STUDIES OF CAPABILITY, STABILITY, TOXICITY AND BIOEFFICACY OF KENAF BASED CELLULOSE NANOFIBER AS LARVICIDE CARRIER FOR MOSQUITO CONTROL

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by

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LIST OF SYMBOLS

% v/v	percentage volume per volume
% w/w	percentage weight per weight
% w/v	percentage weight per volume
°	degree
°C	degree Celsius
cm^{-1}	centimetre $power^{-1}$
K	Kelvin
kg/m^3	kilogram per cubic meter
kV	kilovolt
M	Molar
MΩ	megaohm
m^2/g	square meter per gram
m^3	cubic meter
mA	milliampere
mg/kg bw	milligram per kilogram bodyweight
mg/L	milligram per litre
mcg/L	microgram per litre
mM	millimolar
nm	nanometre
r^2	coefficient of determination
μL	microlitre
ζ	Zeta potential
θ	theta

LIST OF ABBREVIATIONS

AChE	Acetylcholinesterase
AI	Active ingredient
BET	Brunauer–Emmett–Teller
BJH	Barret-Joyner-Halenda
bw	bodyweight
CAS	Chemical Abstracts Service
CNF	Cellulose nanofiber
CrI	Crystallinity index
DO	Dissolved oxygen
DLVO	Derjaguin – Landau – Verwey – Overbeek (DLVO) theory
EC_{50}	the estimated concentration to immobilize 50 % of the daphnids within a stated exposure period
EDL	Electric Double Layer
FESEM	Field Emission Scanning Electron Microscope
GHS	Globally Harmonized System
GI	Gastrointestinal
HPLC	High Performance Liquid Chromatography
IgG	Immunoglobulin G
IS	Internal standard
KCNF	Kenaf cellulose nanofiber
KCNF+T	Kenaf cellulose nanofiber impregnated with temephos
LC_{50}	median lethal concentration
LD_{50}	median lethal dose

LOD	Limit of detection
LOQ	Limit of quantification
LF	Loading fold
PE	Polyethylene
ppm	parts per million
RRT	Relative retention time
RSD	Relative standard deviation
SD	Standard deviation
TEM	Transmission Electron Microscopy
TM	Temephos solution without KCNF
UV-Vis	Ultraviolet-visible (UV-Vis) spectrophotometry

KAJIAN KEUPAYAAN, KESTABILAN, KETOKSIKAN DAN BIOEFIKASI SERAT NANO SELULOSA KENAF SEBAGAI PENGANGKUT LARVISID UNTUK KAWALAN NYAMUK

ABSTRAK

Penyakit bawaan nyamuk semakin meningkat setiap tahun dan menyebabkan jutaan kematian di seluruh dunia. Penyakit ini disebarkan melalui gigitan nyamuk yang dijangkiti virus. Oleh itu, salah satu langkah kawalan yang berkesan untuk mengurangkan kadar jangkitan ialah menggunakan larvisid seperti temefos. Pada masa ini, bahan pengangkut yang digunakan didalam formulasi larvisid menimbulkan kebimbangan terhadap alam sekitar kerana diperbuat daripada sumber yang tidak boleh diperbaharui. Serat nano selulosa dari kenaf dilihat sebagai pengganti lestari untuk pengangkut larvisid. Objektif kajian ini adalah untuk menentukan keupayaan serat nano selulosa kenaf (KCNF) untuk menyerak dan memuat temefos, kestabilan KCNF jejal bersama temefos (KCNF+T) dalam mengekalkan temefos, ketoksikan akut KCNF dan KCNF+T terhadap mamalia dan ketoksikan akut persekitaran KCNF terhadap haiwan akuatik serta menentukan bioefikasi KCNF+T sebagai pengangkut larvisid terhadap larva nyamuk *Aedes aegypti.* Keupayaan KCNF untuk menyerak ditentukan mengguna pakai kaedah OECD TG 318 dan keupayaan muatan ditentukan menggunakan isipadu air yang berbeza yang diletakkan ke atas KCNF kering. Jumlah temefos yang dijejal ke atas KCNF+T diukur menggunakan HPLC. KCNF didapati memiliki keupayaan serakan yang tinggi dengan 50 % keterserakan di dalam enam daripada sembilan keadaan hidrokimia yang diuji dan keupayaan muatan 6 kali ganda dari jisimnya. Jumlah temefos yang dijejal ke atas KCNF didapati 1.94 %w/w menunjukkan efikasi jejal sebanyak 97 %. Kestabilan KCNF+T dalam mengekalkan

temefos lebih daripada 12 bulan tempoh penyimpanan selepas dijejal dan setelah diserakkan di dalam air selama 1, 3 dan 5 bulan juga diuji dengan menggunakan HPLC. Dapatan kajian menunjukkan KCNF+T berupaya mengekalkan 98 % temefos yang dijejalkan selepas tempoh penyimpanan dan sebanyak 30 hingga 7 % temefos kekal selepas diserakkan didalam air. Ketoksikan mamalia diuji ke atas tikus betina Spargue Dawley menggunakan kaedah OECD TG 402, manakala ketoksikan akut persekitaran diuji ke atas kutu air (*Dahphia magna*) dan ikan zebra (*Dania rerio*) menggunakan kaedah OECD TG 202 dan OECD TG 203. Pengujian ketoksikan terhadap mamalia menunjukkan bahawa nilai LD_{50} KCNF dan KCNF+T ialah > 2000 mg/kg bw manakala nilai EC_{50} dan LC_{50} KCNF ialah > 100 mg/L untuk ketoksikan akut persekitaran. Bahan KCNF dan KCNF+T dikategorikan sebagai bahan yang tidak diklasifikasi sebagai berbahaya mengikut klasifikasi GHS. Seterusnya, ujian bioefikasi KCNF+T dijalankan ke atas larva nyamuk *Ae. aegypti* menunjukkan nilai LC_{50} ialah 0.005 mg/L pada 24 dan 48 jam, iaitu dibawah 0.1 mg/L kepekatan yang dicadangkan oleh WHO. Secara keseluruhan, kajian menyimpulkan bahawa KCNF berupaya untuk menyerak dan memuat temefos sebagai pengangkut dengan kestabilan untuk mengekalkan temefos selepas disimpan dan diserakkan serta mempunyai kesan toksik yang rendah terhadap mammalia dan haiwan akuatik.

