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ANTIMICROBIAL AND CYTOTOXIC PROPERTIES OF SOME MALAYSIAN TRADITIONAL VEGETABLES (ULAM)

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ABSTRACT

*Ethanol extracts of 19 Malaysian traditional vegetables (locally known as 'ulam') belonging to 15 families were screened for antimicrobial and cytotoxic activities. Both the disc diffusion (qualitative) and tube dilution (quantitative) assays were employed for the determination of antimicrobial activity against six pathogenic microorganisms, i.e. two Gram-positive bacteria (*Bacillus cereus *and* Bacillus megaterium*), two Gram-negative bacteria* (Escherichia coli *and* Pseudomonas aeruginosa) *and two fungi* (Aspergillus ochraceous *and* Cryptococcus neoformans). *Six extracts, i.e.* Anacardium occidentale, Garcinia atroviridis, Averrhoa bilimbi, Polygonum minus, Diplazium esculentum *and* Etlingera elatior, *showed antimicrobial with minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) values in the respective ranges of 100 → 800 µg/ml and 400 → 800 µg/ml. Seven extracts, i.e.* Anacardium occidentale, Garcinia atroviridis, Sesbania grandiflora, Barringtonia racemosa, Polygonum minus, Kaempferia galanga *and* Etlingera elatior *displayed cytotoxic activity against the HeLa (human cervical carcinoma) cell line with* CD_{50} *values in the range of 10–30 µg/ml. The 'ulam' show potential as 'functional food' in view of the significant therapeutic and nutritive benefits.*

Keywords: Antimicrobial activity, cytotoxic activity, ethanol extracts, functional food, Malaysian traditional vegetables, 'ulam'.

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INTRODUCTION

The biodiversity of Malaysia's plant resources offers some 15,000 species of higher plants of which the traditional vegetables, locally known as 'ulam', comprises about 120 plant species representing many families from herbs to trees (Mansor, 1988). These plants constitute a significant part of the food intake of the local people particularly among the Malay and indigenous communities thereby justifying its importance for scientific study. The 'ulam' are predominantly eaten raw as salads, particularly the leaves, otherwise they may be blanched, sautéed, curried and fried (Bautista *et al.*, 1988; Mansor, 1988). In spite of the popularity of 'ulam' as traditional vegetables, only a few have been studied scientifically and no chemical or pharmacological studies have been undertaken to investigate the 'ulam' as a collective group. Even the few 'ulam' that have been studied individually, were mainly subjected to chemical and nutritional studies thereby generally lacking in information with regard to biological activities. Nutritional studies have indicated that many of the 'ulam' are a rich source of protein, amino acids, dietary fibre, vitamins and minerals (Tee, 1985; Zanariah *et al.*, 1986; Candlish *et al.*, 1987; Bautista *et al.*, 1988). Carotenes, which have been attributed with anticancer properties, are present at high levels in several of the 'ulam' (Tee, 1985; Mansor, 1988; Murakoshi *et al.*, 1992). Separately, ethnobotanical studies reports have ascribed the 'ulam' with a diverse range of medicinal value such as astringent, antiulcer, antiviral, aperient, antiseptic, febrifuge and antiimflammatory properties thus representing an untapped avenue for bio-

logically active compounds (Burkill, 1966; Perry, 1980). Unfortunately, many of these claims of medicinal value have yet to be scientifically substantiated. Therefore, the present study was undertaken in an attempt to establish the antimicrobial and cytotoxic properties of the 'ulam'.

MATERIALS AND METHODS

Plant Materials

The plant materials were randomly procured from various localities in the state of Selangor except for *Melia excelsa* Jack and *Garcinia atroviridis* Griff. ex T. Anders, which were collected at Labu Besar Village, in the state of Kedah, Malaysia (Table 1). The plants were identified by K.M. Salleh and voucher specimens were deposited at herbarium, Faculty of Life Sciences, Universiti Kebangsaan Malaysia.

Extraction

The fresh plant materials were sliced into small pieces and 20 g of each plant was macerated in 200 ml of 80% ethanol at room temperature for seven days. The ethanol extracts were evaporated *in vacuo* at 40°C. Subsequently, the dried residue from each sample was reconstituted in 90% ethanol to give a stock of 100 mg/ml. The final concentration of ethanol in any test medium was always below 0.5% (v/v) to prevent interference with the antimicrobial and cytotoxicity assays.

Microorganisms

The test microorganisms were obtained from the culture collection of the Department of Pharmacognosy, University of Mansoura, Mansoura, Eygpt. The cultures were originally purchased from the American Culture Type Collection (ATCC), Northern Regional Research Laboratories (NRRL) and College of Pharmacy, University of Iowa (UI). The bacterial and

Table 1. Plants screened for antimicrobial and cytotoxic activities.

