



UNIVERSITI PUTRA MALAYSIA

***EFFECTS OF SUPERCRITICAL FLUID-EXTRACTED HIBISCUS
CANNABINUSL. SEED OIL ON COLON CANCER IN VITRO AND IN VIVO***

SITI AISYAH BT ABD GHAFAR

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BERILMU BERBAKTI

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*CANNABINUS*L. SEED OIL ON COLON CANCER *IN VITRO* AND *IN VIVO***

By

SITI AISYAH BINTI ABD GHAFAR

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EFFECTS OF SUPERCRITICAL FLUID-EXTRACTED *HIBISCUS CANNABINUS* SEED OIL ON COLON CANCER *IN VITRO* AND *IN VIVO*

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March 2013

Chairman: Professor Maznah Ismail, PhD

Faculty: Institute of Bioscience

Kenaf (*Hibiscus cannabinus*) from the family *Malvaceae*, is a valuable fiber plant native to India and Africa, and is currently planted as the fourth commercial crop in Malaysia. Kenaf seed oil contains alpha-linolenic acid, phytosterol such as β -sitosterol, vitamin E and other antioxidants with chemopreventive properties. The present study evaluated cytotoxicity towards human colorectal cancer cell line (HT29) and cancer chemopreventive properties of kenaf seed oil from supercritical carbon dioxide fluid extraction (KSO-SFE). Kenaf seed oil was extracted via supercritical carbon dioxide fluid (SFE) at 9 different permutations of parameters based on range of pressure (200-600 bars) and temperature (40-80°C): 200/40, 200/60, 200/80, 400/40, 400/60, 400/80, 600/40, 600/60 and 600/80. All the nine KSO-SFE were screened for cytotoxicity towards human colorectal cancer cell line (HT29) and mouse embryonic fibroblast cell line (NIH/3T3) using MTS assay. KSO-SFE of 600/40 showed the strongest cytotoxicity towards HT29 with IC_{50} of 200 μ g/ml. Nevertheless, IC_{50} for NIH/3T3 was

not detected even at the highest concentration of KSO-SFE employed. Cell cycle analysis exhibited a significant increase in the number of KSO-SFE-treated cells at sub-G1 phase, indicating the induction of apoptosis by the extract. The induction of apoptosis was further confirmed by Annexin V/PI and AO/PI staining. For the chemopreventive properties of KSO-SFE, 60 male Sprague Dawley rats were randomly assigned to 6 groups. All groups were induced with azoxymethane (AOM) except for the negative control (Group 1). They were 1) negative control group, 2) positive control group (without any treatment), 3) vehicle control group (administered with emulsifier (Tween 80), 4) group treated with 500 mg/kg body weight KSO-SFE; 5) group treated with 1000 mg/kg body weight KSO-SFE and 6) group treated with 1500 mg/kg body weight KSO-SFE. The animals were injected subcutaneously once a week for 2 weeks with 15 mg/kg body weight of AOM at 7 weeks of age. Rats were euthanized after 90 days of the experiment. There was no significant difference in weight gain among the groups. Number of aberrant crypt foci (ACF) ranged from 84.4 ± 4.43 to 179.5 ± 12.78 in Group 2, 3, 4, 5 and 6. ACF were reduced by 45.3%, 51.4% and 53.1% in rats fed with 500, 1000 and 1500 mg/kg body weight of KSO-SFE, respectively, compared to the positive control group ($p < 0.05$). For ACF multiplicity, ACF with 4, 5 or more crypts were significantly lower ($p < 0.05$) in rats fed with KSO-SFE compared to the positive control group. The findings indicate that KSO-SFE reduced AOM-induced ACF in Sprague Dawley male rats. The effects of KSO-SFE on fifteen genes involved in colon carcinogenesis were analyzed on AOM-induced rats using GenomeLabGeXP genetic system. It shows that treatment with KSO-SFE increased the expression of tumor suppressor genes (*APC* and *p53*), reduced the expression of tumor marker genes (*COX-*

