UNIVERSITI PUTRA MALAYSIA

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By

YAN SEE WAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

ANTIOXIDANT AND PHYTOCHEMICAL PROPERTIES AND *IN VITRO* CANCER CHEMOPREVENTIVE EFFECTS OF *AVERRHOA BILIMBI* L. EXTRACT ON HUMAN BREAST AND CERVICAL ADENOCARCINOMA

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Chairman: Asmah Rahmat, PhD

Faculty: Faculty of Medicine and Health Sciences

High consumption of fruits has been associated with a reduced risk of various degenerative diseases such as cancer. These protective properties are reckoned to be linked to the micronutrients and non-nutritive phytochemicals present in fruits. This study was conducted to investigate the nutritional compositions, total phenolic and flavonoid contents, antioxidant capacity, antioxidant vitamins, phenolic acid and flavonoid compounds, as well as anticancer potential of two types of fruits endemic to Malaysia: *Averrhoa bilimbi* (bilimbi) and *Averrhoa carambola* (carambola). Commonly consumed among the locals, these underutilized fruits have been popularly practiced as folk medicine since ancient times.

Results from nutritional compositions analyses showed that bilimbi possessed higher fat and total dietary fibre but comparable moisture, ash, carbohydrate and protein content with that of carambola. Results from Folin-Ciocalteu, aluminium chloride colourimetric assays and antioxidant vitamins analyses indicated a higher antioxidant vitamins and flavonoid content in bilimbi while carambola with higher total phenolic. β-carotene bleaching and DPPH radical scavenging assays suggested that carambola displayed stronger antioxidant capacity and was positively correlated with its total phenolic content.

The presence of non-nutritive phytochemicals in both fruits was evaluated by HPLC analysis based on the optimized extraction conditions performed through single-factor experiments. Analysis revealed that optimal extraction conditions were 80% ethanol at 65 ^{o}C for 8 hours in both fruit extracts. Phenolic compounds extracted by the optimal extraction conditions were subjected to HPLC analysis. Epigallocatechin and catechin were identified as the key flavonoids in bilimbi and carambola, respectively while sinapic acid and syringic acid were the predominant phenolic acid in bilimbi and carambola, respectively.

Cytotoxic and anticancer potential of bilimbi and carambola ethanolic extracts were examined on various human cancer cell lines (breast, cervical, colon, liver and ovarian adenocarcinoma) as well as non-malignant Chang Liver cell line. Findings from MTS assay implied a 50% growth inhibitory effect induced by bilimbi extract on cervical cancer cell line (HeLa) and non-hormone dependent breast cancer cell line (MDA-MB-

231) at 70 and 90 μg/ml, respectively without any cytotoxic effect on non-malignant Chang Liver cell line. In contrast, carambola extract was unable to exert any growth inhibitory effect even concentration was increased up to 200 μg/ml. Further investigations to determine apoptogenic potential of bilimbi extract revealed typical morphological features of apoptosis as well as formation of ladder-like pattern in bilimbi-treated HeLa and MDA-MB-231. Apoptosis and antiproliferative effects were further evidenced with the increased in sub G1 population and accumulation of cells with a 2N DNA content in cell cycle analysis, suggesting perturbation at G0/G1 checkpoint. BrdU incorporation cell proliferation assay indicated a decreased in cells synthesis (S phase). Released of cytochrome c coupled with upregulation of caspase-3/7 and caspase-9 expression indicated that molecular mechanism in the induction of apoptosis by bilimbi on HeLa and MDA-MB-231 was through mitochondria-mediated intrinsic pathway, which also involve upregulation of pro-apoptotic Bax protein and downregulation of anti-apoptotic Bcl-2 protein. Upregulation of tumour suppressor p53 protein was detected in HeLa upon exposure to bilimbi, as opposed to MDA-MB-231 which was found to be independent of p53. Hence, bilimbi induced apoptosis through a p53-dependent mitochondrial pathway in HeLa and a p53-independent mitochondrial pathway in MDA-MB-231.

Based on the antioxidant and phytochemicals properties as well as anticancer potential, bilimbi offers a promising candidate for cancer chemoprevention strategy. As a natural product, bilimbi is potentially safe and beneficial for pharmaceutical and health industries worth further research and investigations.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

KANDUNGAN ANTIOXIDAN DAN FITOKIMIA DAN KESAN *IN VITRO* KEMO PENCEGAHAN KANSER EKSTRAK *AVERRHOA BILIMBI* L. PADA ADENOKARSINOMA PAYUDARA DAN SERVIK MANUSIA

Oleh

YAN SEE WAN

Jun 2012

Pengerusi: Asmah Rahmat, PhD

Fakulti: Fakulti Perubatan dan Sains Kesihatan

Kekerapan pengambilan buah-buahan berkait rapat dengan pengurangan risiko panyakit degeneratif seperti kanser. Ciri-ciri perlindungan ini adalah berkaitan dengan mikronutrien dan fitokimia yang terdapat dalam buah-buahan. Kajian ini dijalankan untuk menyiasat komposisi pemakanan, jumlah kandungan fenolik dan flavonoid, kapasiti antioksidan, vitamin antioksida, sebatian asid fenolik dan flavonoid, serta potensi anti kanser dua jenis buah-buahan yang endemik kepada Malaysia: *Averrhoa bilimbi* (bilimbi) dan *Averrhoa carambola* (carambola). Biasanya digunakan di kalangan penduduk tempatan, buah-buahan terpinggir ini kerap diamalkan sebagai ubat tradisional sejak zaman purba.