Family/ Species	Part used	Vernacular name
Anacardiaceae		
Anacardium occidentale Linn.	Leaves	Gajus
Compositae		
Cosmos caudatus H.B & K.	Aerial	Ulam Raja
Gnetaceae		
Gnetum gnemon Linn.	Leaves	Belinjau, Melinjau
Guttiferae		
Garcinia atroviridis Griff. Ex T. Anders	Fruits (Dried)	Asam Gelugor
Lecythidaceae		
Barringtonia racemosa Roxb.	Leaves	Putat
Legunimosae		
Neptunia prostrata Baill.	Leaves	Tangki
Pithecellobium bubalinum Benth.	Seeds	Kerdas
Pithecellobium jiringa (Jack) Prain	Seeds	Jering
Sesbania grandiflora Poir	Leaves	Turi
Meliaceae		
Melia excelsa Jack	Leaves	Setang
Oxalidaceae		
Averrhoa bilimbi Linn.	Fruits	Belimbing Buluh Belimbing Asam
Piperaceae		
Piper sarmentosum Roxb.	Leaves	Kadok
Polygonaceae		
Polygonum minus Huds.	Leaves	Kesom
Polypodiaceae		
Diplazium esculentum Swartz	Aerial	Pucuk Paku
Rutaceae		
Citrus hystrix DC.	Leaves	Limau Purut
Umbelliferae		
Oenanthe javanica DC.	Aerial	Selom
Zingiberaceae		
Alpinia galanga Willd.	Rhizome	Lengkuas
Kaempferia galanga Linn.	Rhizome	Cekur, Kencur
Etlingera elatior (Jack) R.M. Smith	Flower-Shoot	Bunga Kantan

fungal stock cultures were maintained on nutrient agar (NA) and potato dextrose agar (PDA) slants respectively, which were stored at 4°C. Six pathogenic microbial strains were used, i.e. *Bacillus cereus* NRRL 14591B (Gram-positive), *Bacillus megaterium* ATCC 14581 (Gram-positive), *Escherichia coli* UI-190494 (Gram-negative), *Pseudomonas aeruginosa* UI-170494 (Gram-negative), *Aspergillus ochraceous* ATCC 398 (fungi) and *Cryptoccoccus neoformans* UI-350494 (fungi). For the purpose of antimicrobial evaluation, the microorganisms were cultured in the appropriate broths at turbidometrically at a wavelength of 600 nm to 10^5–10^6 colony forming units (CFU) per ml.

Culture of Cells and Cytotoxicity Assay

The HeLa (human cervical carcinoma) cell-line was obtained from RIKEN Cell Bank, Tsukaba, Japan, and cultured in RPMI-1640 (Sigma, USA) medium supplemented with five percent of fetal calf serum (Peasal & Laurel, Germany), 100 IU/ml of penicillin and 100 µg/ml of streptomycin (Sigma, USA) as a complete growth medium (CGM). Cells were maintained in 25 cm^2 flasks (Nunc, Denmark) with 10 ml of CGM in a CO_2 incubator at 37°C until confluence was reached. HeLa cells were cultured in the presence of different concentrations of plant extracts and the concentration which reduced growth by 50% was reported as the CD_{50} as described by Shier (1983).

Qualitative Antimicrobial Assay

Antimicrobial activity of the extracts was qualitatively determined by a modified disc diffusion method method (Bauer *et al*., 1966). A lawn of microorganisms was prepared by pipetting and evenly spreading 100 µl of inoculum (adjusted turbidometrically to 10^5–10^6 CFU/ml) onto agar set in petri dishes, using NA for the bacteria and PDA for fungi. Whatman No.1 filter paper discs of 6 mm diameters were impregnated into the ethanol stock solutions of the plant extracts (100 mg/ml) and dried under sterile conditions to remove the ethnol. The dried discs were then placed on the previously inoculated agar surface. The plates were inverted and incubated for 24 h at 30°C. Antimicrobial activity was indicated by the presence of clear inhibition zones around the discs.

Quantitative Antimicrobial Assay

Extracts that showed positive antimicrobial activity with the disc diffusion assay were subjected to the broth dilution method as described by Hufford and Clark (1988) for the quantitative measurement of microbiostatic (inhibitory) activity. The lowest concentration which completely inhibited visible microbial growth was recorded as the minimum inhibitory concentration (MIC, µg/ml). In order to determine microbiocidal (killing) activity, 100 µl of each test tube showing no turbidity (no growth) were plated onto NA and PDA petri dishes for bacteria and fungi respectively, and reincubated for another 24 h. The minimum lethal concentration (MLC, µg/ml) was measured as the lowest concentration of test extract that decreased the initial inoculum by $> 99.9\%$, i.e. prevented the formation of colonies on agar plates (Kubo *et al*., 1994). Both nystatin and kanamycin (Sigma, USA) were used as positive controls while a negative control, i.e. tube inoculated without extract, was also included for each microorganism.

