# FORMULATION OF NANOEMULSIONS CONTAINING WHEY PROTEIN ISOLATE AND *ECHIUM PLANTAGINEUM* SEED OIL AND THEIR EFFECT ON PROINFLAMMATORY CYTOKINE MODULATION IN THP-1 CELL LINE

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A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Pharmaceutical Technology

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# การตั้งสูตรตำรับนาโนอิมัลชันที่ประกอบด้วยเวย์โปรตีนไอโซเลตและน้ำมันจากเมล็ด *Echium plantagineum* และผลของตำรับต่อการเปลี่ยนแปลงของไซโตไคน์ที่ก่อการอักเสบในเซลล์ ทีเอชพี 1

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กัญญาณัฏฐ์ ชโลธร : การตั้งสูตรตำรับนาโนอิมัลชันที่ประกอบด้วยเวย์โปรตีนไอโซเลตและน้ำมันจากเมล็ด *Echium plantagineum* และผลของตำรับต่อการเปลี่ยนแปลงของไซโตไคน์ที่ก่อการอักเสบในเซลล์ ทีเอชพี 1. (FORMULATION OF NANOEMULSIONS CONTAINING WHEY PROTEIN ISOLATE AND *ECHIUM PLANTAGINEUM* SEED OIL AND THEIR EFFECT ON PROINFLAMMATORY CYTOKINE MODULATION IN THP-1 CELL LINE) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.ดร. วรางคณา วารีสน้อยเจริญ, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ.ดร.สุรีรัตน์ พรธาดาวิทย์, 96 หน้า.

ปัจจุบันมีการนำนาโนอิมัลชันมาประยุกต์ใช้ประโยชน์ในด้านต่างๆ การเตรียมนาโนอิมัลชันด้วย เวย์โปรตีนไอโซเลต มีข้อดีคือสามารถช่วยลดการใช้สารเคมีได้ การศึกษาวิจัยนี้สูตรตำรับนาโนอิมัลชันที่ทำให้เสถียรด้วยเวย์โปรตีนไอโซเลตถูกเตรียมขึ้นโดยใช้วิธีอัลตราโซนิกส์ ทั้งนี้ได้ศึกษาสภาวะที่เหมาะสมของค่าพารามิเตอร์ของวิธีอัลตราโซนิกส์เพื่อให้ได้นาโนอิมัลชัน และทดสอบความเป็นพิษและการต้านการอักเสบในเซลล์ ทีเอชพี 1 จากผลการทดลองพบว่าสูตรที่เหมาะสมคือ นาโนอิมัลชันที่ทำให้เสถียรด้วยเวย์โปรตีนไอโซเลต (20% โดยน้ำหนัก) และประกอบด้วย 5% โดยน้ำหนัก ของน้ำมันที่มีกรดไขมันโอเมก้า-3 จากเมล็ด *Echeium plantagineum* สำหรับพารามิเตอร์ที่เหมาะสมในการเตรียมนาโนอิมัลชันโดยใช้เครื่องอัลตร้าโซนิกส์ที่ความเข้มสูง พบว่าค่าที่เหมาะสมคือ 20% แอมพลิจูด, พลังงานที่ใช้เท่ากับ 1750 จูล และ อุณหภูมิที่ 45 องศาเซลเซียส ซึ่งค่าเฉลี่ยของเส้นผ่านศูนย์กลางของนาโนอิมัลชันมีค่าเท่ากับ 225.83 ± 1.52 นาโนเมตร และ ค่าดรรชนีของการกระจายตัวของขนาดอนุภาคเท่ากับ 0.24 ± 0.01 และมีขนาดประจุของอนุภาคเท่ากับ -31.50 ± 0.20 มิลลิโวลต์ จากผลการทดลองพบว่าที่ความเข้มข้น 5% โดยน้ำหนักของน้ำมันให้ผลของขนาดอนุภาคนาโนอิมัลชันที่เล็กกว่า และมีเสถียรภาพที่ดีกว่าระบบที่ใช้ความเข้มข้น 10% โดยน้ำหนักของน้ำมัน และพบว่านาโนอิมัลชัน (5% น้ำมัน) ที่ความเข้มข้นเวย์โปรตีน 1 มิลลิกรัมต่อมิลลิลิตร ไม่พบความเป็นพิษต่อเซลล์ ทีเอชพี-1 หลังจากถูกบ่มเป็นเวลา 24, 48 และ 72 ชั่วโมง ยิ่งไปกว่านั้นเมื่อบ่มเซลล์ด้วยนาโนอิมัลชันเป็นเวลา 24 ชั่วโมง และ สารก่ออับเสบชนิดไลโปโพลีแซคคาไรด์เป็นเวลา 4 ชั่วโมง พบว่าไซโตไคน์ชนิดก่อการอักเสบ ได้แก่ IL-1β, IL-4, IL-6, IL-8, GM-CSF และ TNF-α มีจำนวนลดลงอย่างมีนัยสำคัญทางสถิติ ($p \leq 0.05$) และไซโตไคน์ชนิด IFN-γ มีจำนวนลดลงอย่างไม่มีนัยสำคัญทางสถิติ ซึ่งผลดังกล่าวอาจเกิดจากการช่วยเสริมฤทธิ์กันระหว่างเวย์โปรตีนไอโซเลต และ น้ำมันชนิดโอเมก้า-3 ที่เป็นส่วนประกอบในตำรับนาโนอิมัลชัน อีกทั้งขนาดอนุภาคในระดับนาโนเมตรช่วยส่งเสริมการผ่านเข้าสู่เซลล์ได้มากขึ้น กล่าวโดยสรุป นาโนอิมัลชันที่ถูกทำให้เสถียรด้วยเวย์โปรตีนไอโซเลต อาจเป็นระบบที่นำมาพัฒนาเพื่อให้ผลในการต้านอักเสบได้ ทั้งนี้ควรจะมีการศึกษาเพิ่มเติมถึงกลไกของนาโนอิมัลชันที่ถูกทำให้เสถียรด้วยเวย์โปรตีนไอโซเลตต่อการต้านการอักเสบ และทำการศึกษาในกายต่อไป

