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Kenaf seed supercritical fluid extract reduces aberrant crypt foci formation in azoxymethane-induced rats

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ABSTRACT

Kenaf (*Hibiscus cannabinus*) a plant of the family *Malvaceae*, is a valuable fiber plant native to India and Africa. Kenaf seeds contain alpha-linolenic acid, phytosterol such as β-sitosterol, vitamin E and other antioxidants with chemopreventive properties. In the present study we examined the hypothesis that kenaf seed 'supercritical fluid extract' (SFE) extract could suppress the early colon carcinogenesis in vivo by virtue of its bioactive compounds. To accomplish this goal, 60 male rats were randomly assigned to 5 groups which were (1) negative control group [not induced with azoxymethane (AOM)]; (2) positive control group (induced with AOM but received no treatment); (3) group treated with 500 mg/kg kenaf seed SFE extract; (4) group treated with 1000 mg/kg kenaf seed SFE extract; (5) group treated with 1500 mg/kg kenaf seed SFE extract. At 7 weeks of age, all rats except the negative control group received 15 mg/kg of AOM injection subcutaneously once a week for 2 weeks. Rats were euthanized at 13 weeks of the experiment. Number of ACF (mean ± SD) ranged from 84.4 ± 4.43 to 179.5 ± 12.78 in group 2, 3, 4, 5. ACF reductions compared with the untreated group were 45.3, 51.4 and 53.1% in rats fed with 500, 1000 and 1500 mg/kg body weight, respectively. There were no significant differences in weight gain among groups. Our finding indicates that kenaf seed SFE extract reduced AOM-induced ACF in Sprague–Dawley male rats.

1. Introduction

Colon cancer, also called colorectal cancer or bowel cancer, includes cancerous growths in the colon, rectum and appendix. As of 2008, it ranked as the third most commonly diagnosed cancer and the third leading cause of cancer death in both men and women in the Western world (American Cancer Society, 2010). In Malaysia, data from the 2005 National Cancer Registry indicated that colon cancer is the most common cancer among men whereas among women it is the third (National Cancer Registry, 2005). Colon carcinogenesis is a multistep process involving three distinct stages, initiation that alters the molecular message of a normal cell followed by promotion and progression that ultimately ends up with a phenotypically altered transformed cell (Vinay et al., 2003). Although epidemiological and experimental documentation that high dietary fat intake increases the risk of colon cancer is strong (Dwivedi et al., 2003; Nkondjock et al., 2003) there appears to be evidence that indicate dietary fat, depending on the source, quantity and fat composition is likely to reduce the incidence of colon cancer (Sambanthamurthi et al., 2000).

Aberrant crypt foci (ACF) are putative preneoplastic lesions that appear on the surface of colon rodents and were described as lesions consisting of large, thick crypts in methylene blue stained specimen colon after subsequent treatment with chemically induced colon carcinogens such as azoxymethane (AOM) (Bird, 1995). These preneoplastic lesions, which occur in the colonic mucosa of rodents, have also been observed at a higher frequency in the colons of patients with sporadic and inherited forms of colon cancer (Bird, 1995; Pretlow et al., 1992a,b; Roncucci et al., 1991; Bunpo et al., 2004). ACF are considered putative preneoplastic lesions because they share many morphological and biochemical characteristics of tumors, including a comparable increase in cell proliferation, higher expression of tumor associated antigens and dysplasia (Pretlow et al., 1994a,b; Sim et al., 1997). The molecular features of ACF defining them as colonic preneoplastic lesions have been studied exten-

Abbreviations: ACF, aberrant crypt foci; ACUC, Animal Care and Use Committee; ANOVA, analysis of variance; ALA, α-linolenic acid; AOM, azoxymethane; KSSE, kenaf seed SFE extract; SD, standard deviation; SFE, supercritical fluid extraction.

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sively (Cheng and Lai, 2003). Using ACF as a model for short term screening assay for colon carcinogenesis in laboratory rodents has so far proven to be a reliable biomarker (Williams et al., 2007; Ozkal et al., 2005; Boateng et al., 2007; No et al., 2007).