STUDIES OF CAPABILITY, STABILITY, TOXICITY AND BIOEFFICACY OF KENAF BASED CELLULOSE NANOFIBER AS LARVICIDE CARRIER FOR MOSQUITO CONTROL

ABSTRACT

Mosquito-borne diseases are increasing every year and causing million deaths worldwide. The diseases are transmitted from the bite of an infected mosquito. Thus, it requires effective control measures to reduce the transmission rate, and one of the control strategies is using larvicide such as temephos. Currently, the carrier materials used in the larvicide formulation raised environmental concerns due to their non-renewable sources. The cellulose nanofiber from kenaf is seen as a sustainable replacement for larvicide carrier. The objectives of this study are to determine the capability of kenaf cellulose nanofiber (KCNF) to disperse and load temephos, the stability of KCNF impregnated with temephos (KCNF+T) in retaining temephos, the acute systemic toxicity of KCNF and KNCF+T in mammals, the acute ecotoxicity of KCNF in the aquatic organisms and the bioefficacy of KCNF+T as a larvicide carrier against *Aedes aegypti* mosquito larvae. The capability of KCNF to disperse is determined using the method adopted from OECD TG 318 and loading capability is evaluated using various water volume loaded on the dry KCNF. The amount of temephos impregnated on KCNF is quantified by HPLC analysis. KCNF is found to have high dispersion capability with more than 50 % dispersibility in six out of nine tested hydrochemical conditions and loading capability 6-fold of its mass. Amount of temephos impregnated on KCNF is found 1.94 % w/w reflecting 97 % impregnation efficacy. The stability of KCNF+T in retaining temephos at more than 12 months of post impregnation storage and after redispersion in water at 1, 3 and 5 months are analysed using HPLC. The result showed that KCNF+T able to retain 98 % of impregnated temephos after the storage and 30 to 7 % of temephos remained after redispersed in water. The acute toxicity is experimented on Sprague Dawley female rats using the OECD TG 402 method while the acute ecotoxicity is observed on water fleas (*Daphnia magna*) and zebrafish (*Dania rerio*) using OECD TG 202 and OECD TG 203 method, respectively. The acute toxicity in mammalian showed that the LD_{50} value of KCNF and KCNF+T were > 2000 mg/kg bw while EC_{50} and LC_{50} of KCNF was > 100 mg/L for acute ecotoxicity. KCNF and KCNF +T are categorised as non-classified hazardous substances for both acute dermal toxicity and acute ecotoxicity according to the GHS classifications. Subsequently, the KCNF+T bioefficiency is tested against *Ae. aegypti* mosquito larvae resulting the LC_{50} value of 0.005 mg/L at 24- and 48- hours, which is below 0.1 mg/L of the recommended concentration by WHO. Overall, the study suggests that the KCNF is capable to disperse and load temephos as a carrier with stability in retaining temephos after storage and redispersion while having low toxicity in mammals and aquatic organisms.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Cellulose is a basic component of the fibril structure from plant cell wall and has high mechanical strength, high strength-to-weight ratio and flexibility (Dufresne, 2013). Nanocellulose is a term referring to cellulose materials broken into smaller size at 1-100 nm in range (Charreau *et al.*, 2013). It has been identified as a potential bionanomaterial because it is renewable, sustainable and one of the most abundant resources on Earth (Klemm *et al.*, 2011; Dufresne, 2013). Nanocellulose can be produced from various plant sources including rubber, empty palm oil fruit bunches and kenaf through various methods such as chemical treatment, using enzyme and mechanical treatments namely ball milling, high pressure homogenisation, grinding, refining, cryo-crushing, and others (Nechyporchuk *et al.*, 2016) . Different synthesis methods produced different types of nanocellulose in which chemical treatment produced cellulose nanocrystal (CNC), while mechanical treatment made cellulose nanofibre (CNF) (Mtibe *et al.*, 2018). Among the two types of nanocellulose, CNF has obtained considerable attention in various industries to be used as bio composite, absorbent, tissue engineering, and carrier material, particularly for drug delivery due to its high surface area, flexibility, low crystalline, high stiffness and strength (Surip *et al.*, 2012; Endes *et al.*, 2016; Dufresne, 2017).

In Malaysia, kenaf has come into attention since 1999 as a potential crop to replace tobacco because of the non-competitive price of tobacco after the implementation of the ASEAN Free Trade Area (AFTA) (Basri *et al.*, 2014). Besides, kenaf plantation has also overcome the issue of deforestation due to the high demand of timber which leads to biodiversity concerns (Basri *et al.*, 2014). As initiated by the National Economic Asian Council which is now known as the National Economic Advisory Council (NEAC), MARDI is responsible in coordinating the research and development of this crop (Basri *et al.*, 2014).