ภาควิชา..วิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม..... ลายมือชื่อนิสิต…….....….

สาขาวิชา...เทคโนโลยีเภสัชกรรม..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก……..…..

ปีการศึกษา....2554..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม……..….

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KUNYANATT CHALOTHORN : FORMULATION OF NANOEMULSIONS CONTAINING WHEY PROTEIN ISOLATE AND *ECHIUM PLANTAGINEUM* SEED OIL AND THEIR EFFECT ON PROINFLAMMATORY CYTOKINE MODULATION IN THP-1 CELL LINE. ADVISOR: ASST. PROF. WARANGKANA WARISNOICHAROEN, Ph.D., CO-ADVISOR: ASST. PROF. SUREERUT PORNTADAVITY, Ph.D., 96 pp.

Nanoemulsions (NEs) are attractive to be employed in various applications. Preparation of NEs using whey protein isolate (WPI) renders benefits due to avoidance of chemical utilization. In this study, the NEs stabilized by WPI was prepared using ultrasonic emulsification. The plant seed oil from *Echium plantagineum* which was rich in omega-3 fatty acid was used. The parameters in the ultrasonication were optimized for generating NEs. In addition, they were investigated on cytotoxic test and anti-inflammatory activities on THP-1 cell line. The WPI-stabilized NEs were obtained at the optimum concentration of 5% (wt.) *E. plantagineum* seed oil and 20% (wt.) WPI using high intensity ultrasonicator. The optimized conditions were 20% amplitude, 1750 Joules of energy and the temperature at 45$^{o}$C. The average size of the obtained WPI-stabilized NEs (5% oil) was 225.83 ± 1.52 nm, polydispersity index (PdI) was 0.24 ± 0.01 and zeta potentials was -31.50 ± 0.20 mV. According to the results, 5% wt. oil WPI-stabilized NEs were smaller and more stable than 10% wt. oil NEs. The results revealed that 5% NEs at 1 mg/ml WPI demonstrated no toxic effect on THP-1 cell line after incubated for 24, 48 and 72 hours. Furthermore, a significant decrease ($p \leq 0.05$) in the pro-inflammatory cytokines IL-1β, IL-4, IL-6, IL-8, GM-CSF and TNF-α were observed on the lipopolysaccharide (LPS)-induced THP-1 cells treated with WPI-stabilized NEs. The pro-inflammatory cytokines of IFN-γ was also reduced but not statistical significant. The findings in this study were possibly due to enhancing effect of WPI and omega-3 oil composed in NEs and the easily uptake of nano-sized droplet into the cell. Conclusively, WPI-stabilized NEs might be promising system for anti-inflammatory treatment. The further investigation would be on anti-inflammatory mechanisms of the WPI-stabilized NEs and *in vivo* study.

Department:...Pharmaceutics and Industrial Pharmacy… Student's Signature……

Field of Study:...Pharmaceutical Technology…..……. Advisor's Signature……

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| α | alpha |
| ~ | approximately |
| β | beta |
| °C | degree Celcius (centrigrade) |
| γ | gamma |
| < | less than |
| µ | micro ($10^{-6}$) |
| µg | microgram |
| µl | microlitre |
| % | percentage |
| ATCC | American Type Culture Collection |
| DHA | docosahexaenoic acid |
| DNA | deoxyribonucleic acid |
| EPA | eicosapentaenoic acid |
| *et al.* | *et alii*, and other |
| etc. | *et cetera* (and other similar things) |
| g | gram (s) |
| GM-CFS | granulocyte macrophage colony-stimulating factor |
| GSH | reduced glutathione |
| h | hour (s) |
| HMG-CoA | 3-hydroxy-3-methylglutaryl-coenzyme A |
| IFN-γ | interferon gamma |