Kenaf (*Hibiscus cannabinus*) a plant of the family Malvaceae, is a valuable fiber plant native to India and Africa (Mohamed et al., 1995). Kenaf is one of the allied fibers of jute and shows similar characteristics. This plant contains various active components including tannins, saponins, polyphenolics, alkaloids, essential oils and steroids, which have long been prescribed in traditional folk medicine in Africa and India (Agbor et al., 2005; Kobaisy et al., 2001). Kenaf seeds yield vegetable oil that is edible for human consumption (Mohamed et al., 1995). Kenaf seed oil (extracted by soxhlet extraction) contains alpha-linolenic acid (ALA), the essential omega-3 fatty acid that is metabolized to eicosapentaenoic acid, a precursor of eicosanoids with anti-inflammatory and antithrombotic activity and also known as chemopreventive agent (Williams et al., 2007; Mohamed et al., 1995; Ruiz et al., 2002). Additionally, kenaf seed oil also contains phytosterol which possesses anti cancer, anti oxidant and lipid lowering cholesterol properties (Kritchevsky and Chen, 2005; Berger et al., 2004; Choi et al., 2003). However, oils that are extracted from organic solvents such as n-hexane or petroleum ether are always doubted for its safe consumption due to the incomplete solvent removal. Hence, supercritical fluid carbon dioxide extraction offers a better way of extraction.

Supercritical fluid extraction is the process of separating one component from another using supercritical fluid as the extracting solvent. A supercritical fluid is any substance at a temperature and pressure above its thermodynamic critical point. It has the unique ability to diffuse through solids like a gas, and dissolve materials like a liquid (Wang and Weller, 2006). Additionally, it can readily change in density upon minor changes in temperature or pressure. Moreover, it also enables the oil extraction to be carried out at low temperature and allows for complete removal of the solvent at the final stage of extraction (Ozkal et al., 2005). Compared with liquid solvents, SFE have several more advantages: (1) the dissolving power of a supercritical fluid solvent depends on its density which is highly adjustable by changing the pressure or/and temperature; (2) supercritical fluid has a higher diffusion coefficient and lower viscosity and surface tension than a liquid solvent, leading to a more favorable mass transfer. These properties make it suitable as a substitute for organic solvents in a process called supercritical fluid extraction (SFE) (Wang and Weller, 2006).

Although kenaf has been widely used traditionally to ease various symptoms and treat diseases, few studies on its chemopreventive activity have been reported. In the present study, we hypothesized that kenaf seed SFE extract may suppress colon carcinogenesis in an animal model. Hence this study was designed to meet two objectives. The first was to extract kenaf seed using supercritical fluid extraction (SFE) and the second was to examine the chemopreventive effect of kenaf seed SFE extract against early chemically induced azoxymethane (AOM) Sprague–Dawley male rats by determining the incidence of aberrant crypt foci (ACF) histologically.

2. Methods and materials

2.1. Preparation of kenaf seed SFE extract

Kenaf seed, V36 was purchased from the Malaysia Kenaf Tobacco Board, Pasir Putih, Kelantan. Kenaf seed was cleaned and dried at constant temperature (50 °C) overnight in oven (FD 115, Fisher Scientific). The final moisture content of the dried seeds was less than 5%. The dried seeds were stored at 4 °C until further use. Extracts of kenaf seed were prepared using the supercritical carbon dioxide fluid extractor (Thar 1000 F, USA) at 600 bars, 40 °C. This was done following a method developed in our lab as described by Chan and Maznah (2009) with slight modifications. In brief, 100 g of seed were ground using a stainless steel blender (Waring Commercial, USA) for 1 min and placed into a 1 l extraction vessel. The desired temperature and pressure were then set. The flow rate of carbon dioxide was set at 25 g/min and regulated by automated back pressure regulator. The extraction started after the desired temperature and pressure were obtained. The whole extraction process lasted for 2.5 h and the yield obtained was calculated.

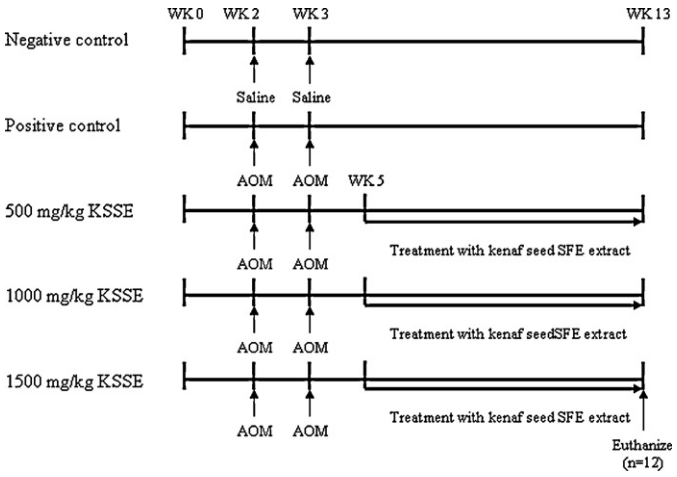

Fig. 1. Experimental design of the study. The rats were randomized into five groups. Group 1 is not treated with AOM, whereas groups 2–5 were injected with 15 mg/kg body weight subcutaneously with AOM at week 2 and 3. The experimental groups were as follow: negative control group (group 1), positive control group (group 2), 500, 1000, 1500 mg/kg fed with kenaf seed SFE extract (KSSE) (groups 3–5).