The World Health Organisation (WHO) reported that vector borne diseases cause 700 000 of deaths every year and the most common vector is mosquito (WHO, 2020a). Mosquitoes such as *Aedes aegypti* (*Ae. aegypti*) are able to spread different kinds of diseases such as dengue, Yellow fever, Zika, Chikungunya and many others (WHO, 2020a). In recent decades, dengue has rapidly spread globally with cases related to this viral disease has been reported to be 8-fold higher since 2000 (WHO, 2020a). WHO estimates 390 million dengue infection per year with 3.9 billion people at risk of the infection (WHO, 2020a). The number of cases is increasing every year and the disease is endemic in more than 100 countries now compared to before 1970 where only nine countries are endemic (WHO, 2020a). In Malaysia, a total of 130101 cases were reported in 2019, an increase of 61.4 % from 2018 with 182 death cases and the number is increasing every year (MOH, 2019). There are many factors identified for the rapid spread of the virus globally and one of the factors is the presence of containers at the local residential areas that could potentially collect water such as old tyres, flowerpots, and roof gutters (Louis *et al.*, 2016). Those containers offer good larval breeding sites which contribute significantly to the spread of the virus (Jansen & Beebe, 2010). WHO recommends integrated vector management (IVM) as an intervention strategy for controlling dengue and other arboviral diseases which encourages optimal use of resources for vector control (WHO, 2020b). One of the methods is through killing mosquito larvae at their breeding sites using larvicide

(WHO, 2004). Mosquito larviciding aims to control mosquito population at the larvae or pupae stage before they become adult mosquitoes and are dispersed (US EPA, 2016). Killing mosquitoes at the breeding site is considered as source reduction and more effective compared to the application of insecticide spray against adult mosquitoes due to the mosquito larvae mainly living underneath the water surfaces (Floore, 2006).

There are several types of larvicide that are mainly organophosphate insecticides, bacterial insecticides, insect growth inhibitors and other materials such as oils and monomolecular films where each of these have different mechanisms to kill the mosquito larvae (US EPA, 2016). The most commonly used larvicide is temephos and this organophosphate has been used as one of the methods for vector control management in Malaysia since 1973 (Seleena *et al.*, 2001). The current commercial temephos in market is known as Abate®1 SG which comprised of 1 %w/w temephos as the active ingredient (AI) and 99 %w/w sand granules (SG) as its inert ingredients which function as the carrier material (WHO, 2011; BASF, 2017). The carrier material is added to the larvicide formulation to enhance active ingredient effectiveness and performance, including distribution, release rate and increasing the bioavailability of the larvicide to the target organism (Floore, 2006; US EPA, 2019). However, the sand granules are prone to sink to the bottom of the water, and unable to float longer in water surfaces (Kase & Branton, 1986). Subsequently, its effectiveness may reduce since most of the mosquito larvae live at the upper part of the water body and can cause toxic effects to non-target aquatic organism such as fish larvae, tadpoles, and aquatic bugs that shares the same habitat with the mosquito larvae (Crivelenti *et al*., 2011; Marina *et al*., 2014; Junges *et al*., 2017).

The innovation of this larvicide carrier to enhance the control of the dengue is consistent with United Nation's third sustainable development goal of good health and well-being (UN, 2015; Schorkopf *et al*., 2016). In addition, the use of nanocellulose as larvicide carrier that is aimed to be environmental-friendly of renewable source with low toxicity against non-target aquatic organisms also addresses the United Nation's twelfth sustainable development goal of responsible consumption and production of to achieve the sustainable management and efficient use of natural resources (UN, 2015). Nanocellulose has been widely recognised as an environmentally friendly carrier in the biomedicine field but research on the use of nanocellulose as a larvicide carrier is still lacking. To date, only one research was found on the use of modified CNF in the microencapsulation of insect repellent against adult mosquitoes (Kadam *et al.*, 2019). The capability of CNF as carrier material has been reported by several researchers in biomedicine using various types of drugs such as paracetamol, itraconazole, bendamustine hydrochloride and tetracycline (Kolakovic *et al.*, 2011; Valo *et al.*, 2011; Bhandari *et al.*, 2017; Iman *et al.*, 2020). It was suggested that CNF as an efficient new carrier material due to nanocellulose is able to improve the drug release behaviour, protect drug from degradation, increase bioavailability through targeted delivery action while having high loading and dispersion capability (Bhandari *et al.*, 2017; Orasugh *et al.*, 2018; Tenhunen *et al.*, 2018). Hence, looking at these characteristics of CNF, there is potential for the use of kenaf CNF (KCNF) as the carrier material for temephos. When this study started in 2018, there were no data available in terms of its safety to human, environment and bioefficacy to determine whether KCNF can be utilised effectively for the purpose of mosquito larviciding.

1.2 Problem statement

Current commercial mosquito larvicide formulation, Abate® 1 SG is comprised of 1 %w/w temephos as active ingredients and 99 %w/w of silica sand granules as the other inert ingredients (WHO, 2011; BASF, 2017). The silica sand granules which act as the carrier material for temephos are made from non-renewable natural minerals. The application of temephos formulated with sand granules is prone to sink to the bottom of water because of its density and poor dispersibility, resulting in inefficient temephos distribution across the water surface where most mosquito larvae occupy (Bradbury *et al.*, 1989; Barresi *et al.*, 2007). Consequently, a higher concentration of AI is needed to achieve the desired bioefficacy of larval mortality due to the passive role of sand granules carrier in releasing temephos to the targeted mosquito larvae (Kase & Branton, 1986; Mulla *et al.*, 2004). In addition, inefficient distribution of temephos formulated with sand granules also increases the possibility of non-target toxicity whereby other organisms may accidentally eat the granules (Muntz *et al.*, 2016). Thus, there is a need to find an alternative to silica sand granules as the carrier material that is sourced from sustainable and renewable natural resource with enhanced effective temephos carrier capability, stability and release while being low toxicity to humans and the environment.

