UNIVERSITI PUTRA MALAYSIA

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

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By

SITI AISYAH BINTI ABD GHAFAR

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

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Chairman: ProfessorMaznah 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 µg/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 *p*53), 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 (*p*21, *cip*1) 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.

Abstraktesisdikemukakankepada Senat Universiti Putra Malaysia
sebagaimemenuhikeperluanuntukijazahDoktorFalsafah

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: ProfesorMaznah Ismail, PhD

Fakulti: InstitutBiosains

Kenaf (*Hibiscus cannabinus*) merupakantumbuhan fiber bernilaitinggidaripadafamili*Malvaceae*, yang berasaldari India danAfrikadansedang di tanamsecarakomersialdi Malaysia.MinyakbijiKenafmengandungiasid α-linolenik, fitosterolseperti β-sitosterol, vitamin E danantioksidanlain yang mempunyaiciri-cirikemopencegahan.

KajianinidijalankanbertujuanuntukmengkajikesanrawatanminyakbijibenihKenafdaripada pengekstrakanbendalirsuperkritikal(KSO-SFE) terhadapkanserkolonsecaramenggunakanselkanserkolonmanusia(HT29) dankemopencegahankanserkolon.Dalamkajianini, minyakbijiKenaftelahdiekstrakmenggunakankaedahpengekstrakanbendalirsuperkritikal (SFE) denganpermutasi9 parameter yang berlainanberdasarkantekananpadajulatdari 200-600 barsdansuhuantara 40-80°C. Parameter yang telahdigunakanadalah200/40,

200/60, 200/80, 400/40, 400/60, 400/80, 600/40, 600/60 dan 600/80. Kesanketoksikan 9 ekstrak KSO-SFE telahdiujikeatasselkanserkolonmanusia (HT29) danselembrionik fibroblast mencit (NIH 3T3) menggunakanasai MTS. Hasilujianmenunjukkan KSO-SFE yang diekstrakpada parameter 600/40 telahmenunjukkanpotensi yang paling tinggidengannilai IC_{50}nya keatassel HT29 adalahpada 200 µg/ml. ManakalanilaiIC_{50}untuksel NIH/3T3 pula tidakdapatditentukan. Rawatan KSO-SFE keatassel HT29 dalamanalisiskitaransel pula telahmenunjukkanbahawapeningkatan yang ketaraterhadappengumpulansel-selpadafasa sub-G1.Iamenunjukkan KSO-SFE mengaruhaktivitiapoptotikkeatassel HT29.Kesanaruhanapoptotikinitelahdikajidenganlebihlanjutmenggunakanasai Annexin V/PI danpewarnaan AO/PI.Untukkajiancirikemopencegahan KSO-SFE, 60 ekortikusspesisSprague Dawleyjantantelahdibahagikansecararawakkepada 6 kumpulaniaitu1) kumpulankawalannegatif[tidakdiaruhdenganazoksimetanaAOM)]; 2) kumpulankawalanpositif(diaruhdenganAOM tetapitidakmenerimasebarangrawatan); 3) kumpulankawalanpembawa (diaruhdenganAOMdandirawatdenganbahanpengemulsi) 4) Kumpulan dirawatdengan500 mg/kg beratbadan KSO-SFE; 5) Kumpulan dirawatdengan 1000 mg/kg beratbadan KSO-SFE; 6) Kumpulan dirawatdengan1500 mg/kg beratbadan KSO-SFE. Padaumur 7 minggu, semuatikuskecualikumpulankawalannegatifmenerimasuntikan AOM pada dos 15 mg/kg beratbadansecarasubkutaneussekaliseminggudalamtempoh 2 minggu. Tikusdibunuhpadaakhirkajianiaitupadaharike 90.Tiadaperbezaanberatbadan yang ketaraditunjukkan di antarakumpulan – kumpulankajian.Kumpulan 2, 3, 4, 5dan 6 mencatatkanbilangan ACF (purata±sisihanpiawai) dalamjulat 84.4 ± 4.43 sehingga

179.5 ± 12.78. Bilangan ACF menunjukkanpengurangansebanyak45.3%, 51.4% dan 53.1%padatikusmasing-masingdiberi 500, 1000 and 1500 mg/kg beratbadanberbandingdengankumpulanyang tidakmenerimaapa-aparawatan ($p<0.05$).Manakalauntukkepelbagaian ACF, ACF dengan 4,5 ataulebih crypts adalahjauhlebihrendah ($p <0.05$) dalamtikus yang diberimakandengan KSO-SFEberbandingdengankumpulankawalanpositif (AOM sahaja). Penemuan kami menunjukkan KSO-SFE mengurangkanbilangan ACF padatikusjantanspesis Sprague Dawley yang diaruh AOM.Kesanpengawalaturan KSO-SFE terhadap gen-gen yang terlibatdalamkarsinogenesiskolontelahdikajipadatikus yang diaruh AOM. Lima belas gen – gentelahdianalisismenggunakansistemgenetikGenomeLabGeXP. Keputusananalisistersebutmenunjukkanbahawa KSO-SFE telahmeningkatkanekspresi gen – gen penghalang tumor (APC dan p53), menurunkanekspresi gen – gen penanda tumor (COX-2 dan β-catenin) dantidakmerubahekspresi gen penindas tumor besar (Lats2) danreseptor TNF apabiladibandingkandengankumpulankawalan ($p<0.05$). KSO-SFE jugatelahmenunjukkankesanaruhankeataslaluanapoptotikdenganmenaikkanekspresi gen – gen caspase (caspase 9, caspase 2 dancaspase 3), gen pro-apoptotik (baxdan bad) danpadamasa yang samamenurunkanekspresi gen anti-apoptotik (bcl-2). Disampingitu, KSO-SFE jugamempengaruhikitaransel di manaiamenaikkanekspresiperencatkitaransel (p21, cip1) danmenurunkanekspresi gen cyclin D1. Kajianinijugadijalankanuntukmenaksirkesanketoksikan KSO-SFE terhadaptikusspesis Sprague Dawley.Kesan KSO-SFE pada dos 500, 1000, 1500 mg/kg beratbadan/harikeatasberatbadan, berat organ, hematologianalisis, klinikalkimia, patologidanekspresi gen – gen yang berkaitdenganketoksikandiujiselama 90 hari.

Tiadakematianataukesanburukhasildaripadarawatandengan KSO-SFE pada parameter yang diukursepanjangtempohkajiankecualiiamerendahkantahapkreateninapadaketiga-tiga dos dantahapkolesterolpada dos 1000 dan 1500 mg/kg. Perubahaninitidakmemberikankesanketoksikan yang signifikan.Kajian yang lebihlanjuttelahdijalankan di peringkatgenomikdenganmenggunakansistemgenetikGenomeLabGeXPpadatisuhati.Keputusanmenunjukkan KSO-SFE tidakmengubah/mengganggueksprési gen – gen metabolismaxenobiotik, kerosakan DNA, penahanankitaransel, dan apoptosis.Berdasarkankajianini, telahdirumuskanbahawa 'No Observed Adverse Effect Level'(NOAEL) untuk KSO-SFE ialahpada dos 1500 mg/kg beratbadan/hari.Sebagaikesimpulan, data yang diperolehidarikajianinimenunjukkanpotensi KSO-SFE sebagaiagenkemopencegahanuntukkanserkolon.

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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:

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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:

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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.

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SITI AISYAH BINTI ABD GHAFAR

Date: 13th March 2013

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