DEVELOPMENT OF FISH SCALE-COLLAGEN CREAM (CoC) FROM WATER-IN-VIRGIN COCONUT OIL EMULSION MEDIUM FOR TOPICAL APPLICATION

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ABSTRACT

This study aimed to develop and characterise the fish scale-collagen cream (CoC) for topical application. Hydrolysed collagen (HC) was extracted from Tilapia fish scales using a combination of hydrothermal extraction and enzymatic hydrolysis. This HC (molecular weight, Mw ≈ 1 kDa) was incorporated in water-in-oil (w/o) emulsion of Span 60:Tween 60/water/virgin coconut oil (VCO) system at water volume fractions (φ_w) of 0.74, 0.83, and 0.94 via high shear homogenisation technique. The developed CoC were then characterised by optical polarising microscope (OPM), field emission scanning electron microscopy (FESEM), particle size analyser, differential scanning calorimetry (DSC), and rheometer. Up to 20 wt% HC has been successfully loaded in all emulsion systems and the optimum loading achieved at HC content as low as 5 wt%. The best CoC system with great physical characteristic, fine texture (305 nm) and uniformly disperse in VCO phase was produced by emulsion at the lowest φ_w (0.94). The developed CoC has been found to be thermally stable with T_c and T_m range between -20 to -30 ℃ and 10 to 20 ℃, respectively. The rheological properties of CoC demonstrated shear thinning behaviour and thus greatly meet the needs of topical applications. The permeation study of *ex vivo* rat skin showed that HC in emulsion droplets (CoC system) easily pass through the skin. Cytotoxicity study through *in vitro* fibroblast cells and *in vivo* toxicity to the mice showed that HC at 25 and 50 mg/kg body weight did not give chronic toxic to the mice. In conclusion, CoC from water-in-VCO emulsion system was succesfully developed. As an implication, the developed CoC is potentially used as a carrier for HC in topical application.

PEMBANGUNAN KRIM KOLAGEN- SISIK IKAN DARIPADA MEDIUM EMULSI AIR-DALAM-MINYAK KELAPA DARA UNTUK APLIKASI TOPIKAL

ABSTRAK

Kajian ini bertujuan untuk membangunkan dan mencirikan krim kolagen-sisik ikan (CoC) untuk aplikasi topikal. Kolagen terhidrolisis (HC) telah diekstrak daripada sisik ikan Tilapia menggunakan gabungan pengekstrakan hidrotermal dan rawatan hidrolisis enzimatik. HC ini (berat molekul, Mw ≈ 1 kDa) telah digabungkan di dalam emulsi air-dalam-minyak (w/o) bagi sistem Span 60:Tween 60/air/minyak kelapa dara (VCO) pada beberapa pecahan isipadu air (φ_w), iaitu 0.74, 0.83 dan 0.94 melalui teknik penghomogenatan ricih tinggi. CoC yang dibangunkan kemudiannya dicirikan oleh mikroskop pengutuban optik (OPM), mikroskopi elektron pengimbasan pancaran medan (FESEM), penganalisis saiz zarah, kalorimetri pengimbasan pembezaan (DSC), dan reometer. Sehingga 20% berat HC berjaya dimuatkan ke dalam semua sistem emulsi dan pemuatan yang optimum dicapai pada kandungan HC serendah berat 5%. Sistem CoC yang terbaik dengan ciri fizikal yang bagus, tekstur yang halus (305 nm) dan tersebar secara seragam di dalam fasa VCO telah dihasilkan oleh emulsi pada φ_w yang paling rendah (0.94). CoC yang dibangunkan telah didapati stabil secara termal pada julat T_c and T_m dengan masing-masing antara -20 hingga -30 ℃ dan 10 hingga 20 ℃. Sifat reologi CoC menunjukkan perilaku penjarangan ricih dan ini sangat menjadi keperluan dalam aplikasi topikal. Kajian *ex vivo* penelapan kulit tikus menunjukkan HC di dalam titisan emulsi (sistem CoC) dengan mudah melalui kulit. Kajian kesitotoksikan melalui *in vitro* sel-sel fibroblas dan *in vivo* ketoksikan kepada mencit menunjukkan HC pada 25 dan 50 mg/kg berat badan tidak memberikan kesan toksik yang kronik pada mencit. Kesimpulannya, CoC daripada sistem emulsi air-dalam-VCO telah berjaya dibangunkan. Sebagai implikasi, CoC yang dibangunkan berpotensi digunakan sebagai pembawa kepada HC dalam aplikasi topikal.