RESULTS AND DISCUSSION

Preliminary screening of the crude ethanol extracts of 19 'ulam' from 15 families for antimicrobial activity was performed qualitatively using the disc diffusion assay. Six of these 'ulam' extracts (32%), i.e. *Anacardium occidentale, Garcinia atroviridis, Averrhoa bilimbi*, *Polygonum minus*, *Diplazium esculentum* and *Etlingera elatior* yielded clear inhibition zones around the discs (Table 2). Of these six, more were active against Gram-negative bacteria (six extracts, 32%) than against Gram-positive bacteria or fungi, i.e. four extracts (21%) each. The efficacy of the extracts, particularly against Gram-negative bacteria, is very promising as a source of antibiotics in view of the difficulty encountered by most antibiotics in penetrating the structurally complex cell wall (Rang and Dale, 1993). Three (16%) extracts were active against *Bacillus cereus*, *Escherichia coli* and *Cryptococcus neoformans*, respectively; five (26%) extracts against *Pseudomonas aeruginosa*, well-noted for its extraordinary insusceptibility to most antibiotics; and two (11%) extracts against *Aspergillus ochraceous*.

A majority of the six 'ulam' extracts with antimicrobial activity also displayed both antibacterial and antifungal activities and thus seems to reflect broad-spectrum antimicrobial activity. The extracts of both *Averrhoa bilimbi* and *Polygonum minus* showed antibacterial activity against *Pseudomonas aeruginosa*.

MIC and MLC values of the above six 'ulam' extracts were determined for the quantitative measurement of antimicrobial activity, and also to distinguish between microbiostatic and microbiocidal action. The

MIC values did not always correlate well with the diameter of inhibition zones particularly for *Bacillus megaterium*, *Escherichia coli* and *Cryptococcus neoformans*. This inconsistency may perhaps be attributed to the parameters of varying agar height, extract content of the discs and inoculum density. Therefore, the tube dilution assay was chosen for the accurate quantitative determination of antimicrobial activity as suggested by Hufford and Clark (1988).

The results of Table 2 may be described as follows:

(1) The extract of *Anacardium occidentale* recorded MIC values of 200 µg/ml against *Pseudomonas aeruginosa*; 400 µg/ml against *Aspergillus ochraceous* and 800 µg/ml against *Bacillus cereus*, *Bacillus megaterium* and *Cryptococcus neoformans*. The MLC values obtained from *Anacardium occidentale* were 800 µg/ml against *Bacillus cereus*, *Pseudomonas aeruginosa* and *Aspergillus ochraceous*; and more than 800 µg/ml against *Bacillus megaterium* and *Cryptococcus neoformans*.

(2) *Garcinia atroviridis* exhibited MIC values of 100 µg/ml against *Pseudomonas aeruginosa* (same as the kanamycin control value), 400 µg/ml against *Bacillus cereus* (lower than the kanamycin control value), and 800 µg/ml against *Escherichia coli* and *Cryptococcus neoformans*. The MLC values shown were 400 µg/ml against *Pseudomonas aeruginosa*, 800 µg/ml against *Bacillus cereus* and *Cryptococcus neoformans*, and more than 800 µg/ml against *Escherichia coli*.

(3) *Averrhoa bilimbi* displayed MIC and MLC values of 200 µg/ml and 400 µg/ml, respectively, against *Pseudomonas aeruginosa*. The MIC and MLC from *Polygonum minus* were 400 µg/ml and more than 800 µg/ml, respectively, against *Pseudomonas aeruginosa.*

(4) *Diplazium esculentum* afforded MIC values of 200 µg/ml against *Bacillus cereus* (lower than kanamycin control value); 400 µg/ml against *Escherichia coli* and *Aspergillus ochraceous*; and 800 µg/ml against *Bacillus megaterium.* MLC values were 800 µg/ml against *Bacillus cereus* and *Aspergillus ochraceous*; and more than 800 µg/ml against *Bacillus megaterium* and *Escherichia coli*.