However, the dispersibility of non-modified surface of CNF including from kenaf, has yet to be explored. Moreover, the stability of CNF in retaining incorporated material over the storage period has not been described in current available literature. In terms of CNF toxicity, there is still limited information to conclude its safety, whereby various toxicity effects were reported, ranging from no significant toxic effect to inflammatory response. In addition, no study on acute dermal toxicity was reported to date, although exposure of CNF to the skin is likely to occur since the skin is the largest organ of mammals. On the other hand, ecotoxicity of CNF from kenaf against Daphnia and adult zebra fish has not been reported. While the application of CNF as carrier material in the biomedicine field already has gained considerable attention amongst researchers, the application of CNF as a pesticide carrier is still limited. Therefore, this study intends to investigate utilisation of CNF from kenaf as a potential larvicide nanocarrier.

1.3 Objectives

The study aims to explore the potential use of KCNF as temephos carrier material for the control of mosquito larvae. Hence, the study objectives are to:

i. Determine capability of the KCNF to disperse and load temephos.

ii. Determine stability of the KCNF in retaining its nano size and loaded temephos.

iii. Assess acute systemic toxicity of the KCNF and KCNF+T in mammalian model.

iv. Assess acute ecotoxicity of the KCNF in freshwater aquatic organisms.

v. Evaluate bioefficacy of the KCNF+T against the *Ae. aegypti* mosquito larvae.

1.4 Hypotheses

The study's alternative hypotheses are:

$H_{i:}$ The KCNF has high dispersion capability with equal or more than 50 % dispersibility while quantity of the temephos loaded in the KCNF+T is above 1 %w/w.

$H_{ii:}$ The KCNF is able to retain its nano size with one of its dimensions remained in 1- 100 nm in size, temephos amount impregnated on the KCNF+T is not less than 95 % from the initial impregnation over 12 months of storage period and residual temephos retained on the KCNF+T more than 3 months after redispersion in water.

$H_{iii:}$ The KCNF and KCNF+T do not cause skin irritation and the LD_{50} value of KCNF and KCNF+T acute dermal toxicity are higher or equal to 2000 mg/kg bw.

$H_{iv:}$ The EC_{50} and LC_{50} value of KCNF acute ecotoxicity are higher or equal to 100 mg/L.

H_v: The LC_{50} of KCNF+T against *Ae. aegypti* larvae is below or equal to 0.1 mg/L at 24^{th} hours of post exposure.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Recent advances in nanotechnology have benefited the natural fibre as a source of nanocellulose (Dufresne, 2013). Natural fibres from a plant such as kenaf can be used as a source to produce two types of nanocellulose, namely cellulose nanofiber (CNF) and cellulose nanocrystal (CNC), depending on the different synthesis methods (Mtibe *et al.*, 2018). Currently available products in the market that use nanocellulose are mobile phone casing, spare tire cover, ultra-absorbent gel, cosmetics product and now research is ongoing worldwide to find any other potential use of nanocellulose (John *et al.*, 2010; Kargarzadeh *et al.*, 2018; Manikkam, 2018). Discover Natural Fibres Initiative (DFNI) has reported that world natural fibre production increased from 28 million metric tons to 32 million metric tons in 10 years with kenaf/jute/allied fibres comprised of 7.8 % of the total worldwide production in 2018 (Townsend, 2019).

Transparency Market Research (2018) reported that the global market for nanocellulose is estimated to reach USD 700 million by the end of 2023 and demands for cellulose nanofiber (CNF) was more than 50 % of the market shares in 2014 compared with CNC (Transparency Market Research, 2018). Manufacturers that produced CNF commercially include CelluForce Inc., UPM-Kymmene Oyj, Nippon Paper Industries Co., Ltd., American Process Inc., and Daicel FineChem Ltd. (Transparency Market Research, 2018). On the other hands, several research centres have produced CNF for research and development purpose on their pilot-scale plant such as NANOTECH (Universiti Malaya, Malaysia), UMaine PDC (University of

Maine, US), and InnventiaAB (Sweden) (An *et al.*, 2017). Thus, CNF has more economic importance than CNC.

While cellulose nanomaterials (CNM) are emerging in their field and provide an alternative material replacement for sustainable development with progressive growth of nanocellulose material production in the market, there is a potential human health risk from the exposure to nanocellulose material either from raw material during processing or as end product regardless of any route of entry to the human body (Endes *et al.,* 2016).

In moving forward, this review will focus on the CNF in terms of its isolation process, properties, use as carrier material and its safety in mammalian model in terms of oral and dermal toxicity as well as toxicity against aquatic organisms. The review will describe about kenaf plantation in Malaysia, its biology and cellulose content, isolation process of kenaf CNF, its nano physicochemical properties and role of the CNF as a carrier material. Subsequently, the review will explain on the dengue fever and its vector including properties and toxicity of temephos as larvicide to control the dengue vector. Eventually, toxicity of the CNF against mammalian and aquatic organisms mainly invertebrate and vertebrate species also will be cover which include the non-modified and modified surface CNF. Description on the method used in the toxicity evaluation, Globally Harmonised System (GHS) for chemical classification and the larvicidal bioefficacy method also will be explain.

2.2 Kenaf (*Hibiscus cannabinus* L.) plantation in Malaysia

Kenaf (*Hibiscus cannabinus* L.) is a non-wood plant mainly cultivated for its fibre and has various usage from its stalk to the seed (Ayadi *et al.*, 2017). Kenaf stalk is rich in fibre comprised of bast and core fibre while its leaves and seed contain naturally allelopathic chemical and edible oil (Webber & Bledsoe, 2002). Historically, kenaf was used as cordage plant to produce twine, rope and sackcloth due its strength and resistance to fungus (Cook, 1960 as cited in Ayadi *et al*., 2017) while potential used of kenaf in pulp and paper industry as well as for livestock feed was first introduced by Killinger (1969). In decades, usage of kenaf has been expanded to medicinal benefits, reinforced composite material, textile, construction, automotive, environmental cleaning, and alternative for plant-based-milk (Ramesh, 2016; Ayadi *et al.*, 2017; Karim *et al.*, 2020).