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LIST OF ABBREVIATIONS

cm	Centimeter
C-N	Carbon-Nitrogen
C=O	Carbon=Oxygen
cP	Centipoise
CoC	fish scale-collagen cream
cos δ	Cosine delta
DSC	Differential scanning calorimetry
FTIR	Fourier-transform infrared spectroscopy
gm	Gram
G’	Storage modulus
G”	Loss modulus
GPC	Gel permeation chromatography
HAp	Hydroxyapatite
HLB	Hydrophilic-lipophilic balance
HPLC	High-performance liquid chromatography
kg	Kilogram
kV	Kilovolt
LVR	Linear viscoelastic region
M	Molar
min	Minutes
mL	Milliliter
mm	Millimeter

$NaNO_3$	Sodium nitrate
N-H	Nitrogen-Hydrogen
Nm	Nanometer
OPM	Optical polarising microscope
o/w	Oil-in-water
Pa	Pascal
PEG	Polyethylene glycol
P-O	Phosphate-Oxygen
PO_4^{3-}	Phosphate ion
Rpm	Revolutions per minute
S	Second
SEM	Scanning electron microscope
sin δ	Sine delta
tan δ	Tangent delta
UV	ultraviolet
VCO	Virgin coconut oil
w/o	Water-in-oil
wt.%	Weight percentage
(w/v)	Weight per volume
Mg	Microgram
µL	Microliter
φ_w	Water phase volume
^oC	Degree celcius
%	Percentage

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Collagen is a naturally existing protein representing 25–35% of the total protein mass in the human body and makes up approximately 80% of the dry weight of the skin. It is the most abundant protein in the human body and becomes the main components of extracellular matrix which gives the skin its structure and firmness (Gelse, Poschl, & Aigner, 2003; Krieg & Aumailley, 2011). An abundance of collagen in the body provides a smooth, healthy, and young appearance to the skin as it works hand-in-hand with another protein called elastin by making supporting nets all along the cellular structures (Chang, Shefelbine, & Buehler, 2012). Figure 1.1 shows a structure of skin consists of a few layers which are stratum corneum, viable epidermis, dermis, and hypodermis.