| | |
|---|---|
| IL | interleukin |
| k | kilo ($10^3$) |
| l | litre (s) |
| LPS | lipopolysaccharide |
| m | milli ($10^{-3}$) |
| M | molar (s), micromole (s) per litre |
| min | minute (s) |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| mV | millivolt |
| NEs | nanoemulsions |
| NF-kB | nuclear factor kappa-light-chain-enhancer of activated B cells |
| nm | nanometer |
| o/w | oil-in-water |
| PBS | phosphate buffer saline |
| PCR | polymerase chain reaction |
| pH | potential of hydrogen |
| PIT | phase inversion temperature |
| ROS | reactive oxygen species |
| S.D. | standard deviation |
| SDA | stearidonic acid |
| SEM | standard error of mean |
| rpm | round per minute |
| RT | room temperature |
| THP-1 | the human monocytic leukemia-1 |

| | |
|---|---|
| TNF | tumor necrosis factor |
| UV-Vis | ultraviolet-visible |
| WPC | whey protein concentrate |
| WPH | whey protein hydrolysate |
| WPI | whey protein isolate |
| w/o | water-in-oil |
| w/w | weight by weight |

# CHAPTER I

# INTRODUCTION

## 1. Background and significance of the study

Nanoemulsions (NEs) are known as isotropic mixtures of oil and water stabilized by surfactants. NEs can form fine oil-in-water (o/w) or water-in-oil (w/o) systems with the droplet size usually ranging from 20 to 500 nanometers (nm) (Siah *et al.,* 2012). Their properties depend not only on thermodynamic condition but also on preparation method (Forgiarini *et al.,* 2001). It has been well documented that droplet size plays an important role in emulsion properties such as drug solubility, dissolution, and bioavailability after oral administration (Shafiq *et al.,* 2007). NEs can protect active ingredients against extreme conditions and especially enzymatic hydrolysis (Madene *et al.,* 2006). The extremely small droplet size of NEs leads to better absorption into the body, thus increasing bioavailability of encapsulated compounds (Wen-Ting *et al.,* 2005). From a previous study, the NEs (79.5 nm) containing 1% curcumin showed 85% inhibitory effect on 12-*0*-tetradecanolphorbol-13-acetate (TPA)-induced edema of mouse ear while the corresponding emulsion (618 nm) had less (43%) anti-inflammatory effect (Xiaoyong *et al.*, 2008). NEs have been reported to make the plasma concentration profiles and bioavailability of oral drugs more reproducible. From a study of Sheikh *et al.* (2007), the absorption of ramiprilat-containing NEs resulted in 2.94-fold increase in bioavailability as compared to conventional capsule and 5.4-fold to that of drug suspension. Hence, NEs would be an efficient and more patient compliance approach in comparison to ordinary tablets and capsules (Siah *et al.*, 2012).

There are many ways available for the production of NEs including “low-energy emulsification” methods such as phase inversion temperature technique. This method has several limitations due to requiring a large amount of surfactants and a limited selection of surfactant-cosurfactant combination, and not being applicable to large scale industrial production (Seekkuarachchi, Tanaka and Kumazawa, 2006). On the other hand, “high-energy emulsification” method such as high pressure homogenization and microfluidization can produce emulsion with narrower and smaller in size distribution (Pinnamaneni, Das and Das, 2003). One of the main problems of producing very fine emulsions with high-energy emulsification techniques is that microfluidization is unfavourable in specific circumstance such as higher pressure and longer emulsification time. It leads to “over-processing” which is re-coalescence of emulsion droplets and an increase in emulsion droplet size (Lobo and Svereika, 2003; Olson, White and Richter, 2004; Jafari, He and Bhandari, 2006).

Recently, ultrasound emulsification also known as acoustic emulsification has been widely used for preparing NEs, mostly on an analytical laboratory scale (Nicolas, Jean and Patrick, 2008). The amount of surfactant required to provide a stable emulsion is significantly reduced and the energy consumption is considerably lower than other classical mechanical devices (Tadros et al., 2004). Ultrasonic emulsification is believed to occur through two mechanisms. Firstly, the application of an acoustic field produces interfacial waves which become unstable, eventually resulting in the eruption of the oil phase into the water medium in the form of droplets. Secondly, the application of low frequency ultrasound causes acoustic cavitation, that is, the formation and subsequent collapse of micro bubbles by the pressure fluctuations of a simple sound wave. The intense effect (disruption and

mixing) of shock waves explains the very small droplet size (Jafari *et al.,* 2006). In addition to quick droplet rupture due to cavitation, this phenomenon is said to favor the formation of electric charges able to adsorb onto the interface, playing a role in emulsion stabilization (Canselier *et al.,* 2002). Furthermore this method is more convenient in term of operation, ease of maintenance, and aseptic process. The most influential process parameters such as frequency, amplitude, sonication time, power input, viscosity, etc. are those affecting cavitation phenomena (Canselier *et al.,* 2002; Jafari *et al.,* 2006; Kentish *et al.,* 2008).

