NUTRITIONAL AND TOXICOLOGICAL STUDIES OF PLANT-BASED MILK FROM KENAF (*Hibiscus cannabinus* L.) SEEDS

By

NUR SYAMIMI BINTI ZAINI

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Chair : Norhasnida Binti Zawawi, PhD
Faculty : Food Science and Technology

Kenaf (*Hibiscus cannabinus* L.) is an herbaceous fibre crop that belongs to the Malvaceae family with exceptional economic values. Kenaf seed has been reported to contain rich protein content, high unsaturated fatty acids, and plenty of beneficial bioactive compounds. This suggests that kenaf seeds may exert favourable health impacts such as antihypercholesterolemic and anti-oxidative effects on humans. Derived from the underexploited kenaf seeds, a novel non-dairy beverage namely plant-based kenaf seed milk (KSM) can be produced. Being a novel food product, nutritional and safety evaluations of KSM are essential to ensure safe human consumption. Therefore, this study aimed: (i) to assess the nutritional and antinutritional composition of KSM (whole seed and dehulled seed varieties), (ii) to investigate the acute (14-day) oral toxicity effects of a single-dose KSM (whole seed variety) consumption at a dose of 9.2 ml/kg body weight, and (iii) to investigate the subacute (28-day) oral toxicity effects of daily KSM (whole seed variety) consumption at doses of 3.1, 6.1, and 9.2 ml/kg body weight. KSM (whole and dehulled seed varieties) was significantly higher in total carbohydrates and lower in crude protein than SM. KSM (whole seed variety) contained significantly lower crude fat content than SM, whereas crude fat in KSM (dehulled seed variety) was the highest. Glucose, fructose, and sucrose contents in KSM (whole seed variety) were significantly lower than in SM. KSM (dehulled seed variety) did not contain glucose but contained the highest fructose and sucrose. Potassium, phosphorus, and magnesium were the major minerals in KSM (whole and dehulled seed varieties), with phosphorus and magnesium present in significantly higher amounts than in SM. The total phenolic content and antioxidant activities of KSM (whole and dehulled seed varieties) were significantly higher than those of SM. KSM (whole and dehulled seed varieties) also presented lower lipid peroxidability than SM throughout the 3-day storage at chilled temperature. Qualitative analysis of chemical compounds by ultra-high performance liquid chromatography-mass spectrometry revealed that KSM shared similarities in the presence of several chemical components with

SM especially apigenin, citric acid, gluconic acid, palmitic acid, oleic acid, linoleic acid, and 13-Hydroxyoctadecadienoic acid. KSM (whole seed variety) was significantly lower in phytates, oxalates, and total saponins, and higher in total tannins than those of SM. Dehulling of kenaf seed significantly increased all antinutritional factors in KSM (dehulled seed variety). In the acute oral toxicity study, KSM (whole seed variety) was administered at a single high dose of 9.2 ml/kg body weight and animals were observed closely for 14 days. Next, in the subsequent subacute toxicity study, KSM (whole seed variety) was given at doses 3.1 ml/kg body weight (low), 6.1 ml/kg body weight (medium), and 9.2 ml/kg body weight (high) to different animal groups (n = 5) for consecutive 28 days. All treatments resulted in no toxicity signs in terms of growth, feed and water intakes, organ weights and histopathology, and mortality. Haematological parameters were unaffected by acute and subacute KSM consumption. Serum biochemical analysis revealed that subacute KSM consumption induced a significant favourable reduction in alanine aminotransferase level at the medium dose by 40%. Additionally, low-density lipoprotein cholesterol and total cholesterol increased significantly at the low dose (by approximately twice) and high dose (by 33.33%), respectively. Nonetheless, KSM was observed to have equivalent effects with SM in improving high-density lipoprotein cholesterol levels, at both low and medium doses. Hence, the oral lethal dose (LD_{50}) and the no-observed-adverse-effect-level (NOAEL) of KSM were greater than 9.2 ml/kg (or 1533 mg/kg) body weight. Further subchronic and chronic oral toxicity studies and human trials are recommended as there is the potential of developing KSM as a plant-based milk alternative.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

KAJIAN NUTRISI DAN TOKSIKOLOGI SUSU BERASASKAN TUMBUHAN DARIPADA BIJI KENAF (*Hibiscus cannabinus* L.)