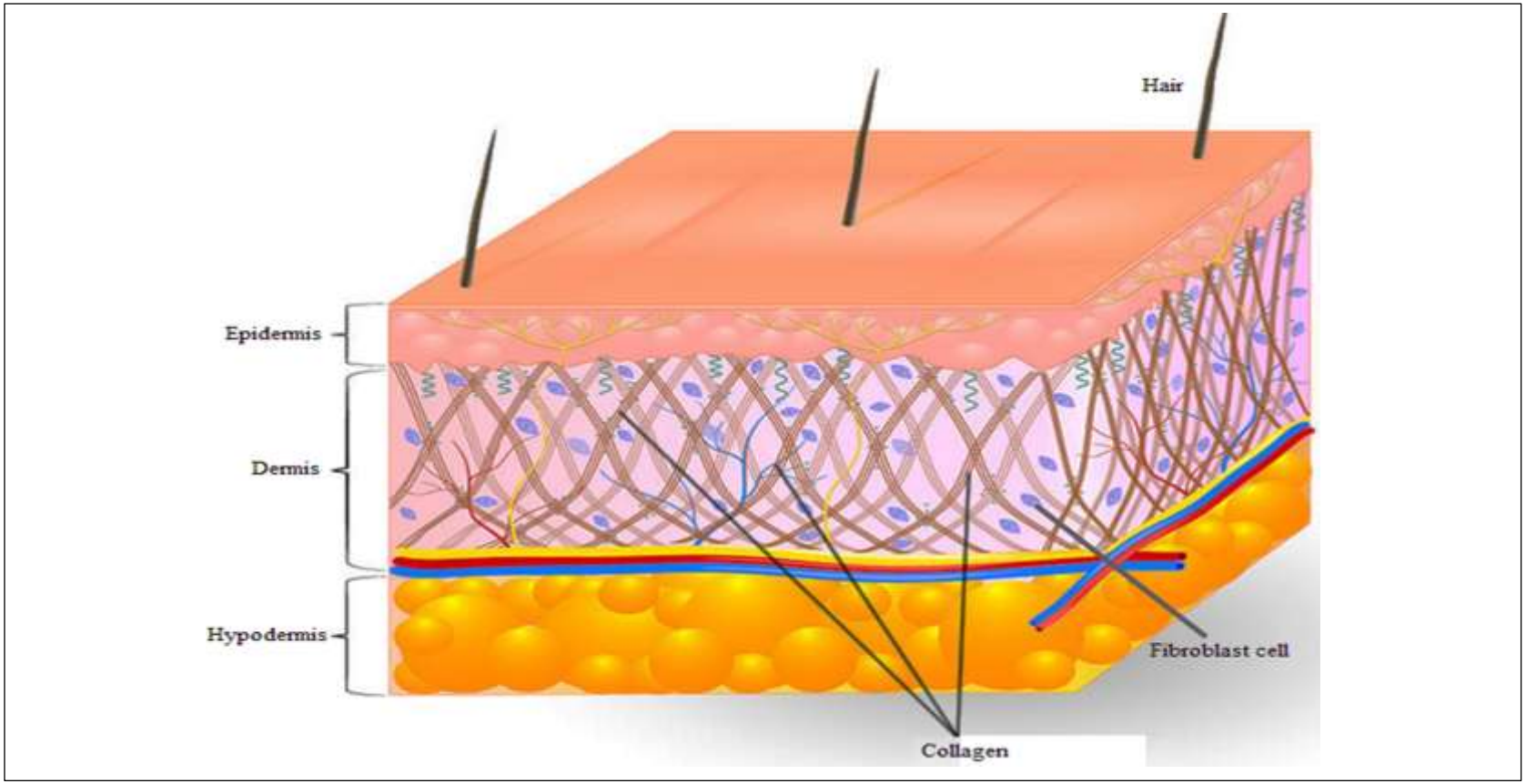

Figure 1.1. The different layers of structure skin which are consist of epidermis, dermis, and hypodermis. Adapted from Marine Matrix (n.d)

Collagen levels in the body reach a turning point at 20 years old in which factors of intrinsic and extrinsic ageing have damaged the collagen fibre under the skin and reduced the ability of the fibroblast cell to produce new collagen. Fibroblasts are the most common cells of connective tissue specifically located in the dermis layer (Figure 1.1) which are responsible for producing and organising collagen fibres in the body. When the collagen in the dermal layer of the skin begin to break down and the rate of collagen production starts to slow down, the surface of the skin loses some of its elasticity and firmness. The most noticeable signs of damaged collagen are the appearance of wrinkles, fine lines, and saggy skin (Rodriguez, Barroso, & Sanchez, 2018). Everyone wants to look young or youthful and nobody enjoys the effect of growing older on their looks especially in the facial skin. Today, most of the men and women have spent hundreds of ringgit on cosmetic products to make sure they are always looking youthful (Khan, 2010).