2 and β -catenin) and did not change the expression of large tumor suppressor and *TNF* receptor genes compared to the positive control group ($p < 0.05$). KSO-SFE has also shown to activate the apoptotic pathway by up regulating the expression of caspases (caspase 9, caspase 2 and caspase 3) and pro-apoptotic genes (*bax* and *bad*), and down regulating the expression of anti-apoptotic gene (*bcl-2*). Treatment with KSO-SFE also affects the cell cycle genes with increased expression of cell cycle inhibitor (*p21*, *cip1*) and decreased expression of *cyclin D1*. Assessment of toxicity of KSO-SFE at 500, 1000 and 1500 mg/kg body weight/day towards Sprague Dawley rats was also performed. The parameters for toxicity include body and organ weight, haematology, clinical chemistry, pathology and expression of toxicity-related genes. No mortality or treatment-related adverse effects were observed at all doses throughout the period of 90 days. All the parameters were in normal range. Low creatinine level at all doses and low total cholesterol level at 1000 and 1500 mg/kg body weight of KSO-SFE were noted but insignificant. Further analysis using GenomeLabGeXP genetic system on the liver tissues showed the expression of genes involved in xenobiotic metabolism, DNA damage, cell cycle arrest and apoptosis was at normal level. In short, The No Observed Adverse Effect Level (NOAEL) for KSO-SFE at 1500 mg/kg body weight/day. In conclusion, data from this study demonstrate the potential of KSO-SFE as a chemopreventive agent against colon cancer.

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**KESAN MINYAK BIJI *HIBISCUS CANNABINUS* L. YANG DI EKSTRAK
OLEH BENDALIR SUPERKRITIKAL TERHADAP KANSER KOLON IN
VITRO DAN IN VIVO**

Oleh

SITI AISYAH BT ABD GHAFAR

Mac 2013

Pengerusi: Profesor Maznah Ismail, PhD

Fakulti: Institut Biosains

Kenaf (*Hibiscus cannabinus*) merupakan tumbuhan fiber bernilai tinggi daripada famili *Malvaceae*, yang berasal dari India dan Afrika dan sedang ditanam secara komersial di Malaysia. Minyak biji Kenaf mengandungi asid α -linolenik, fitosterol seperti β -sitosterol, vitamin E dan antioksidan lain yang mempunyai ciri-ciri kemopencegahan.

Kajian ini dijalankan bertujuan untuk mengkaji kesan rawatan minyak biji benih Kenaf daripada pengekstrakan bendalir superkritikal (KSO-SFE)

terhadap kanser kolon secara menggunakan sel kanser kolon manusia (HT29)

dan kemopencegahan kanser kolon. Dalam kajian ini,

minyak biji Kenaf telah diekstrak menggunakan kaedah pengekstrakan bendalir superkritikal

(SFE) dengan permutasi 9 parameter yang berlainan berdasarkan tekanan pada julat dari

200-600 bars dan suhu antara 40-80°C. Parameter yang telah digunakan adalah 200/40,

200/60, 200/80, 400/40, 400/60, 400/80, 600/40, 600/60 dan 600/80. Kesanketoksikan 9 ekstrak KSO-SFE telah diujikeatasselkanser kolon manusia (HT29) dan selembriolik fibroblast mencit (NIH 3T3) menggunakan asai MTS. Hasil ujian menunjukkan KSO-SFE yang diekstrak pada parameter 600/40 telah menunjukkan potensi yang paling tinggi dengan nilai IC_{50} nya keatassel HT29 adalah pada 200 μ g/ml. Manakala nilai IC_{50} untuk sel NIH/3T3 pula tidak dapat ditentukan. Rawatan KSO-SFE keatassel HT29 dalam analisis kitaransel pula telah menunjukkan bahawa peningkatan yang ketara terhadap pengumpulan sel-sel pada fasa sub-G1. Ia menunjukkan KSO-SFE mengaruh aktiviti apoptotik keatassel HT29. Kesan aruhan apoptotik ini telah dikaji dengan lebih lanjut menggunakan asai Annexin V/PI dan pewarnaan AO/PI. Untuk kajian cirikemopencegahan KSO-SFE, 60 ekor tikus spesies Sprague Dawley jantan telah dibahagikan secara rawak kepada 6 kumpulan iaitu 1) kumpulan kawalan negatif [tidak diaruh dengan azoksimetana (AOM)]; 2) kumpulan kawalan positif (diaruh dengan AOM tetapi tidak menerima sebarang rawatan); 3) kumpulan kawalan pembawa (diaruh dengan AOM dan dirawat dengan bahan pengemulsi) 4) Kumpulan dirawat dengan 500 mg/kg berat badan KSO-SFE; 5) Kumpulan dirawat dengan 1000 mg/kg berat badan KSO-SFE; 6) Kumpulan dirawat dengan 1500 mg/kg berat badan KSO-SFE. Pada umur 7 minggu, semua tikus kecuali kumpulan kawalan negatif menerima suntikan AOM pada dos 15 mg/kg berat badan secara subkutaneus sekali seminggu dalam tempoh 2 minggu. Tikus dibunuh pada akhir kajian iaitu pada hari ke 90. Tiada perbezaan berat badan yang ketara ditunjukkan di antara kumpulan – kumpulan kajian. Kumpulan 2, 3, 4, 5 dan 6 mencatatkan bilangan ACF (purata \pm sisihan piawai) dalam julat 84.4 ± 4.43 sehingga