2.2. Animal management

Ethics approval for animal use in this study was obtained from the Animal Care and Use Committee (ACUC), Faculty of Medicine & Health Sciences, Universiti Putra Malaysia (ACUC no: UPM/FPSK/PADS/BRUUH/00256). Sixty male Sprague–Dawley rats (at 4 weeks of age) weighed between 90 and 150 g were divided randomly into five groups ($n = 12$) after 2 weeks of acclimatization period. They were kept in a well-ventilated room at the animal house under room temperature, 29–32 °C, 70–80% humidity with a 12 h light/dark cycle. The animals were fed with a basal diet for 13 weeks. The animals were cared for according to the guidelines of the Animal Care and Use Committee (ACUC) Faculty of Medicine & Health Sciences Universiti Putra Malaysia. They were divided into groups as follow (refer to Fig. 1): negative control group (group 1), positive control group (group 2), fed with 500, 1000 and 1500 mg/kg kenaf seed SFE extract (groups 3–5), respectively. Groups 3–5 were treated with kenaf seed SFE extract via gavage daily in the morning for 13 weeks. Water and food were given *ad libitum*. Body weight was recorded weekly.

2.3. Administration of carcinogen

At 7 weeks of age, rats received the first azoxymethane (AOM) injection (Sigma) followed by the second injection in the following week. This subcutaneous administration of AOM was given at a dosage of 15 mg/kg to all treatment groups with the exception of negative control (as shown in Fig. 1).

Table 1
Effect of kenaf seed SFE extract on AOM induced aberrant crypt foci (ACF) incidence in colon and body weight in Sprague–Dawley male rats.

Group	ACF counts			Liver weight (g)	Body weight (g/13 weeks)
	Proximal colon	Distal colon	Total		
−ve control	0	0	0	7.87 ± 1.01[a]	322.45 ± 52.76[a]
+ve control	77.5 ± 4.4[a]	101.2 ± 7.1[a]	179.5 ± 12.8[a]	8.43 ± 1.89[a]	307.64 ± 56.90[a]
500 mg/kg	42.5 ± 3.2[b]	57.3 ± 4.7[b]	98.1 ± 7.0[b]	8.38 ± 1.67[a]	308.80 ± 57.90[a]
1000 mg/kg	36.4 ± 2.1[b,c]	51.0 ± 3.0[b,c]	87.0 ± 4.09[b]	7.46 ± 1.34[a]	314.44 ± 50.24[a]
1500 mg/kg	29.6 ± 2.0[c]	43.7 ± 1.2[c]	84.4 ± 4.43[c]	8.78 ± 1.21[a]	336.25 ± 32.87[a]

Values are means ± SD ($n = 12$). Means in the same column with different letters differ significantly ($p < 0.05$) using Duncan Multiple Range Test.

2.4. Colon sample collection and counting of ACF

At the end of treatment period, the rats were sacrificed after anesthetized with diethyl ether. Enumeration of ACF was performed as described by Bird (1987). Colons were then removed and flushed with PBS. They were split open longitudinally and fixed overnight on a strip filter paper with RCL_2®. The colons were separated into two portions, proximal and distal and were stained with 0.2% methylene blue staining solution for 20 min. The subjected colons were then decolorized with 70% methanol for 4–6 min. Colons that retain staining were counted (Masako et al., 2005). Each portion was examined, and the total ACF was categorized based on multiplicity: (1) small (1–3 crypts per focus), (2) medium (4–5 crypts per focus), and (3) large (>5 crypts per focus).

2.5. Statistical analyses

Statistical analysis were performed using Statistical Package for Social Science (SPSS) version 13 and significant were accepted at $p < 0.05$. Data presented in the study were analyzed using ANOVA and values were given as mean ± SD and means were separated using Duncan Multiple Range Test.

3. Results

3.1. Yield of kenaf seed SFE extraction

6.9 kg dry weight of kenaf seed yielded on an average of 15–20% of extract after 150 min of extraction.