Most surfactants are conceivably good candidates to stabilize NEs by electrostatic or steric repulsion mechanisms. Proteins as natural emulsifiers can be used to improve the stability and provide specific physicochemical properties of NEs. They can form an interfacial membrane that plays an important role in stabilizing the droplets against flocculation and coalescence during long-term storage (David, 2004).

Whey protein has been previously reported for good emulsifying and stabilizing properties to facilitate the formation and stability of O/W emulsions (Morr and Ha, 1993). Susanne, Inta and Gerald (2001) and Ana *et al.,* (2007) have found that emulsion stabilized by whey protein isolate (WPI), whey protein with ≥90% protein, was stable against coalescence and provided smaller droplet sizes ($d_{43}$: 1.5-2 µm). Besides, WPI provides biological and pharmacological activities (Josee, Claude and Pierre, 2007) such as immunomodulatory activities (Brix *et al.,* 2003), anti-allergic and anti-inflammation (Kimber *et al.,* 2002).

It is known that depletion of intracellular antioxidant such as glutathione (GSH) can lead to the accumulation of intracellular reactive oxygen species (ROS)

(Teramoto *et al.,* 1999) and activation of nuclear factor-κB (NF-κB) pathway. The activation of NF-κB pathway will increase the production of pro-inflammatory cytokines especially interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α). Due to the high content of cysteine in WPI, it is proposed to reduce TNF-α *via* inhibition of ROS-induced activation of NF-κB and reducing the concentration of intracellular ROS (**Figure 1**). The cysteine-rich whey protein was reported to significantly reduce ear inflammation, ear redness and thickness in atopic contact dermatitis mouse model (Beaulieu, Dupont and Lemieux, 2007) by reducing TNF- α, and significantly inhibiting neutrophil extravasation (Beaulieu *et al.*, 2009).

Apart from emulsifier selected for the NE formulation, the oil types are also important. The oil could play a role in physicochemical properties of NEs and in biological effect. The use of omega-3 oil from fish is well-known for parenteral emulsion (Martina *et al.,* 2007). Dietary omega-3 alpha-linolenic acid (18:3ω3) can be converted into eicosapentaenoic acid (EPA) (20:5ω3) and to docosahexaenoic acid (DHA) (22:6ω3), which exhibit anti-inflammatory effect. The anti-inflammatory effect of omega-3 comes from the modulation of the composition of the cell membranes involved in the inflammatory process (Robbert, 1998) and competitive inhibition of the pro-inflammatory interleukins (IL-1β, IL-6 and IL-12), TNF-α, and inflammatory prostaglandin (PGE2) (Joseph and Jeffrey, 2006) (**Figure 1**). The *Echium plantagineum* L., Boraginaceae, contains 16% of stearidonic acid (SDA), a highly unsaturated omega-3 fatty acid, which is an intermediate in the biosynthesis of EPA and DHA (Berti *et al.*, 2007). *E. plantagineum* seed oil can be used as a source of dietary omega-3 oil to reduce the consumption of omega-3 oil from fish and

discard fishy odor (Guerrero *et al.*, 2000). Recently, the comparison of Atlantic salmon fed with fish oil, *E. plantagineum* seed oil (SDA oil) and canola oil at the same concentration was reported that absolute amounts of DHA and total omega-3 were significantly greater in muscle tissue of Atlantic salmon fed with fish oil and *E. plantagineum* seed oil treatments compared to those fed with canola oil (Matthew, Peter and Chris, 2007).

In this present work, NEs containing WPI (emulsifier) and *E. plantagineum* seed oil will be prepared by using ultrasonication. The NEs will be characterized and *in vitro* tested for modulatory effect on proinflammatory cytokines (such as IL-1β, IL-6, TNF-α) in human monocytic leukemia THP-1 cell line. The findings should provide the formulation for nutraceutical and pharmaceutical applications.