Nowadays, collagen has been extensively incorporated in cosmetic products as it has been proven that collagen can act as a messenger and triggers the synthesis reorganisation of new collagen in the body (Okawa et al., 2012; Ohara et al., 2010; Iwai et al., 2006). It is believed that taking collagen can facilitate the biosynthesis of natural collagen in the body and thus improved the structure of epidermal appearance (Schagen, 2017; Song & Li, 2017). Therefore, most of the previous researches have focused on the isolation of collagen from tissues that are rich in fibrous possessing collagen such as skin and tendons. Many reports indicated that the main source of collagen in the world is derived from pig skin (46%), followed by bovine skin (29.4%), bones (23.1%), and other sources (1.5%) (Gelatin Manufacturers of Europe [GME], 2008). These sources cause the limitation for Muslim consumers to use it due

to halal issues. Fish-based collagen is the most potential alternative source to replace bovine or porcine collagen in terms of its biocompatibility, possesses weak antigenicity, and biodegradable (Tang & Saito, 2015; Yamada, Yamamoto, Ikeda, Yanagiguchi, & Hayashi, 2014). The collagen has received an increasing attention among researchers as collagen alternative source since there are more than 30% of fish-processing wastes consist of fish skin, scale, and bones that are very rich in Type I collagen fibre (Kittiphattanabawon, Benjakul, Visessanguan, Nagai, & Tanaka, 2005; Ikoma, Kobayashi, Tanaka, Walsh, & Mann, 2003).

A wide variety of collagen products is available in the cosmetic field to suit with the customers' needs. However, collagen-based products in the forms of lotions, cream, or gel that is to be applied to the skin need to be absorbed deep into the dermis layer. Therefore, this study focuses on incorporating the low molecular weight of fish scale collagen in cosmetic products. Collagen was extracted from Tilapia (Oreochromis niloticus) fish scales using hydrothermal extraction according to the patented method conducted by Zainol, Aziz, and Ahmad (2011). In order to produce low molecular weight collagen, the extracted collagen was further hydrolysed with the enzyme.

However, low molecular weights of hydrolysed collagen tend to agglomerate due to strong Van der Waals interactions of fine particles, thus reducing its penetration capability into the skin when administrated topically (Dumitriu & Popa, 2013; Chai et al., 2010; Baert et al., 2007). Therefore, in this study, an approach of an emulsion system is used in stabilising hydrolysed collagen to keep them separated. The fish scale-collagen cream (CoC) was prepared by dispersing the water-based fish

scale-collagen droplet in virgin coconut oil (VCO) through high shear homogenisation technique. The droplets in the emulsion are stabilised by non-ionic surfactant, which is the surface active molecule that adsorbs to the surface of droplets, forming a thin coating around the droplets that inhibits their agglomeration by generating repulsive forces between them (Chung & McClements, 2014). Figure 1.2 shows a schematic illustration of the formation of CoC droplets. The small size of water-based collagen droplet gives a lot of advantages especially in the topical application which allows the easiest diffusion through the epidermal barrier when considering the application onto the skin.

The performance of the CoC suitability to be used for topical application was investigated by analysing the permeability of hydrolysed collagen through the membrane and the toxicity effect. Great characteristics owned by CoC might be an innovative approach for delivering collagen to the dermis. Samson, Basri, Masoumi, Karjiban, and Malek (2016) reported that nanoemulsion containing copper peptide increases the permeability of copper peptide through the membrane. Therefore, CoC is supposed to be easily absorbed when applied into the skin. The preparation of CoC to be used in the topical application is still new in the cosmetic industry and only a few research works have been reported.