Oleh

NUR SYAMIMI BINTI ZAINI

Disember 2022

Pengerusi : Norhasnida Binti Zawawi, PhD
Fakulti : Sains dan Teknologi Makanan

Kenaf (*Hibiscus cannabinus* L.) merupakan tanaman gentian herba yang tergolong dalam keluarga Malvaceae dengan nilai ekonomi yang sangat baik. Biji kenaf telah dilaporkan mengandungi kandungan protein yang tinggi, asid lemak tak tepu yang tinggi, dan banyak sebatian bioaktif yang bermanfaat. Ini menunjukkan bahawa KSM boleh memberikan kesan kesihatan yang menggalakkan seperti kesan antihiperkolesterolemik dan anti-oksidatif ke atas manusia. Diperolehi daripada benih kenaf yang kurang dieksploitasi, minuman bukan tenusu baharu iaitu susu berasaskan biji tumbuhan kenaf (KSM) boleh dihasilkan. Sebagai produk makanan baru, penilaian nutrisi dan keselamatan KSM adalah penting untuk memastikan penggunaan manusia yang selamat. Oleh itu, kajian ini bertujuan: (i) untuk menilai komposisi pemakanan dan antinutrisi KSM (varieti biji dengan kulit dan biji yang dibuang kulit), (ii) untuk menyiasat kesan ketoksikan oral akut (14 hari) KSM (varieti biji dengan kulit) pada dos tunggal 9.2 ml/kg berat badan, dan (iii) untuk menyiasat kesan ketoksikan oral subakut (28 hari) KSM (varieti biji dengan kulit) pada dos 3.1, 6.1, dan 9.2 ml /kg berat badan. KSM (varieti biji dengan kulit dan biji yang dibuang kulit) adalah jauh lebih tinggi dalam jumlah karbohidrat dan lebih rendah dalam protein kasar daripada SM. KSM (varieti biji dengan kulit) mengandungi kandungan lemak kasar yang jauh lebih rendah daripada SM, manakala lemak mentah dalam KSM (varieti biji yang dibuang kulit) adalah yang tertinggi. Kandungan glukosa, fruktosa, dan sukrosa dalam KSM (varieti biji dengan kulit) adalah jauh lebih rendah daripada SM. KSM (varieti biji yang dibuang kulit) tidak mengandungi glukosa tetapi mengandungi fruktosa dan sukrosa tertinggi. Kalium, fosforus, dan magnesium adalah mineral utama dalam KSM (varieti biji dengan kulit dan biji yang dibuang kulit), dengan fosforus dan magnesium hadir dalam jumlah yang jauh lebih tinggi daripada SM. Jumlah kandungan fenolik dan aktiviti antioksidan KSM (varieti biji dengan kulit dan biji yang dibuang kulit) adalah jauh lebih tinggi daripada SM. KSM (varieti biji dengan kulit dan biji yang dibuang kulit) juga menunjukkan kebolehoksidaan lipid yang lebih rendah daripada SM sepanjang penyimpanan

3 hari pada suhu sejuk. Analisis kualitatif sebatian kimia oleh kromatografi cecair berprestasi tinggi-spektrometri jisim mendedahkan bahawa KSM berkongsi persamaan dengan kehadiran beberapa komponen kimia dengan SM terutamanya apigenin, asid sitrik, asid glukonat, asid palmitik, asid oleik, asid linoleik, dan asid 13-Hydroxyoctadecadienoic (13-HODE). KSM (varieti biji dengan kulit) jauh lebih rendah dalam fitat, oksalat, dan jumlah saponin, dan lebih tinggi dalam jumlah tannin berbanding SM. Pembuangan kulit biji kenaf meningkatkan semua faktor antinutrisi dengan ketara dalam KSM (varieti biji yang dibuang kulit). Dalam kajian ketoksikan oral akut, KSM (varieti biji dengan kulit) diberikan pada dos tunggal sebanyak 9.2 ml/kg berat badan dan haiwan diperhatikan dengan teliti selama 14 hari. Seterusnya, dalam kajian ketoksikan oral subakut, KSM (varieti biji dengan kulit) diberikan pada dos 3.1 ml/kg berat badan (rendah), 6.1 ml/kg berat badan (sederhana), dan 9.2 ml/kg berat badan (tinggi) kepada kumpulan haiwan yang berbeza (n = 5) selama 28 hari berturut-turut. Semua rawatan tidak menghasilkan tanda ketoksikan dari segi pertumbuhan, makanan dan pengambilan air, berat organ dan histopatologi, dan kematian. Parameter hematologi tidak terjejas oleh konsumasi KSM akut dan subakut. Analisis biokimia serum mendedahkan bahawa konsumasi KSM secara subakut menyebabkan pengurangan yang menggalakkan dalam tahap alanine aminotransferase sebanyak 40% dengan ketara pada dos sederhana. Selain itu, kolesterol lipoprotein berketumpatan rendah dan jumlah kolesterol masing-masing meningkat dengan ketara pada dos rendah (lebih kurang dua kali ganda) dan dos tinggi (sebanyak 33.33%). Walau bagaimanapun, KSM diperhatikan mempunyai kesan yang setara dengan SM dalam meningkatkan tahap kolesterol lipoprotein berketumpatan tinggi, pada kedua-dua dos rendah dan sederhana. Oleh itu, dos maut oral (LD_{50}) dan tahap tiada kesan buruk diperhatikan (NOAEL) KSM adalah lebih tinggi daripada 9.2 ml/kg (atau 1533 mg/kg) berat badan. Kajian ketoksikan oral subkronik dan kronik serta kajian manusia secara lanjut adalah disyorkan kerana terdapat potensi untuk membangunkan KSM sebagai susu alternatif berasaskan tumbuhan.