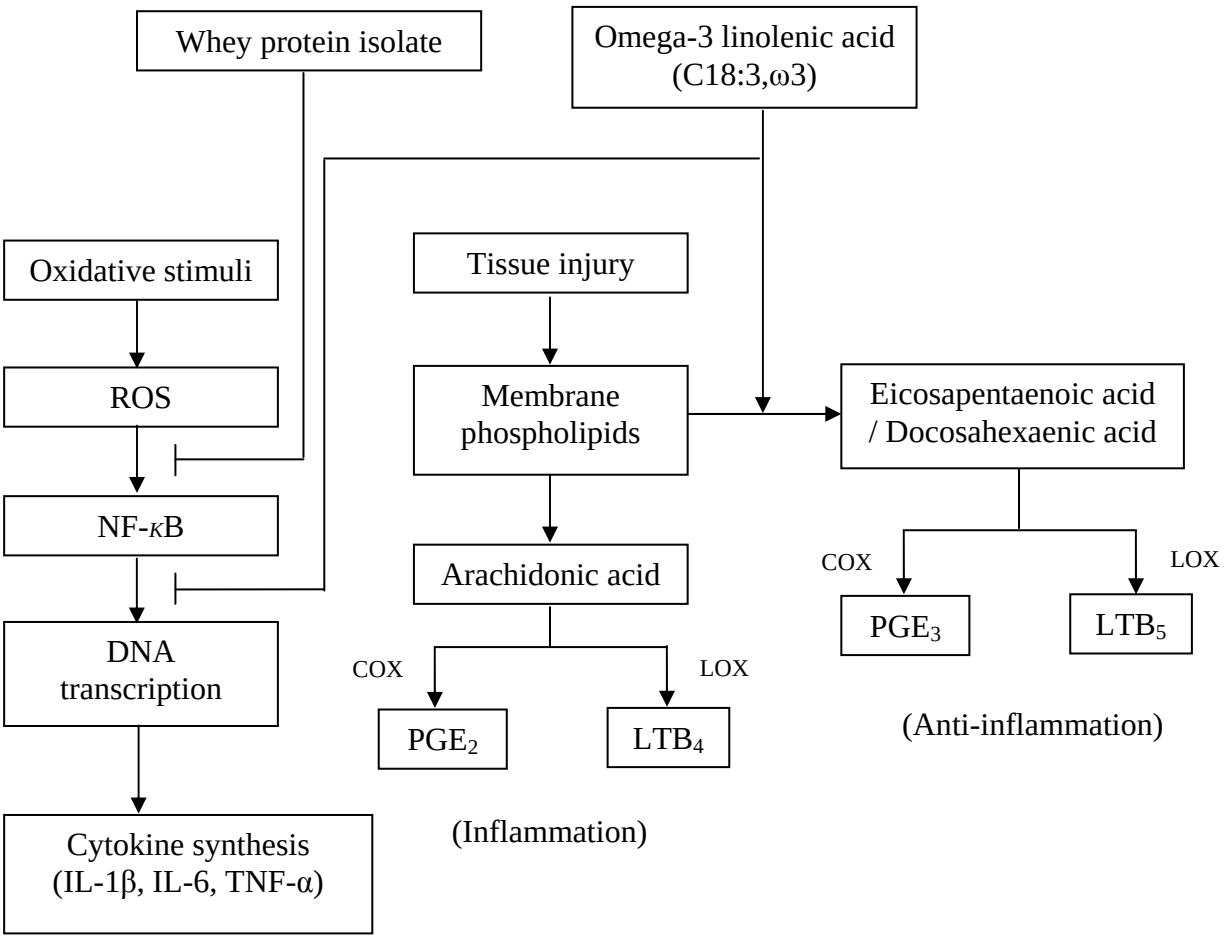



**Figure 1** The modulation of anti-inflammatory effect of whey protein isolate and omega-3 fatty acid. (ROS = reactive oxygen species, NF-*k*B = nuclear factor-*k*B, IL-1β = interleukin 1-β, IL-6 = interleukin 6, TNF-α = tumor necrosis factor-α, COX = cyclooxygenase, LOX= lipoxygenase, $PGE_2$ = prostaglandin $E_2$, $PGE_3$ = prostaglandin $E_3$, $LTB_4$ = leukotriene 4, $LTB_5$ = leukotriene 5)

## 2. Objectives of the study

2.1 To optimize and characterize nanoemulsions (NEs) containing whey protein isolate (WPI) and *E. plantagineum* seed oil by ultrasonication method.

2.2 To investigate a modulatory effect of NEs on pro-inflammatory cytokines in human monocytic leukemia THP-1 cell line

## 3. Hypothesis

3.1 The ultrasonication technique provide stable WPI-stabilized NEs

3.2 Nanoemulsions containing WPI and *E. plantagineum* seed oil cause a modulatory effect on pro-inflammatory cytokines in human monocytic leukemia THP-1 cell line.

# CHAPTER II

# LITERATURE REVIEW

## 1 Nanoemulsions

### 1.1 Overview of nanoemulsions

Nanoemulsions (NEs) are heterogeneous systems consisting of two immiscible liquids normally oil droplets dispersed in aqueous media and stabilized by surfactant molecules (**Figure 2**) (Chen *et al.,* 2011). NEs have increasing attention to be emerged in various applications such as foods, medicines and cosmetics fields due to their unique structure and properties, i.e. much smaller droplet size (20-500 nm) with larger surface area. NEs can be used to incorporate non-polar functional components into aqueous-based medicine, cosmetic, food and beverage products (Sadurni *et al.*, 2005; Chen *et al.*, 2011). In addition, decreasing the particle sizes as in a NEs, results in greater cellular penetration and bioavailability of the encapsulated compounds (Sonneville, Simonnet, and Alloret, 2004). Moreover, NEs are kinetically stable without any apparent flocculation or coalescence during the long term storage (Singh, and Vingkar, 2008). This is because the relatively small particle size means that Brownian motion effects dominate gravitational forces and the attractive forces acting between the droplets decrease with decreasing particle size (Cheng and David, 2010).