3.2. Body weight and organ weight of rats fed with kenaf seed SFE extract

There were no significant differences ($p < 0.05$) in the body weight between the control and rats fed with various concentrations of kenaf seed SFE extract (500, 1000, 1500 mg/kg). The final liver and kidney weights were also taken during termination after 9 weeks of treatment. There was no significant difference in liver or kidney weight among the groups. In addition, no toxicopathological findings were observed in liver and kidney of any rat examined.

3.3. Number of ACF in colon

Number of aberrant crypt foci incidence in the proximal and distal part of colon of the positive control group was significantly higher ($p < 0.05$) than in rats fed with kenaf seed SFE extract at all doses (Table 1). However, higher number of ACF was primarily observed in the distal colon, regardless of the treatment. Among the rats fed with various doses of kenaf seed SFE extract, there were significant differences ($p < 0.05$) in the number of ACF in both the proximal and distal colon. A significantly ($p < 0.05$) lower ACF was observed in the rats fed with 1500 mg/kg kenaf seed SFE extract compared to the positive control group. The highest percent reduction of ACF among treatment groups was observed in the group fed with 1500 mg/kg kenaf seed SFE extract (53.1%). Whereas aberrant crypt foci reduction in rats fed with 500 and 1000 mg/kg kenaf seed SFE extract was 45.3 and 51.4%, respectively.

3.4. Crypt multiplicity

The size of ACF is expressed as the number of aberrant crypt/ACF or crypt multiplicity. There were significant differences ($p < 0.05$) in the ACF with four aberrant crypts per focus among rats fed with kenaf seed SFE extract and the control group (Fig. 2). Aberrant crypt foci with four, five or more crypts were significantly lower ($p < 0.05$) in rats fed with kenaf seed SFE extract compared to the control group. ACF with more than five crypts were not detected in rats treated with kenaf seed SFE extract.

4. Discussion

Kenaf seed yields new edible oil suitable for human consumption (Mohamed et al., 1995; Nyam et al., 2009). There are many efforts to find optimal extraction method with the highest yield of oil possible and yet taking into consideration the safety of the oil. Supercritical carbon dioxide extraction (SFE) seems by far the best way for kenaf seed extraction. Yield of the oil extracted is one of the important criteria to be considered for commercialization of products such as drugs or specialty oil. Oil yield from this study ranged between 15 and 20% after 2.5 h of extraction from the starting material of 6.9 kg dried kenaf seed. Because of its high seed oil content (20%), it has been suggested that kenaf might be a profitable oil seed crop if consistent seed yield of 1200 kg/ha could be obtained (Dempsey, 1975). Currently in Malaysia the seed yield is approximately 700 kg/ha (Mardi, 2004).

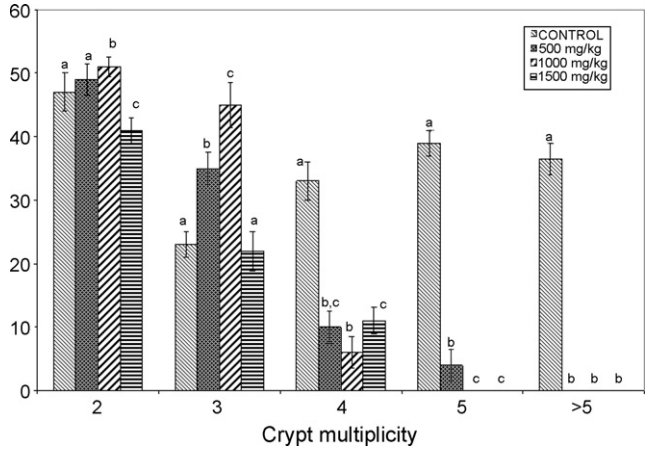

Fig. 2. Effect of treatment of kenaf seed SFE extract on number of crypt per focus in colon of male Sprague–Dawley rats. The data shown are means ± SD. Means in the same group with different letters differ significantly ($p < 0.05$) using Duncan Multiple Range Test. Number of crypts greater than or equal to 4 was significantly higher in control group. Aberrant crypt foci consisting four or more crypts have been reported to progress into putative malignant lesions.

The present study shows that kenaf seed SFE extract reduced the AOM-induced formation of colonic ACF in the Sprague–Dawley rats in a dose-dependent manner. From data shown (Table 1), kenaf seed SFE extract reduced ACF counts significantly ($p < 0.05$) compared to the untreated group. Regarding the distribution pattern of ACF in the colon, all four groups including the untreated group, showed a higher density of ACF in the distal colon. The findings are consistent with those of Cheng and Lai (2003).

