EFFECTS OF SUPERCritical FLUID-EXTRACTED HIBiscus CANNABINUSL. SEED OLd ON COLON CANCER IN VITRO AND IN VIVO

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

By

SITI AISYAH BINTI ABD GHAFA"
Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirements for the degree of Doctor of Philosophy.

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

By

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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\textsubscript{50} of 200 µg/ml. Nevertheless, IC\textsubscript{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.
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 GHAFAH

Mac 2013

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Kenaf (*Hibiscus cannabinus*) merupakantumbuhan fiber bernilaitinggidadaripadafamili *Malvaceae*, yang berasaldari India dan Afrikadansedang ditanamsekarakomersialdi Malaysia. MinyakbijiKenafmengandungiasid α-linolenik, fitosterolseperti β-sitosterol, vitamin E dan antioksidanlain yang mempunyaiciri-cirikemopencegahan.

Kajianinidijalankanbertujuanuntukmengkajikesanrawatanminyakbiji benih Kenaf daripada pengekstrakan bendalir superkritikal (KSO-SFE) terhadap kanser kolon secaramenggunakan sel kanser kolon manusia (HT29) dan kemopencegahankanserkolon. Dalammajinini, minyakbiji Kenaf telah diekstrak menggunakan kanaedahtenggekstrakan bendalir superkritikal (SFE) dengan permutasi 9 parameter yang berlainan berdasarkan parameter pada julat dari 200-600 bars dan suhu antara 40-80°C. Parameter yang telah digunakan adalah 200/40,
Kesanketoksikan 9 ekstrak KSO-SFE telah diuji ke atas kanser kolon manusia (HT29) dan selembarik fibroblast mencit (NIH 3T3) menggunakan assay MTS. Hasil ujian menunjukkan KSO-SFE yang diekstrak pada parameter 600/40 telah menunjukkan potensi yang paling tinggi dengan nilai IC\textsubscript{50}nya ke atas sel HT29 adalah pada 200 µg/ml. Manakala nilai IC\textsubscript{50} untuk sel NIH/3T3 pula tidak dapat ditentukan. Rawatan KSO-SFE ke atas sel HT29 dalaman analisis kitaran sel pula telah menunjukkan bahawa peningkatan yang ketaraterhadap pengumpulan sel-sel pada fase sub-G1. Ia menunjukkan KSO-SFE mengaruh aktiviti apoptotik ke atas sel HT29. Kesana ruhan apoptotik tilelah dikaji dengan lebih lanjut menggunakan Annexin V/PI dan pewarnaan AO/PI. Untuk kajian ciri kemopencegahan KSO-SFE, 60 ekor tikus spesis Sprague Dawley jantan telah dibahagikan kepada 6 kumpulan iaitu: 1) Kumpulan kawalan negatif [tidak diaruh dengan azoximetana AOM]; 2) Kumpulan kawalan positif (diaruh dengan AOM tetapi tidak menerima bahan pengemulsi); 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 subkutan several dalam tempoh 2 minggu. Tikus dibunuh pada akhir kajian pada hari ke 90. Tiada perbezaan berat badan yang ketara di antara kumpulan – kumpulan kajian. Kumpulan 2, 3, 4, 5 dan 6 mencatatkan bilangan ACF (purata±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% padatikus 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 ataupun lebih crypts adalah jauh lebih rendah (p <0.05) dalam tikus yang diberi KSO-SFE berbanding dengan kumpulan awalan positif (AOM sahaja). Penemuan kami menunjukkan KSO-SFE mengurangkan bilangan ACF padatikus spesis Sprague Dawley yang diaruh AOM. Kesimpulan dari analisis KSO-SFE terhadap gen-gen yang terlibat dalam karsinogenesiskolontelah dikaji pada patikus yang diaruh AOM. Lima belas gen – gentelah dinilaisi semenggunakan sistem genetik GenomeLabGeXP. Keputusan analisis tersebut menunjukkan bahawa KSO-SFE telah menurunkan 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 receptor TNF apabila dibandingkan dengan kumpulan awalan (p<0.05). KSO-SFE juga telah menunjukkan kesan atu pada laluan apoptosis dengan menaikkan ekspresi gen – gen caspase (caspase 9, caspase 2 dan caspase 3), gen pro-apoptotik (bax dan bad) dan padamasa yang samamunurunkan ekspresi gen anti-apoptotik (bcl-2). Disamping itu, KSO-SFE juga mempengaruhi kitaran sel di mana ia menurunkan ekspresi gen cyclin D1. Kajian ini juga telah dilakukan untuk menaksir kesan ketoksikan KSO-SFE terhadap kumpulan Sprague Dawley. Kesannya adalah KSO-SFE pada dos 500, 1000, 1500 mg/kg berat badan/hari ke atas berat badan, berat organ, hematologi, klinik, kimia, patologi, dan ekspresi gen – gen yang berkait dengan ketoksikan selama 90 hari.
Tiadakematianatakesanburukhasildaripadarawatandengan KSO-SFE pada parameter yang diukursepanjangtempohkajiankecuali merendahkan kreateniapadaketicagatiga dos dantahapkolesterol pada dos 1000 dan 1500 mg/kg. Perubahaninitidakmemberikankesanketoksikan yang signifikan. Kajian yang lebihlanjuttelahdijalankan di peringkat genomik dengan menggunakan sistem genetik GenomeLab GeXP padatisuhi. Keputusanmenunjukkan KSO-SFE tidak mengubah/menggangg ekspresi gen – gen metabolisme xenobiotik, kerosakan DNA, penahanan kitaransel, dan apoptosis. Berdasar 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 diperolehidari 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.

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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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