Kenaf fibre used as a natural resource for renewable and biodegradable fibre was a main economic drive of its plantation (Ramesh, 2016). With awareness and education towards sustainable development value, the transition over petroleum-based fibre such as polyester to natural fibre like kenaf has gained interest among industries and consumers (Thyavihalli Girijappa *et al.*, 2019). Changing the fibre raw material from chemical-made and non-renewable source to bio-based and renewable material has been in progress due to fibre from natural origin is depicted as CO_2 neutral (Van Dam, 2009). The current main producers of kenaf are India, China and Thailand, which account 95 % of the world production (Van Dam, 2009; Faruq *et al.*, 2013).

In Malaysia, kenaf was introduced in early 1970 and research on kenaf has started in 1999 as a replacement for tobacco plantation after country policy changes on ASEAN Free Trade Area (AFTA) in 2010 (Basri *et al.*, 2014). Kenaf was selected as a tobacco replacement plant in Malaysia because it is suitable to grow in a temperate climate, high rainfall, fast growth and produced good fibre quality (Abdul Khalil *et al.*, 2010; Faruq *et al.*, 2013). Research on various kenaf accessions was carried out to find suitable kenaf genotypes that could adapt to Malaysia tropical climate and produced high biomass (Basri *et al.*, 2014).

Among the nine types of reported kenaf accessions potentially to grow in Malaysia, V36 accession was the most recommended kenaf genotype, due to its high growth rate and biomass production (Basri *et al.*, 2014). In 2019, Malaysia had produced 7183 tons of dried kenaf stem from 885.2 hectares harvested crop, contributing to MYR 4.4 million gross income (LKTN, 2019). Details of the kenaf cultivation and production across the states in Malaysia is presented in Table 2.1.

Table 2.1 Kenaf cultivation and production by state in Malaysia for 2019 (LKTN, 2019).

State	Harvested crop* (ha)	Production of kenaf dried stem** (ton)
Kelantan	207.9	2,221.7
Terengganu	225.0	1,141.9
Pahang	411.0	3,427.4
Perak	5.0	43.3
Johor	3.8	73.4
Perlis	22.0	141.3
Melaka	8.0	120.7
Kedah	2.5	13.3
Total	**885.2**	**7,183.0**

*Gross income is MYR 5000/ha.
**From V36 kenaf seed.

2.3 Kenaf (*Hibiscus cannabinus* L.)

2.3.1 Kenaf plant biology

Kenaf or its scientific name, *Hibiscus cannabinus* L. is a dicotyledons plant under the Malvaceae family which is in the same category as okra (*Hibiscus esculentus*), cotton (*Gossypium hirsutum* L.), and hibiscus (*Hibiscus hibiscum* L.) (Izran *et al.*, 2014). The *Hibiscus* genus is divided into six sections which are *Furcaria, Alyogen, Abelmoschus, Ketmia, Calyphyllia*, and *Azanza* (Ayadi *et al.*, 2017). The herbaceous and short-photoperiod plant of kenaf is categorised in the *Furcaria* section (Basri *et al.*, 2014). As shown in Figure 2.1, the kenaf plant comprises stalk, leaf, flower, fruit, seed and roots (Ayadi *et al.*, 2017). The stalk is the main source for the fibre, comprised of bast and core fibres, also known as long and short fibre (Ramesh, 2016). Bast fibre is referred to as the fibre's outer part from the stalk which produced high-quality pulp while core fibre is the fibre from the inner part of the stalk and produced low-quality pulp (Abdul Khalil *et al.*, 2010). The bast and core fibres are indicated in Figure 2.2. Even though bast fibre produced high-quality pulp, it only contributes 25-40 % of total kenaf dry weight while another 75-60 % is made up of core fibre (Abdul Khalil *et al.*, 2010). Kenaf is an easily grown plant under vast environmental condition, and its height can grow up to 1.5 - 4.5 meters tall within 4-5 months with minimal fertiliser, pesticide and water required (Abdul Khalil *et al.*, 2010; Ramesh, 2016).

Kenaf flowering period will influence the plant's composition. Late flowering will produce higher fibre content, useful for pulp processing while early flowering is useful for forage due to high protein content (Ramesh, 2016). The flowering period is within two months for early flowering, two to three months for intermediate flowering

Figure 2.1 Photograph of the kenaf (*Hibiscus cannabinus* L.) farming.

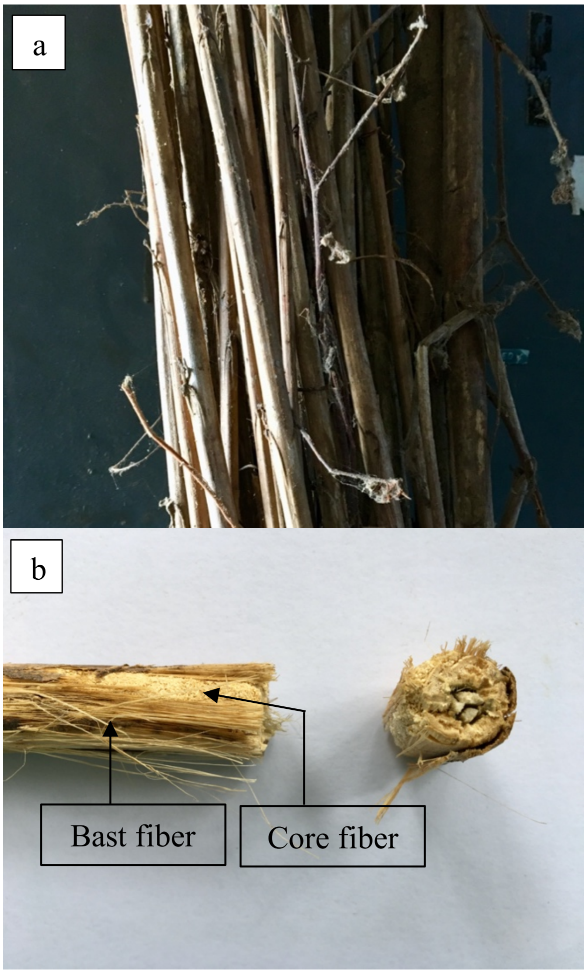


Figure 2.2 Photograph of the kenaf's dried stalk, (a) external shape at 0.25X magnification and (b) peel and cross section stalk exposing the core fibre at 0.75X magnification.