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

Norhasnida Binti Zawawi, PhD
Senior Lecturer
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Chairman)

Roselina Binti Karim, PhD
Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Member)

Ahmad Faizal Bin Abdull Razis, PhD
Associate Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Member)

____

ZALILAH BINTI MOHD SHARIFF, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 13 April 2023

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Signature:	
Name of Chairman of Supervisory Committee:	Dr. Norhasnida Binti Zawawi
Signature:	
Name of Member of Supervisory Committee:	Prof. Dr. Roselina Binti Karim
Signature:	
Name of Member of Supervisory Committee:	Assoc. Prof. Dr. Ahmad Faizal Bin Abdull Razis

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LIST OF ABBREVIATIONS

α	Alpha
β	Beta
γ	Gamma
λ	Lambda (symbol for wavelength)
ω	Omega
°Brix	Degree Brix
°C	Degree celsius
%	Percentage
µ	Micro
µmol	Micromoles
g	Gram
L	Litre
M	Molar
N	Normality
p-value	Probability value
R^2	Coefficient of determination
Rpm	Rotation per minute

CHAPTER 1

INTRODUCTION

1.1 Background

Milk is a liquid food that is rich in essential nutrients such as protein, lipids, carbohydrates, calcium, phosphorus, and B vitamins, all of which are important for promoting growth and bone health (Chalupa-Krebzdak et al., 2018). Milk products can be divided into two categories: those derived from animals and those derived from plants. Among the many types of animal-based milk or mammal milk available are cow's milk, goat milk, and human milk, to name a few. Plant-based milk is water extracts that are obtained as a consequence product of grinding raw materials (seeds, nuts, legumes, cereals, and grains) with water to obtain a smooth texture and a creamy consistency. Plant-based milk has a consistency and colour similar to those of animal-based milk (Jeske et al., 2017). The flavour is based on the types of flavonoids that are present in the crop. For example, isoflavones are responsible for the bitter and beany taste of legumes such as soybeans and chickpeas (Dini, 2019). Nuts, such as almonds, peanuts, and hazelnuts, naturally produce plant-based milk with a rich nutty flavour (Sethi et al., 2016). Plant-based milk, such as soybean milk and almond milk are termed 'functional foods' because they provide a wide range of health advantages, rather than simply providing the sensation of satiety and quenching thirst. Plant-based milk is low in saturated fat content and is absent of lactose and cow's milk protein. Furthermore, bioactive components are also present in abundant quantities in plant-based milk, which includes polyphenolic compounds, phytosterols, isoflavones, and essential fats, all of which have a wide range of beneficial effects (e.g., antidiabetic, anticancer, neuroprotective, cardioprotective) on the health (Dini, 2019; Aydar et al., 2020). The market's demand for functional beverages is increasing year after year, and this trend is expected to continue. Novel plant-based milk derived from underutilized seeds, beans, cereals, pseudocereals, and legumes is constantly being developed and commercialized to accommodate customers' demands (Sethi et al., 2016; Zandona et al., 2021). However, some raw materials contain allergenic components such as soybeans, almonds, peanuts, and kidney beans, among others (Jeske et al., 2017; Paul et al., 2020). For this reason, other alternatives to plant-based milk must be explored to minimize allergies.

