bertholletia excelsa, Acalypha indica, Piper sarmentosum, Oenanthe javanica, Mirabilis jalapa, Premna odorata, Cerbera manghas, Codiaeum variegatum, Plectranthus amboinicus, Centella asiatica* and *Morinda elliptica*) with MIC values from 0.005 - 0.1 mg/ml. More plant extracts were active against VSV than HSV-1. The selective antiviral activity of some plant extracts against either HSV-1 or VSV implicates the involvement of different mechanisms of action exploiting the difference in nucleic acid composition of the viruses.

In the case of the anti HSV-1 species, only *Freycinetia malaccensis* showed cytotoxicity (CD_{50}: 0.1 mg/ml) whereas 7 of the anti-VSV extracts (in order of decreasing activity, *Cerbera manghas, Morinda elliptica, Acalpyha indica, Centella asiatica, Codiaeum variegatum, Mirabilis jalapa* and *Piper sarmentosum*) showed cytotoxicity ranging from CD_{50} 0.001-0.1 mg/ml. Since VSV is a RNA-type virus, the concomitant anti-VSV and cytotoxic activities may involve a

related mode of action, most probably via protein interaction. Co-existing antiviral and cytotoxic activities were found in the extracts of three species (5%), i.e. *Eugenia michelii, Mentha arvensis* and *Polygonum minus*.

Of the 18 plants showing cytotoxicity, only 3 species (*Acalypha indica, Cerbera manghas* and *Morinda elliptica*) showed significant activity below the cut-off value of 0.02 mg/ml suggested by Wall *et al.* (1987) and all three species exhibited anti-VSV activity. The strongest cytotoxic activity was shown by the fruits of *Cerbera manghas* (CD_{50}: 0.001 mg/ml). The fruits of *Cerbera manghas* always exhibited stronger cytotoxic (20 times) and anti-VSV activities (twice the activity) than its leaves. This suggests that a higher concentration of the bioactive compound(s) is present in the fruits of *Cerbera manghas* than the leaves.

The *in vitro* cytotoxicity displayed by the plant extracts tested is an initial indicator of *in vivo* antitumour activity. However, since a wide range of phytocompounds are capable of exhibiting non-specific cytotoxicity, plant extracts with significant cytotoxic activity should be further assayed using animal models to confirm antitumour activity, and/or a battery of various cell lines to detect specific-cytotoxicity. This step is necessary to eliminate cytotoxic compounds with little value for further investigation as anticancer agents.

CONCLUSION

The results of this preliminary study scientifically substantiate to a certain extent the pharmacological activities of 33 plants used in Malaysian indigenous medicine and point out some plants with potential for further investigation. In addition, these results may also contribute towards the documentation of pharmacological profiles of Malaysian plants for conservation efforts and protection of biodiversity rights. Inadequate recording of the pharmacological activities of Malaysian plants may lead to the commercial exploitation of traditional knowledge by foreign parties without any benefit to the country as experienced by India in the case of the Neem tree and turmeric (Agarwal and Narain 1996).

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