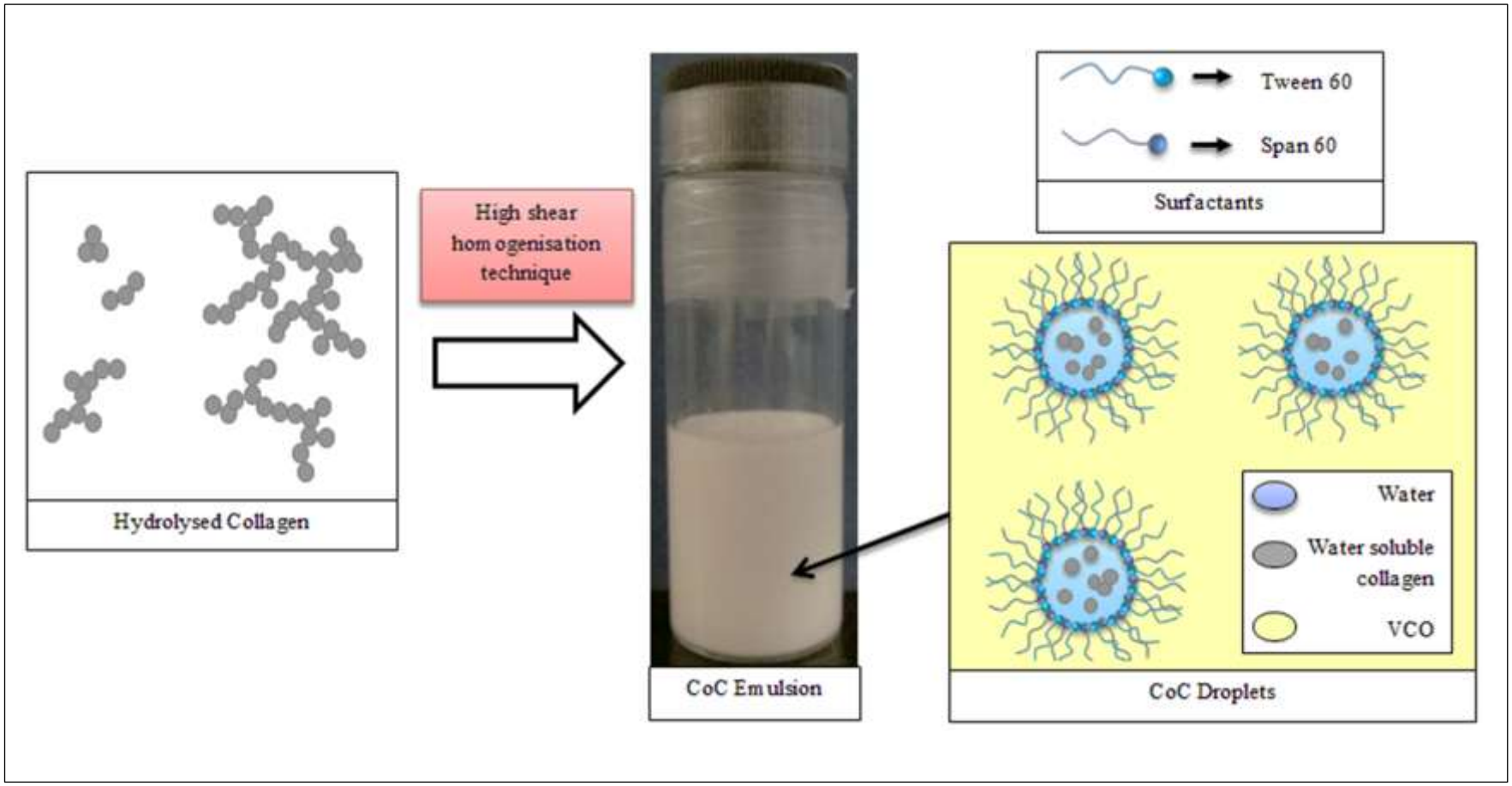

Figure 1.2. Schematic illustration of the formation of fish scale-collagen cream (CoC) droplets

1.2 Problem Statement

The main source of collagen in the world is derived from pig and cow skin followed by other sources such as marine organism, chicken, sheepskin, duck feet, and frog skin (Rodriguez et al., 2018; Silvipriya et al., 2015). However, the outbreak of bovine spongiform encephalopathy (BSE) and the spreading foot-mouth disease (FMD) has urged critical considerations on the safety of using collagen extracted from land-based animals especially pig and cow (Silvipriya et al., 2015; Yuan, Wang, Lin, Chou, & Li, 2014). It is possible that these diseases can be transferred to human beings. Besides safety issues, the halal issue is the major concern for Muslim consumers when they are using collagen-related products. These sources cause the limitation of consumption due to religious reason especially in Malaysia, a country which has the highest majority of the Muslim community. Both Islam and Judaism forbid using any pork-related products, while Hindus do not consume any cow-related product (Pranoto, Lee, & Park, 2007). This problem has encouraged researchers to explore alternative sources of collagen to make it acceptable to all users.