Kenaf or *Hibiscus cannabinus* L., from the Malvaceae family, is an annual herbaceous tropical plant that is rich in fibre, oil, and energy. In Malaysia, research on kenaf has been extensively carried out since 1999 to study its potential as another industrial crop in the country. National Economic Advisory Council first started the project on kenaf to study the kenaf potential as a new industrial crop in Malaysia to replace tobacco, which then replaced National Tobacco Board (NTB) with National Kenaf and Tobacco Board (NKTB) in 2010

(Hill, 2013). Malaysian Agricultural Research and Development Institute (MARDI), Malaysian Rubber Board (MRB), Malaysian Palm Oil Board (MPOB) and Universiti Putra Malaysia (UPM) are in charge of the research and development (R&D) of kenaf since 2000 (Tan et al., 2017). The processing of kenaf plants for their fibre and leaves results in the waste of the other parts of the plants, with kenaf seeds being one of the by-products (Mariod et al., 2017). Only 2% of the total seeds produced are used to grow seedlings for a hectare of kenaf plantation and the remaining are typically discarded after processing (Rajashekher et al., 1993; Chan, 2019).

In recent decades, the utilization of kenaf seeds has become great attention in the food industry, alongside the automotive, pharmaceutical, and cosmetics industries. Several types of research have been carried out in the past few years in an attempt to transform kenaf seeds into kenaf seed-based food products. For example, kenaf seed oil (KSO) can be extracted from kenaf seeds by solvent extraction or supercritical fluid extraction that has the potential as an edible oil (Cheng et al., 2016). KSO contains bioactive compounds that possess biological activities such as anti-oxidation (Chan & Ismail, 2009), anti-inflammatory (Nyam et al., 2016), and anticancer (Foo et al., 2012; Abd Ghafar et al., 2013; Wong et al., 2014; Yazan et al., 2016). Furthermore, from the extraction of KSO, defatted kenaf seed meal (DKSM) can be derived. DKSM has the potential to be marketed as flour with a higher protein, carbohydrate, total phenolic and flavonoid contents, and antioxidant activity as compared to wheat, rice, and sweet potato flours (Chan et al., 2013). Additionally, when soaked kenaf seeds are ground with water, kenaf seeds can produce a milk-like solution just like in the production of soybean milk (Karim et al., 2020).

1.2 Problem statements

Plant-derived food products are frequently deemed as ‘natural’ and ‘safe’, but some plant species with potential as protein sources naturally contain toxic constituents which could cause health problems, especially after prolonged consumption (Liener, 1970). Furthermore, despite considerable R&D initiatives that have been conducted on KSO and DKSM, currently, there is limited research regarding the development, physicochemical composition, and health aspects of food products from kenaf seed milk (KSM). Only in the past few years, there have been recent studies conducted to innovate food products from KSM such as tofu, cheese, and milk beverage (Siti Nur Shafiqah, 2018; Ibrahim et al., 2020; Karim et al., 2020). R&D of KSM-based food products is expected to contribute to an increased trend in the valorization and commercialization of this underutilized seed (Antonio Teixeira et al., 2018). What is more, consumers would have an even wider variety of choices in terms of plant-based food and beverage products in the market (Sethi et al., 2016; Wolf et al., 2020).

The safety of KSM for future human consumption should be fully explored by conducting safety analyses before introducing innovative food products derived from KSM to the market. Regulation (EC) No 258/97 also recommended that novel foods that have not had a history of safe use should be evaluated for their safety (EC, 1997). Even though numerous parts of the kenaf plant (e.g., leaves, seeds) have a traditional history of usage as food in Africa, where it grows in abundance, it is important to scientifically demonstrate the safety of kenaf seed and kenaf seed milk (OECD, 1993; de Boer & Bast, 2018). When toxic effects arise after ingestion, the maximum tolerable dose or concentration of the test substance needs to be determined, which could be carried out through a series of toxicological assessments (acute, subacute, subchronic, chronic) as per the Organisation for Economic Cooperation and Development (OECD) guidelines. Moreover, not only must it be demonstrated that KSM is safe, but its nutritional and antinutritional values should also be highlighted, as nutritional information of food is one of the most important drivers of human well-being (Chen & Antonelli, 2020). Therefore, these led to the objectives of this study.

1.3 Research objectives

General objective:

To evaluate the nutritional composition and toxicity of plant-based milk from kenaf (*Hibiscus cannabinus* L.) seeds.

Specific objectives:

1. To assess the nutritional and antinutritional composition of KSM (whole seed and dehulled seed varieties).

2. To investigate the acute (14-day) oral toxicity effects of a single dose KSM (whole seed variety) consumption at a dose of 9.2 ml/kg body weight.

3. To investigate the subacute (28-day) oral toxicity effects of daily KSM (whole seed variety) consumption at doses of 3.1, 6.1, and 9.2 ml/kg body weight.

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