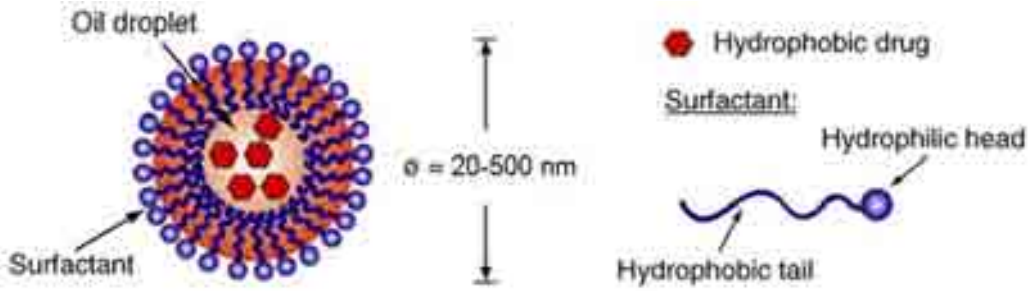



**Figure 2** Schematic diagram of drug solubilization in oil-in-water nanoemulsions (Chen *et al.*, 2011).

### 1.2 Preparation of nanoemulsions

A variety of preparation methods have been developed to prepare NEs. These may be classified in two fundamental types: high-energy emulsification and low-energy emulsification (Acosta, 2009).

#### 1.2.1 High-energy emulsification

The high-energy emulsification method utilizes high (mechanical) energy such as high pressure homogenizers, microfluidizer and ultrasonication. The mechanical processes that generate nanometric sized emulsions include initial drop creation, followed by disruptive forces that breakup the oil and water phases and lead to the formation of these initial droplets and surfactant absorption at their interface to ensure steric stabilization (Leong, *et al.*, 2009; Hareesh *et al.*, 2010)**.**

##### 1.2.1.1 High pressure homogenization

High pressure homogenization forms NEs owing to the disruptive forces created by high pressure (**Figure 3**). The final particle size achieved by a homogenizer is important because it determines the stability, appearance, texture, and

bioavailability of the final product (Acosta, 2009). The major factors that impact the particle size are homogenizer type and homogenizer operating conditions such as pressure, numbers of cycle, and temperature (Cheng and David, 2010). Typically, the mean droplet diameter tends to decrease with increasing homogenization pressure and number of passes. Unfortunately, for certain emulsifiers (typically biopolymers) higher pressures and longer emulsification times may lead to “over-processing”, resulting in an increase in particle size (Jafari, Assadpoor, He, and Bhandari, 2008). The diagram of work flow of high pressure homogenizes is illustrated in **Figure 3**.

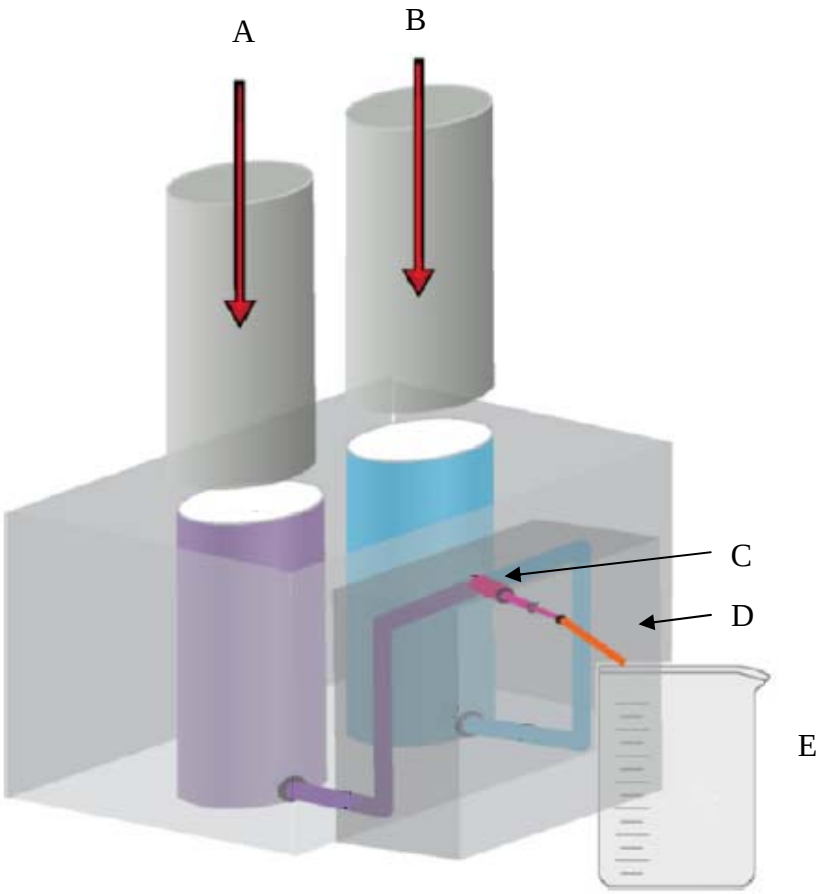



**Figure 3** Schematic representation of a high pressure jet homogenizer to prepare emulsions by forcing two immiscible liquids (disposed on compartments A and B) to flow and mix in the T-mixer (C) located just before homogenization. After mixing and homogenization, the emulsion are conducted out from the homogenization chamber (D) and received on vessel E (Herley and Lina, 2011).