Keputusan analisis komposisi pemakanan menunjukkan bahawa bilimbi memiliki lemak dan serat yang tinggi tetapi kandungan air, abu, karbohidrat dan protein yang standing dengan carambola. Keputusan kaedah Folin-Ciocalteu, ujian kolorimetrik aluminium klorida dan vitamin antioksida analisis menunjukkkan bahawa bilimbi mengandungi vitamin antioksida dan flavonoid yang lebih tinggi manakala carambola mengandungi jumlah fenolik yang lebih tinggi. Ujian pelunturan β-karotena dan pemerangkapan radikal DPPH mencadangkan bahawa carambola memaparkan kapasiti antioksidan yang kuat dan berkait positif dengan jumlah kandungan fenolik.

Kehadiran fitokimia dalam kedua-dua buah-buahan dinilai oleh analisis HPLC berdasarkan keadaan pengekstrakan optima yang ditentukan melalui uji kaji satu faktor. Analisis menunjukkan bahawa keadaan pengekstrakan optima adalah 80% etanol pada 65 ^{o}C selama 8 jam pada kedua-dua ekstrak buah-buahan. Sebatian fenolik kemudian diekstrak dalam keadaan pengekstrakan optima melalui analisis HPLC. Epigallocatechin dan catechin masing-masing dikenal pasti sebagai flavonoid utama dalam bilimbi dan carambola manakala asid sinapic dan asid syringic masing-masing dikenal pasti sebagai asid fenolik utama dalam bilimbi dan carambola.

Potensi sitotoksik dan anti kanser ekstrak etanol bilimbi dan carambola disaring pada beberapa sel kanser manusia (adenokarsinoma payudara, serviks, kolon, hati dan ovari) serta sel normal Chang Liver. Keputusan analisis MTS menunjukkkan bahawa ekstrak bilimbi merencat pertumbuhan sel kanser serviks (HeLa) dan payudara (MDA-MB-231) sebanyak 50% masing-masing dengan nilai 70 dan 90 μg/ml tanpa kesan sitotoksik ke

atas sel normal Chang Liver. Sebaliknya, ekstrak carambola tidak mampu merencat pertumbuhan sebarang sel kanser walaupun kepekatan ekstrak ditingkatkan sehingga 200 μg/ml. Siasatan lanjut untuk menentukan potensi apoptosis ekstrak bilimbi menunjukkan bahawa kesan ekstrak bilimbi ke atas HeLa dan MDA-MB-231 menunjukkan ciri-ciri morfologi lazim apoptosis serta pembentukan pola DNA ladder. Kesan apoptosis dan anti proliferasi juga dibuktikan melalui peningkatan fasa sub G1 dan peningkatan sel dengan kandungan 2N DNA dalam analisis kitaran sel yang menyebabkan pembantutan proses kitaran sel di fasa G0/G1. Ujian sel proliferasi BrdU menunjukkan pengurangan sintesis sel (fasa S). Pengaktifan sitokrom c serta peningkatan caspase-3/7 dan caspase-9 menunjukkan bahawa mekanisme induksi apoptosis oleh bilimbi pada HeLa dan MDA-MB-231 di peringkat molekul adalah melalui laluan intrinsik mitokondria yang juga melibatkan peningkatan protein pro apoptosis Bax dan penurunan protein anti apoptosis Bcl-2. Pendedahan HeLa kepada ekstrak bilimbi menyebabkan peningkatan protein *tumour suppressor* p53, sebaliknya, induksi apoptosis oleh bilimbi pada MDA-MB-231 didapati tidak melibatkan protein *tumour suppressor* p53. Oleh itu, induksi apoptosis oleh bilimbi pada HeLa adalah bergantung kepada p53 dan melalui laluan intrinsik mitokondria manakala pada MDA-MB-231 adalah tidak bergantung kepada p53 dan melalui laluan intrinsik mitokondria.

Berdasarkan ciri-ciri antioksidan dan fitokimia serta potensi anti kanser, bilimbi berupaya digunakan dalam strategi kemo pencegahan kanser. Sebagai produk semulajadi, bilimbi adalah selamat dan bermanfaat untuk industri farmaseutikal dan kesihatan serta amat sesuai untuk penyelidikan dan siasatan lanjut.

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"*Success is not final, failure is not fatal; it is the courage to continue that counts*"

~ Winston Churchill ~

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I certify that a Thesis Examination Committee has met on 11 June 2012 to conduct the final examination of Yan See Wan on her thesis entitled "Antioxidant and Phytochemical Properties and *In Vitro* Cancer Chemopreventive Effects of *Averrhoa bilimbi* L. Extract on Human Breast and Cervical Adenocarcinoma" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1988. The Committee recommends that the students be awarded the degree of Doctor of Philosophy.

Members of the Thesis Examination Committee were as follows:

Norhaizan binti Mohd. Esa, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Patimah binti Ismail, PhD
Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Fauziah binti Othman, PhD
Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Ranjana Prasad Bird, PhD
Professor
University of Windsor
Canada
(External Examiner)

_____
SEOW HENG FONG, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 23 July 2012

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Asmah Rahmat, PhD
Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Rajesh Ramasamy, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

Noorjahan Banu Mohamed Alitheen, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

_____
BUJANG BIN KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

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YAN SEE WAN

Date: 11 June 2012

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