(5) *Etlingera elatior* recorded MIC values of 200 µg/ml against *Pseudomonas aeruginosa*; 400 µg/ml against *Bacillus megaterium* (lower than kanamycin control value); 800 µg/ml against *Escherichia coli*; and more than 800 µg/ml against *Cryptococcus neoformans.* The MLC values obtained were 400 µg/ml against *Pseudomonas aeruginosa*, and more than 800 µg/ml against *Bacillus megaterium* and *Escherichia coli.*

Most of the extracts showed MLC values close to the MIC values, i.e. with MLC/MIC ratios of two and below (Table 2) indicating microbiocidal action (Varisai *et al.*, 1992). A high MLC/MIC ratio of four was obtained from three extracts, viz. *Anacardium occidentale* and *Garcinia atroviridis* against *Pseudomonas aeruginosa*; and *Diplazium esculentum* against *Bacillus cereus*, thereby suggesting prominent microbiostatic activity.

Table 2. Antimicrobial and cytotoxic activities of 'ulam' extracts.

	Cell line/Microorganism												
	B. cereus		*B. megaterium*		*E. coli*		*P. aeruginosa*		*A. ochraceous*		*C. neoformans*		HeLa
Plant	[a]MIC	[b]MLC	MIC	MLC	MIC	MLC (µg/ml)	MIC	MLC	MIC	MLC	MIC	MLC	[c]CD_{50}
A. occidentale	800	800 (*1)	800	> 800 (*n.d.)	–	–	200	800 (*4)	400	800 (*2)	800	> 800 (*n.d.)	10
G. atroviridis	400	800 (*2)	–	–	800	> 800 (*n.d.)	100	400 (*4)	–	–	800	800 (*1)	30
S. grandiflora	–	–	–	–	–	–	–	–	–	–	–	–	30
A. bilimbi	–	–	–	–	–	–	200	400 (*2)	–	–	–	–	–
B. racemosa	–	–	–	–	–	–	–	–	–	–	–	–	10
P. minus	–	–	–	–	–	–	400	> 800 (*n.d.)	–	–	–	–	30
D. esculentum	200	800 (*4)	800	> 800 (*n.d.)	400	> 800 (*n.d.)	–	–	400	800 (*2)	–	–	–
K. galanga	–	–	–	–	–	–	–	–	–	–	–	–	10
E. elatior	–	–	400	> 800 (*n.d.)	800	> 800 (*n.d.)	200	400 (*2)	–	–	> 800	n.d. (*n.d.)	10
[d] Antibiotic Control	> 400	–	> 400	–	200	–	100	–	6.25	–	6.25	–	–

[a] MIC (µg/ml) = minimum inhibitory concentration, i.e. the lowest concentration to completely inhibit microbial growth.
[b] MLC (µg/ml) = minimum lethal concentration, i.e. the lowest concentration to completely kill all microorganisms.
[c] CD_{50} (µg/ml) = cytotoxic dose at 50%, i.e. the concentration to reduce growth of HeLa cells by 50%.
[d] Kanamycin was used for bacteria and nystatin for fungi.
n.d. = undetermined because MIC value exceeded 800 (µg/ml).
*MLC/MIC ratio.

From the 19 plant extracts screened for cytotoxicity, seven (37%) showed suppresive activity towards the HeLa cell line, i.e. *Anacardium occidentale, Garcinia atroviridis, Sesbania grandiflora, Barringtonia racemosa, Polygonum minus, Kaempferia galanga* and *Etlingera elatior* (Table 2). The CD_{50} value of 10 µg/ml was exhibited by four extracts (21%), i.e. *Anacardium occidentale, Barringtonia racemosa, Kaempferia galanga* and *Etlingera elatior*. These 'ulam' extracts would be considered as potential antitumour agents because the CD_{50} values were below the cut-off point of 20 µg/ml as suggested by Wall *et al*. (1987). On the other hand, low cytotoxicity, i.e. CD_{50} of 30 µg/ml, was shown by the extracts from *Garcinia atroviridis, Sesbania grandiflora* and *Polygonum minus*.

The 'ulam' have been underutilised as a food and therapeutic source despite ethnopharmacognostic and nutritional reports of high medicinal and nutritive values. Therefore, the therapeutic value of the 'ulam' as shown in this present study coupled with the high nutritive value reported in previous studies qualifies them as 'functional food', i.e. food that perform sustenance, prophylactic and disease-treatment roles (Hathcock, 1993). Thus, the practicality of prevention and amelioration of disease through regular consumption of the 'ulam' is noteworthy in the Malaysian context since the 'ulam' are a well established segment of the dietary intake. Furthermore, awareness of the health and nutritional benefits of the 'ulam' would also lead to overall systematic cultivation of the 'ulam' on a commercial scale which would overcome shortage of supply of particularly the less popular species. However, before the 'ulam' are aggressively promoted as 'functional food', extensive studies should be carried out to ascertain safety factors such as allergenecity, toxicity and presence of deleterious compounds.

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