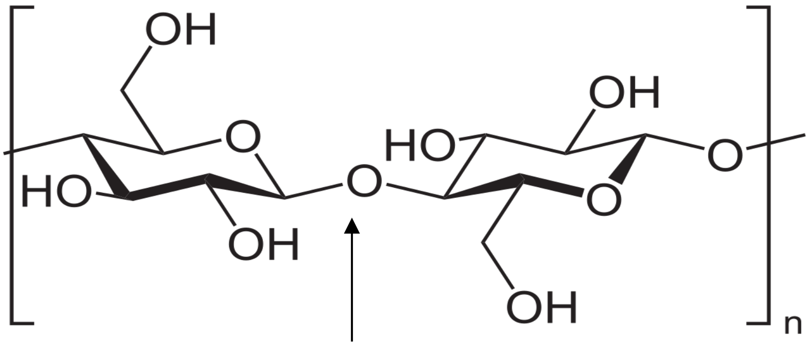
and three to five months for late flowering (H'ng *et al.*, 2009). In Malaysia, early flowering varieties are Q-Ping and KK60, whereas V12, V19, V36, V132, and NS are intermediate varieties. The V133 and TK varieties are late flowering group (H'ng *et al.*, 2009).

Physical morphology characterisations between kenaf accessions were different between each other's with basal diameter, plant height, leaf number, leaf area and photosynthesis were 11-16 mm, 157-247 cm, 67-81, and 994-1452 cm^2 $plant^{-1}$ respectively among 40 kenaf accessions as studied by Hossain *et al.*(2012). However, microscopic observation of kenaf tissue structure between the nine kenaf varieties showed a similar vessel distribution, ray parenchyma and fibre size despite of the physical differences among the varieties (H'ng *et al.*, 2009).

According to the study by Abdul Khalil *et al.* (2010), kenaf's cell wall structure contains primary and secondary wall layers. The secondary layers are further divided into the outer layer (S_1), middle layer (S_2) and the inner layer (S_3) with shape varies from round to polygonal. The S_2 layer of kenaf bast fibre is thicker than the S_2 layer of kenaf core fibre, contributing to higher cellulose content in the kenaf bast fibre (Abdul Khalil *et al.*, 2010). In addition to that, kenaf bast fibre length was also reported to be three times longer than kenaf core fibre (Abdul Khalil *et al.*, 2010).

2.3.2 Kenaf cellulose content

Kenaf is mainly formed by cellulose, hemicellulose and lignin, and considered a non-wood lignocellulosic material (Izran *et al.*, 2014). According to Ashori *et al.* (2006) and Abdul Khalil *et al.* (2010), kenaf as a whole contained 49 – 53 % of alpha cellulose, 19 -21 % of lignin and 2 - 4 % ash content with kenaf bast fibre contained higher alpha cellulose (55-56 %) than kenaf core fibre. Alpha cellulose is the highest degree of cellulose polymerisation and provides stability in the function material (Gooch, 2006). Cellulose (Figure 2.3) is a homo-polysaccharide consisting of a D-glucose unit connected by β(1-4)-glycosidic bonds (Dufresne, 2013). Abundant cellulose in kenaf can be isolated either through enzymatic, chemical, and/or mechanical processes to obtain nanocellulose (Endes *et al.*, 2016). Nanocellulose can further be divided into longer fibres, composed of the amorphous and crystalline region referred to as cellulose nanofibers (CNF) and cellulose nanocrystal (CNC) which only contained crystalline region (Endes *et al.*, 2016).



β(1-4)-glycosidic bonds

Figure 2.3 Chemical structure formula of cellulose shows two D-glucose units connected by β(1-4)-glycosidic bonds (Dufresne, 2013).

2.4 Kenaf as an alternative renewable source of cellulose nanofiber (CNF)

2.4.1 Synthesis of KCNF

Synthesis of the KCNF is following a top-down approached starting from its raw kenaf stalk which is a millimetre in size, followed by retting process or delignification to remove the lignin and obtained cellulose in micrometre and further processing to defibrillate the cellulose into nanometre (Dufresne, 2013; Karimi *et al.*, 2014). According to Kian *et al.* (2019), in general, CNF isolation from bast fibre involved three distinct stages which are the disintegration of microfibrillated cellulose into CNF as pre-treatment, the principal treatment that used mechanical processing to defibrillate the nanocellulose and the post-treatment to segregate between coarse and fine fractions of the CNF. Cellulose fibre isolation into nanocellulose can be carried out using a chemical or combination of both chemical and mechanical treatment. Production of kenaf CNF using chemical treatment was carried out by Shi *et al.* (2011) and Zaini *et al.* (2013) while the combination of both chemical and mechanical treatment was performed by many researchers (Jonoobi *et al.*, 2009, 2010, 2011; Karimi *et al.*, 2014; Nurul Atiqah *et al.*, 2019).