High demand for fish consumption has led to a large number of wastes. About 50%–70% of total wastes (scales, heads, and skeletons) is generated from fish processing industry (Huang, Hsiao, & Chai, 2011; Kittiphattanabawon et al., 2005). When these wastes were disposed of improperly, they can cause environmental pollution with an unpleasant odour. The odour will attract flies and animals to feed on the wastes and can also lead to the growth of microorganism (Nagai & Suzuki, 2000). It was reported that the fish wastes consist of 5% of fish scales are major fish industry residues (Wang & Regenstein, 2009). Generally, these fish scales contain valuable

materials such as collagen and hydroxyapatite, which have commercial value in the industry (Kongsri, Janpradit, Buapa, Techawongstien, & Chanthai, 2013). Instead of disposing of these wastes, fish scales have the potential for conversion into useful products of higher value. Fish scales-derived collagen is one of the potential alternatives to non-halal collagen.

The need for halal collagen has increased in its demand due to the awareness of Muslim community toward products with halal status (Husain, Ghani, Mohammad, & Mehad, 2012). Collagen extracted from Tilapia fish scales is not only halal but safe and cheaper than animal-based collagen. High demand for fish scales as a raw material for the production of collagen will increase the economy of the fisherman or fish industries. In addition, the usage of fish scales to derive collagen will not only reduce pollution but reclaim the fish waste to high value-added materials. The water-based CoC has the potential to be used as a halal cosmetic product that will have a great impact on Muslim society.

Collagen-based products have become common in cosmetic applications (Li, Fukunaga, Takenouchi, & Nakamura, 2005). However, the result may just clean, protect, and moisturise the skin but not improving the appearance and health of the skin (Juncan & Vonica-Gligor, 2016; Lupo, 2001). The development of cosmetics should be based on the efficacy and safety of targeted components of the skin. In cosmetic preparation such as cream, it was intended that the particle size would be small to ensure the easy diffusion under the skin. If not, it will act like applying an icing cream on the top of the cake. In this study, an approach of colloidal carrier system which is emulsion was used as a carrier to deliver collagen into the skin. The

small size of CoC droplets gives a lot of advantages especially in the topical application which allows the easiest diffusion through the epidermal barrier when considering the application on to the skin.

Another problem should be concern about the cosmetic preparation is the ingredients that were used which consists of many harmful chemicals. Cosmetic products are typically loaded with preservatives which are chemical substances to allow them to remain stable over a long period and prevent microbial growth (Sasseville, 2004). However, it was reported that the preservatives could cause skin irritation, disruption of the secretion of hormones, and cancer (Charnock & Finsrud, 2007; Darbre, 2003; Oishi, 2002). In addition, individuals who are sensitive to these preservatives need to find preservative-free products. In this study, the CoC was prepared using the lowest percentage of chemical substances which is only 5 wt.% of non-ionic surfactant. The non-ionic surfactant is uncharged molecule so it is safe, biocompatible and not affected by pH changes in any medium. Fish scale-collagen cream (CoC) developed with free preservative is suitable for all type of skin.

In Malaysia, the coconut or *Cocos nucifera* is the fourth important industrial crop after palm oil, rubber, and rice. Our country remains as one of the top ten coconut producing countries in the world. However, a few research has been done on different ways of utilising coconut and on increasing the income of farmers. The VCO is traditionally used to enhance the beauty and promote the growth of hair, refine and moisturises the skin conditions and also being used to treat many skin disorders (Mansor, Che Man, Shuhaimi, Abdul Afiq, & Ku Nurul, 2012). The presences of fatty acids (caprylic and lauric) in VCO protect the skin and contain many antioxidants that

make it suitable for all types of skin conditions. The benefits of VCO on the skin have attracted us to use it in our study. In this research, VCO was used as a medium to disperse the hydrolysed collagen particles to prepare fish scale-collagen cream (CoC). The development of CoC will widen the use of VCO. The results of the research can provide an alternative use of VCO for economic sustainability. It will contribute to the use of coconut in Malaysia.