179.5 ± 12.78. Bilangan ACF menunjukkan pengurangan sebanyak 45.3%, 51.4% dan 53.1% pada tikus masing-masing diberi 500, 1000 and 1500 mg/kg berat badan berbanding dengan kumpulan yang tidak menerima apa-apa rawatan ($p < 0.05$). Manakala untuk kepelbagaian ACF, ACF dengan 4,5 atau lebih crypts adalah jauh lebih rendah ($p < 0.05$) dalam tikus yang diberi mak dengan KSO-SFE berbanding dengan kumpulan kawalan positif (AOM sahaja). Penemuan kami menunjukkan KSO-SFE mengurangkan bilangan ACF pada tikus jantan spesies Sprague Dawley yang diaruh AOM. Kesan pengawalan KSO-SFE terhadap gen-gen yang terlibat dalam karsinogenesis kolon telah dikaji pada tikus yang diaruh AOM. Lima belas gen – gen telah dianalisis menggunakan sistem genetik GenomeLab GeXP. Keputusan analisis tersebut menunjukkan bahawa KSO-SFE telah meningkatkan ekspresi gen – gen penghalang tumor (APC dan p53), menurunkan ekspresi gen – gen penanda tumor (COX-2 dan β -catenin) dan tidak merubah ekspresi gen penindas tumor besar (Lats2) dan reseptor TNF apabila dibandingkan dengan kumpulan kawalan ($p < 0.05$). KSO-SFE juga telah menunjukkan kesanaruh keatas laluan apoptotik dengan menaikkan ekspresi gen – gen caspase (caspase 9, caspase 2 dan caspase 3), gen pro-apoptotik (bax dan bad) dan pada masa yang sama menurunkan ekspresi gen anti-apoptotik (bcl-2). Disamping itu, KSO-SFE juga mempengaruhi kitaran sel di mana ia menaikkan ekspresi perencat kitaran sel (p21, cip1) dan menurunkan ekspresi gen cyclin D1. Kajian ini juga dijalankan untuk menaksir kesan ketoksikan KSO-SFE terhadap tikus spesies Sprague Dawley. Kesan KSO-SFE pada dos 500, 1000, 1500 mg/kg berat badan/hari keatas berat badan, berat organ, hematologi analisis, klinik kimia, patologi dan ekspresi gen – gen yang berkait dengan ketoksikan diuji selama 90 hari.

Tidak kematian atau kesan buruk hasil dari perawatan dengan KSO-SFE pada parameter yang diukur sepanjang tempoh kajian kecuali iai merendahkan tahap kreatinin pada ketiga-tiga dos dan tahap kolesterol pada dos 1000 dan 1500 mg/kg. Perubahan ini tidak memberikan kesan ketoksikan yang signifikan. Kajian yang lebih lanjut telah dijalankan di peringkat genomik dengan menggunakan sistem genetik GenomeLab GeXP pada hati. Keputusan menunjukkan KSO-SFE tidak mengubah/mengganggu ekspresi gen – gen metabolisme xenobiotik, kerosakan DNA, penahanan kitaran sel, dan apoptosis. Berdasarkan kajian ini, telah dirumuskan bahawa 'No Observed Adverse Effect Level' (NOAEL) untuk KSO-SFE ialah pada dos 1500 mg/kg berat badan/hari. Sebagai kesimpulan, data yang diperoleh dari kajian ini menunjukkan potensi KSO-SFE sebagai agen kemopencegahan untuk kanser kolon.

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I certify that an examination Committee has met on (13th March 2013) to conduct the final examination of SitiAisyahBtAbdGhafar on her Doctor of Philosophy thesis entitled “**Effects of supercritical fluid extracted *hibiscus cannabinus* seed oil on *in vitro* and *in vivo* colon cancer**” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulation 1981. The committee recommends that the student be awarded the Doctor of Philosophy degree.

Members of the examination committee are as follows:

MdZuki bin Abu Bakar @ Zakaria, PhD

Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Fatimah bintiMdYusoff, PhD

Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Internal Examiner)

Fauziahbinti Othman, PhD

Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Ranjana Prasad Bird, PhD

Professor

Vice-President, Research
University of Northern British Columbia
Canada
(External Examiner)

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

Date:

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:

Maznah Ismail, PhD
Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Latifah Saiful Yazan, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

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

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

Date:

DECLARATION

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

SITI AISYAH BINTI ABD GHAFAR

Date: 13th March 2013

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