In the pre-treatment process, kenaf bast was cut or ground into smaller pieces before subjected to alkali treatment using sodium hydroxide (NaOH) or potassium hydroxide (KOH) (Kian *et al.*, 2019). The alkali treatment or delignification process was carried out to remove the lignin and hemicellulose compound of the kenaf natural fibre as carried out by Jonoobi *et al.* (2011), Shi *et al.* (2011), Zaini *et al.* (2013), Karimi *et al.* (2014) and Nurul Atiqah *et al.* (2019). The researchers used 4 to 25 wt% of sodium hydroxide (NaOH) to remove the non-cellulosic component of the kenaf bast fibre. Jonoobi and Karimi used NaOH with a combination of 0.1 wt%

anthraquinone solution to enhance the delignification process and protect the fibres from degradation (Jonoobi *et al.*, 2009, 2011; Karimi *et al.*, 2014). The delignification was carried out at temperature 80 – 160 °C for 1 to 2 hours. However, Nobuta *et al.* (2016) found that alkaline treatment caused damage to the cellulose fibres, and they suggested using sodium chlorite ($NaClO_2$) to remove the lignin. Upon delignification, bleaching was carried out to dissolute the remaining lignin on the fibres via an oxidation reaction (Kian *et al.*, 2019). Jonoobi *et al.* (2009, 2011) and Karimi *et al.* (2014) used three steps of bleaching process that used sodium chlorite ($NaClO_2$), acetic acid (CH_3COOH) and hydrogen peroxide (H_2O_2) while Shi *et al.* (2011) and Zaini *et al.* (2013) used 10 % H_2O_2 and acetate buffer with aqueous chloride respectively.

Before proceeding with principle treatment of CNF isolation using mechanical disintegration, some researchers performed biological and chemical pre-treatment to enhance the fibrillation process while reduced the energy needed during mechanical disintegration (Nechyporchuk *et al.*, 2016). The pre-treatments include enzymatic hydrolysis, carboxylation using 2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO)-mediated oxidation, periodate-chlorite oxidation, sulfonation, carboxymethylation, quaternisation, and solvent-assisted pre-treatments (Nechyporchuk *et al.*, 2016). For instance, Narkpiban *et al.* (2019) used xylanase treatment combined with microfluidization, which led to successive isolation of kenaf fibre from its bark with no undesired elemental contamination. Jonoobi *et al.* (2010) on the other hand, had carried out acetylation on the kenaf cellulose, which facilitated the isolation of CNF before carried out a mechanical process such as cryo-crushing and high-pressure homogenisation.

Mechanical disintegration of chemically and enzymatically treated cellulose is carried out to obtain desirable nanocellulose. Examples of mechanical processes are ball milling, high-pressure homogenisation, grinding, refining, cryo-crushing, steam explosion and many more (Nechyporchuk *et al.*, 2016). In a study by Nuruddin *et al.* (2016), they isolate CNF from chemically treated kenaf fibre using ball milling with 12.7 mm ball diameter at a different time interval. Nobuta *et al.* (2016) only used the grinding method to produced CNF while Jonoobi *et al.* (2009, 2010, 2011) used a combination of disintegration, refining, cryo-crushing and high-pressure homogenisation for 40 times at 500 bar in the isolation of kenaf CNF from chemically treated cellulose pulp. Usage of supermasscolloider combined with a grinder was reported by Karimi *et al.* (2014). In contrast, Shi *et al.* (2011) centrifuged the CNF to segregate coarse and finer CNF for two times. They centrifuge the CNF at a rotating speed of 6500 rpm and 7600 rpm respectively and sonicated using probe sonicator at 40 % amplitude. For post-treatment of kenaf CNF isolation, only Nobuta *et al.* (2016) reported the step where they filtered the kenaf CNF using filter paper.

However, chlorine-based chemical for cellulose bleaching, homogeniser, microfluidiser and grinding as mechanical treatment were deemed conventional methods, using a lot of energy and creating an environmental issue (Nechyporchuk *et al.*, 2016; Nurul Atiqah *et al.*, 2019). Therefore, Nurul Atiqah *et al.* (2019) has introduced a new isolation method recently where they used a total chlorine-free bleaching treatment comprised of oxygen, ozone, and hydrogen peroxide to remove the remaining lignin after alkaline-anthraquinone treatment. Subsequently, the bleached fibres underwent supercritical carbon dioxide (SC-CO2) as pre-treatment and followed by mild acid treatment using 5 % oxalic acid to obtain CNF (Nurul

Atiqah *et al.*, 2019). According to Nurul Atiqah *et al.* (2019), kenaf CNF was successfully isolated using the new proposed eco-friendly method with compromised nano physicochemical characteristics.

2.4.2 Physical and chemical properties of KCNF

Kenaf CNF stem a exhibited smoother and smaller diameter of surface morphology compared to raw kenaf stem (Jonoobi *et al.*, 2011). The smoother surface and smaller diameter of treated kenaf fibre are due to the removal of hemicellulose and lignin throughout the treatment process from raw kenaf fibre to CNF (Kargarzadeh *et al.*, 2012; Karimi *et al.*, 2014). According to Karimi *et al.* (2014), the diameter of CNF depends on the treatment methods which range from 6 to 35 nm. Fibre size from kenaf CNF was reported to be 100 to 500 nm in length and 2 to 5 nm in diameter and therefore, nanocellulose has a higher surface area due to its high aspect ratio (Surip *et al.*, 2012; Zaini *et al.*, 2013).

The surface area and porosity of nanomaterials are important parameters in toxicity assessment and influenced the loading capability of the CNF. This is because a high surface-to-volume ratio may increase surface reactivity and dissolution rate, altered bioavailability, and caused changes to the toxicity profile of nanomaterial (Oberdörster *et al.*, 2005). Currently, researchers reported the specific surface area of the tested CNF from BET analysis result of 26.8 to 79 m^2/g (Gebald *et al.*, 2011; Zhao *et al.*, 2015).