#### 1.2.1.2 Microfluidization

The microfluidizer has been used in food and pharmaceutical industries to make pharmaceutical emulsions, flavor emulsions or homogenized milk (Jafari, *et al.*, 2008). Microfluidization uses a high-pressure pump to force the emulsions through many microchannels into the interaction chamber which creates a very high shearing force to obtain a fine emulsion (**Figure 4**). Previous studies have shown that the

particle size distribution produced by microfluidizers tends to be narrower and smaller than those produced by other homogenization devices (Andreas *et al.,* 2008; Chen *et al.,* 2011). However, the microfluidizer is very expensive and is prone to significant loss in efficiency due to high equipment wear rates (Jafari, Assadpoor, He, and Bhandari, 2008).

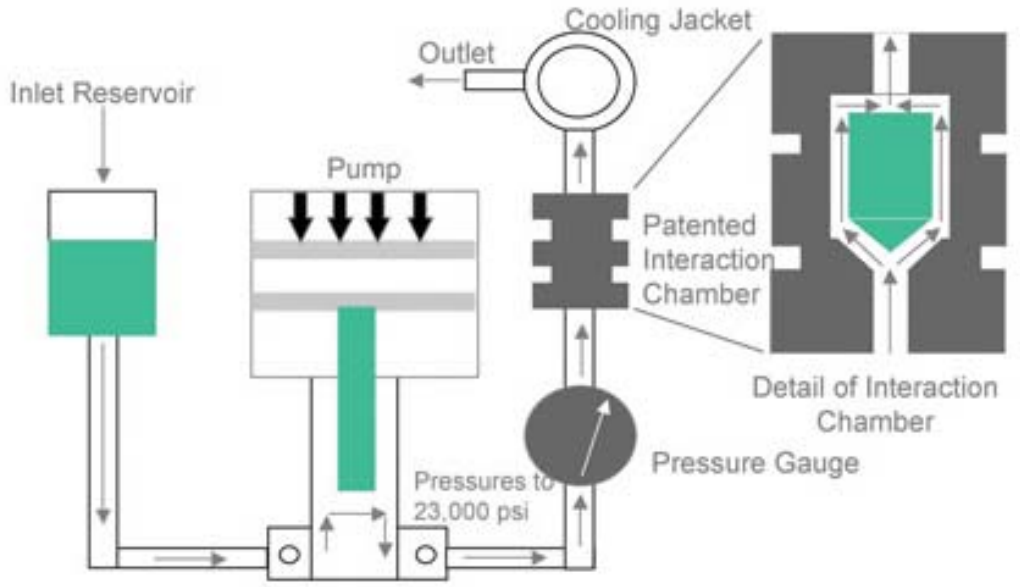



**Figure 4** Schematic of work flow of microfluidizer to produce a fine emulsion (Irwin, 2003).

#### 1.2.1.3 Ultrasonication

Ultrasonic homogenizer provides an alternative to other high-energy emulsification because they are easy to clean and are capable of achieving required power densities (Leong, Wooster, Kentish, and Ashokkumar, 2009).

In an ultrasonicator, the cavitational bubble is formed by converting the electricity into the acoustic wave. The microtip then creates the cavitation to the

samples (**Figure 5**) (Leong, Wooster, Kentish, and Ashokkumar, 2009). **Figure 6** shows the steps of emulsification by ultrasonicator. The first step involves a combination of interfacial acoustic waves and Rayleigh-Taylor instability, leading to the eruption of dispersed phase droplets into the continuous phase. The second step consists of breaking droplets up through cavitation near the interface; the intense effect (disruption and mixing) of shock waves explains the very small droplet size. The most influential parameters affecting cavitation phenomena include power amplitude, input energy and temperature.

Although the procedure is simple, (Chen *et al.*, 2011) ultrasonic homogenization is currently only suitable for small batches (Solans *et al.*, 2005). However, the acoustically formed emulsions were all found to be stable and more homogeneous as compared to mechanical methods (Tadros *et al.*, 2004). The performance of this process can be improved by optimizing the parameters such as the power density, amplitude sonotrodes, irradiation time and temperature (Tal, 2007).