The number of ACF, also termed as "crypt multiplicity", is one of the important parameters in evaluating ACF progression. ACF with a multiplicity of four and more crypts, correlate better with tumor incidence than the total amount of ACF (Bird, 1995; Pretlow et al., 1992a,b). The current study found that a clear reduction of more than 70% of crypts multiplicity (four and more crypts) when the animals were treated with kenaf seed SFE extract compared to control group.

The number of ACF and multiplicity per focus was reduced by kenaf seed SFE extract indicates that the observed effects were probably a result of the action of bioactive components such as vitamin E, phytosterol, α-linolenic acid and other antioxidants present in kenaf seed SFE extract (Mohamed et al., 1995; Nyam et al., 2009). This is further observed as kenaf seed SFE extract were given at three different levels of doses.

A study done by Shivapurkar et al. (1995) showed that vitamin E reduced a significant number ($p < 0.05$) of AOM-induced colon tumors. Vitamin E seems to hold promise as a potential cancer preventive agent and should be further investigated. In addition, there are some other studies that have primarily focused on the potential benefits of the individual components of vitamin E such as tocotrienols and tocopherols and their roles as biological antioxidant, indicating that chemopreventive properties of tocopherol and tocotrienol may be due to their additive and/or synergistic effect (Fraser and Bramley, 2004). Tocotrienols as one of the vitamin E constituents were involved in inducing apoptosis and growth inhibition in human breast cancer cell (MCF7). This was due to increase in NK cells and B-lymphocytes. NK cells have been associated with having cytotoxic activity against tumor cells (Guthrie et al., 1997; Nesaretnam et al., 1995, 1998, 2002). Other studies also showed that rats fed high vitamin E diets at 600 and 100 mg/kg, respectively, had lower incidence of colorectal tumors and carcinoma in 1,2 dimethylhydrazine induced colon cancer (Cook and McNamara, 1980; Sumiyoshi, 1985).

Phytosterol is one of the bioactive components in kenaf seed SFE extract. They have a structure similar to cholesterol but with some modifications. These modifications involve the side chain and include the addition of double bond and/or methyl or ethyl group (Awad and Fink, 2000). The most common dietary phytosterol are β-sitosterol, campesterol and stigmasterol. β-Sitosterol (70%) are the most abundant phytosterol in hexane-extracted kenaf seed oil (Mohamed et al., 1995). Phytosterol especially β-sitosterol has been proven to show protective role from chemically induced colon cancer (Raicht et al., 1980). Usually the development of colon cancer is preceded by an increase in cell proliferation in the colon mucosa for example hyperplasia. This condition is considered to be a risk factor for development of colon cancer (Lipkin et al., 1985). Several investigators examined the effect of dietary phytosterol on colonocyte proliferation in mice and rats indicated that dietary phytosterol reduce the proliferative compartment of the crypt and cell proliferation (Janezic and Rao, 1992; Awad et al., 1997; Dreshner et al., 1982).

Other minor bioactive components present in kenaf seed SFE extract that might help in reducing ACF formation are α-linolenic acid the essential ω-3 fatty acid that is metabolized to eicosapentaenoic acid, a precursor of eicosanoids with anti-inflammatory and antithrombotic activity (Ruiz et al., 2002) and is also known as chemopreventive agent (Williams et al., 2007). A recent study also found that kenaf seed SFE extract exerts high antioxidant activities which may also help in reducing ACF formation (Chan and Maznah, 2009).

On the other hand, because of the following limitations in our current study, further investigation is necessary. First, the chemopreventive ability of kenaf seed SFE extract was studied in the early stages of carcinogenesis using preneoplastic lesions (ACF), which may not fully covered our hypothesis. Studying chemopreventive property via observing the reduction in tumor formation may provide a more clearly chemopreventive ability of kenaf seed SFE extract, which require more than 30 weeks of experimental period. Additionally, the inclusion of biomarkers such as biochemical, molecular and genetic biomarker studies are needed in interpreting ACF inhibition to fully elucidate the spectrum of chemopreventive mechanism of kenaf seed SFE extract.

Kenaf seed SFE extract which was used in this study contains several bioactive components such as phytosterols, vitamin E, α-linolenic acid and other antioxidant that significantly reduced ($p < 0.05$) AOM induced ACF formation as well as crypts/focus in male Sprague–Dawley rats compared to control group. Based on the results of this experiment, it shows that kenaf seed SFE extract may be able to reduce colon cancer risk and may therefore act as an effective chemopreventive agent.

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