A hydrogen bond is an interaction of a hydrogen atom with an electronegative atom, such as nitrogen, oxygen or fluorine, from another molecule or chemical group (Fan *et al.*, 2012). The presence of hydroxyl groups in the cellulose lead to the formation of inter- and intra-molecular hydrogen bonds between the cellulose unit which eventually will determine solubility and hydroxyl reactivity of CNF as carrier material (Kondo, 1997; Fan *et al.*, 2012) As for cellulose material, different hydrogen bonds produced wavenumber range from 662 cm^{-1} to 3332 cm^{-1} (Kondo, 1997). Hydroxyl (-OH) stretching bond presented by cellulose has wavenumber of 3332 cm^{-1}, hemicellulose with the presence of C=O stretching of carbonyl group has wavenumber of 1724 cm^{-1} and wavenumber of 1506 cm^{-1} and 1239 cm^{-1} correspond to phenol and aryl compound in lignin respectively (Fan *et al.*, 2012). The chemical bonds are consistent with several studies where FTIR analysis showed wavenumber range 3300-3400 cm^{-1} for cellulose OH stretching bond, 1731 – 1738 cm^{-1} for hemicellulose C=O stretching bond and 1239-1250 cm^{-1} for lignin C-O stretching aryl group (Karimi *et al*., 2014, Kargarzadeh *et al*., 2012, and Jonoobi *et al*., 2011). However, only Karimi *et al*. (2014) reported the absorbance peak at wavenumber 1425-1435 $cm^{-1,}$ which resembles C=C stretching of the aromatic group of lignin. All studies compared the presence of the hydrogen bonds with raw material and nanocellulose, which showed the absence of hemicellulose and lignin peak after treatment indicating successful removal of these two groups.

The crystalline phase and crystallinity degree of CNF are the important parameters influencing the reactivity, toxicity of the material, and release rate of incorporated material (Van Duong & Van den Mooter, 2016; Rasmussen *et al.*, 2018). Jonoobi *et al.* (2015) reviewed that the crystallinity percentage of nanocellulose is higher than its raw material, attributed to the efficient removal of non-cellulosic components of the fibres during the isolation processes. They noted that a sharper peak demonstrates higher crystallinity of nanocellulose at 2θ = 22.7°. In another study conducted by Jonoobi *et al.* in 2010 and 2011, found that the crystallinity index (CI) of nanocellulose fibre is 62 % and 67 % for kenaf core and kenaf bast respectively.

Zeta potential is an analysis to determine nanomaterial surface charge in colloidal solution (Kumar & Dixit, 2017). Charge on the nanomaterial surface will attract opposite ions to the nanomaterial surface and formed an Electrical Double Layer (EDL) where strongly bound ions are called stern layer, and loosely bound ions are called diffused layer (Alsharef *et al.*, 2017; Kumar & Dixit, 2017). The voltage measurement between the edge of the diffused layer and the surrounding liquid (slipping plane) is defined as Zeta potential (Analytik, 2020). Zeta potential value will be useful in anticipating the particle stability in a solution where value 0 to less than ± 30 mV is considered as flocculation/coagulation to incipient stability, more than ± 30 mV to ± 60 mV is regarded as moderate to good stability and more than ± 60 mV is excellent stability (Kumar & Dixit, 2017). Current available studies reported on the Zeta potential value of CNF from the natural fibre was in the range of -23 to -31 mV (de Morais Teixeira *et al.*, 2010; Filipova *et al.*, 2018). However, only the Zeta potential of CNC was reported for kenaf, ranging from 8.7 to - 95.3 mV depending on the hydrolysis time (Kargarzadeh *et al.*, 2012). Previous studies showed that Zeta potential value from the natural fiber is categorised under intermediate stability in solution (de Morais Teixeira *et al.*, 2010; Filipova *et al.*, 2018).

2.4.3 The dispersion capability of KCNF

Like many other nanomaterials, KCNF tends to agglomerate due to adherence of particle-particle collisions from Brownian motion affected its dispersion capability. Understanding the agglomeration and dispersion capability of the nanomaterial is significantly important since it will affect the nanomaterial environmental fate and eventually, ecotoxicity of nanomaterial can be estimated (OECD, 2017b). The dispersion capability of nanomaterial is expressed as nanomaterial particles to remain disperse and stable over a certain period (OECD, 2017b). Dispersion stability was studied in terms of nanocellulose colloidal behaviour in aqueous media and explained based on the Derjaguin – Landau – Verwey – Overbeek (DLVO) theory of the surface-charged groups electrical double layers (EDL) repulsion and Van der Waals attractive energy. This theory explains that dispersion of any fiber particles will remain stable as long as EDL repulsion force between the particles stronger than Van der Waals attractive energy (Derjaguin & Landau, 1941; Verwey & Overbeek, 1947). Chemical influences, such as pH and concentration of electrolytes, can reduce the thickness of the EDL of colloids and trigger aggregation (Fukuzumi *et al.*, 2014). Dispersion stability of CNF such as kenaf in aqueous media can be influenced by pH and ionic strength for instance $Ca(NO_3)_2$ as the electrolyte. The pH of the aqueous medium can affect different level of hydroxyl group dissociation on the nanocellulose surface (Mendoza *et al.*, 2018). It may change CNF surface charge through protonation or deprotonation of the hydroxyl group, thus effect fibril-fibril interaction and induced fibril aggregations (Fall *et al.*, 2011; Mendoza *et al.*, 2018).

On the other hand, the presence of salt plays a second important role in the dispersion stability of nanomaterial in aqueous media (Fall *et al.*, 2011). According to DLVO theory, ion from salt relates directly to the thickness of EDL where a high concentration of ionic strength reduced the thickness of EDL surrounding nano particle which eventually decreases nanoparticle surface charge, causing particle aggregation (Mendoza *et al.*, 2018). Another factor that influenced the dispersion