The knowledge developed in this study will provide a great opportunity for our country to lead in the production of a halal, environmentally-friendly, and high-quality product with low manufacturing cost. Besides that, other academic institution, research organisations, and relevant industries from local and abroad will be lining up to make a collaboration. Overall, the collagen prepared at lab scale will be commercialised at industrial scale and therefore will create profit to both university and nation.

1.3 Research Objectives

The objectives of this research are:

i. To extract collagen from Tilapia fish scales by a combination of hydrothermal treatment and enzymatic hydrolysis.
ii. To stabilise hydrolysed collagen in water-in-virgin coconut oil emulsion using high shear homogenisation technique.
iii. To characterise phsycochemical properties of fish scale-collagen cream (CoC).

iv. To study the transdermal permeation ability of hydrolysed collagen in CoC using Franz diffusion cell.

v. To investigate the toxicity and biocompatibility of hydrolysed collagen and CoC.

1.4 Scope of Study

This study is divided into four stages in which the first stage is the extraction and enzymatic hydrolysis treatment to produce low molecular weight collagen of Tilapia fish scales, second stage is to prepare CoC by dispersing the hydrolysed collagen in emulsion system, the third stage is the transdermal penetration ability of hydrolysed collagen, and the last stage is *in vitro* and *in vivo* toxicity study of hydrolysed collagen and CoC.

First stage: The study deals with the optimisation of controlled parameters of the preparation of Tilapia fish scale collagen. The laboratory works are limited to these crucial parameters: the extraction temperatures at 80 ^{o}C for eight hours (for collagen isolated from fish scales), the enzymatic hydrolysis at 55 ^{o}C for one hour (for reactivity of enzyme), and the effects of enzyme concentrations from 0.5–2.0 wt.% (for hydrolysis of collagen). For the characterisations of hydrolysed collagen, the following equipment was utilised: gas permeation chromatography (GPC), Fourier transform infrared spectroscopy (FTIR), scanning electron microscope (SEM), Brookfield digital viscometer, pH meter, and amino acid analyser.

Second stage: Hydrolysed collagen was incorporated into an emulsion system (Span 60:Tween 60/water/VCO) at different water volume fractions (φ_w) of 0.75, 0.84, and 0.93. The concentrations of hydrolysed collagen loaded into the system are 5, 10, 15, and 20 wt.%. The system was homogenised at 19,000 rpm for 15 minutes and the characterisation of the CoC was characterised by the following equipments: optical polarising microscope (OPM), scanning electron microscope (SEM), zetasizer nanoparticle analyser, pH meter, differential scanning calorimetry (DSC), and rheometer. The preliminary stability of the sample preparation was evaluated by physical observation for 24 hours after centrifuged.

Third stage: The ability of the hydrolysed collagen on transdermal penetration was determined by employing the Franz diffusion cell model. Winstar rat skin was selected as a membrane of penetration. Samples were performed at 1, 2, 3, 4, and 6 hours only. The amount of total hydrolysed collagen that can pass through the rat skin was measured by the bicinchoninic acid (BCA) protein assay.

Fourth stage: *In vitro* experiment – Fibroblast cells were seeded in 96-well plates at a density of 1×10^4 cells/well. The 0.002, 0.02, 0.2, and 2 mg/mL of hydrolysed collagen solution (solution was prepared by dissolving the hydrolysed collagen in Dulbecco's Modified Eagle's medium) were added, respectively, and incubated for 24 hours. Then, cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colourimetric assay. *In vivo* – Healthy three-month-old ICR white mice were used in this study. The sub-acute toxicity was determined by subcutaneous injection and topical administration on mice, respectively in seven days of treatment.