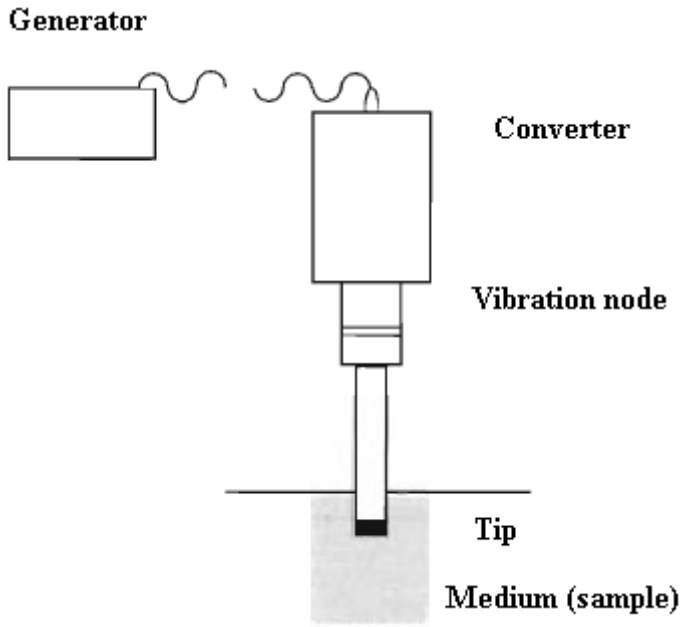



**Figure 5** Experimental set-up for ultrasonication (Canselier, 2002).

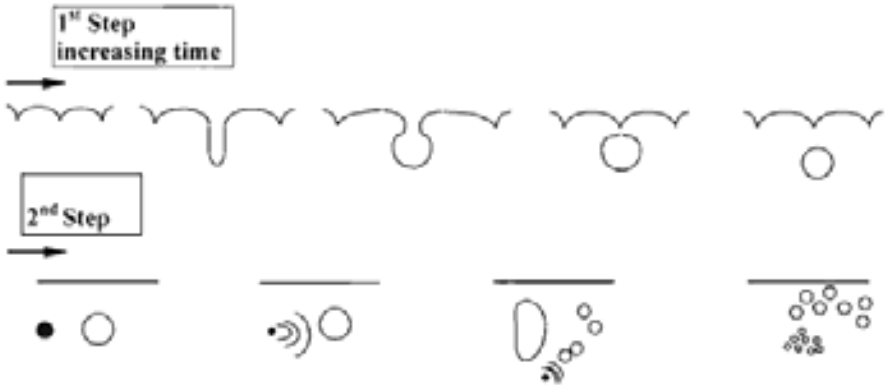



**Figure 6** Steps of acoustic emulsification: droplet formation ($1^{st}$) and size reduction ($2^{nd}$) (Canselier, 2002).

### 1.2.2 Low-energy emulsification

Low-energy emulsification method such as phase inversion temperature (PIT) or emulsion inversion point (EIP) and solvent displacement method. For spontaneous emulsification by the solvent displacement method, also called the ‘‘Ouzo effect’’,

NEs are formed due to very rapid diffusion of an organic solvent (e.g., acetone or ethanol) from the oil phase to the aqueous phase (Anton, Benoit, and Saulnier, 2008; Hareesh *et al.,* 2010).

For the low-energy PIT method, NEs are formed by heating above PIT temperature to inverse the phase of emulsion and then cooling to induce a re-inversion (Johanna *et al.,* 2011). The factors that affect this system are temperature, and electrolyte concentration (Sole *et al.,* 2006). However, these methods have several limitations such as requiring a large amount of surfactants and a careful selection of surfactant-cosurfactant combination**,** and are not applicable to large scale industrial productions (Seekkuarachchi, Tanaka, and Kumazawa, 2006).

### 1.3 Nanoemulsion in medical areas

Nanoemulsions have been used for the transdermal delivery of drugs because of their high drug-loading capacity and enhanced skin permeability. High drug concentration gradients and the small diameters of nanodroplets can also be used for the intravenous delivery of lipophilic drugs that cause venous irritation by encapsulating the drugs in the oil phase, thus preventing the drug exposure to the vessel endothelium (Chen *et al.,* 2011). In addition, NEs have recently been reported to improve the sublingual and intranasal delivery of drugs because of their mucoadhesive properties and ability to enhance the permeability of the mucous layer (Kumar *et al.,* 2008).

Recent studies showed that the bioavailability of non-polar components was increased when encapsulated into NEs compared to the conventional emulsions

(Cheng and David, 2010). It was possibly due to small particle size and high surface area of NEs droplet. The nano-scale sizes of NEs allow them to pass easily into the cells and also penetrate through capillaries. These benefits result in enhancing drug efficacy and less toxicity to the cells which solve the severe side effect problems from conventional formulation.

**2 Inflammation**

Inflammation is the complex biological response of vascular tissue to harmful stimuli, such as pathogens, damaged cells, or irritants (Esper *et al.,* 2006). It is a protective attempt to remove the injurious stimuli as well as to initiate the healing process for the tissue. Inflammation is characterized by five cardinal signs including redness, increased heat, swelling, pain, and loss of function. These five signs appear when acute inflammation occurs on the body's surface. The cell-derived mediators act in parallel with a cellular biochemical cascade systems to initiate and propagate the inflammatory response (**Table 1**).

One of the most important soluble mediators of inflammation is interleukin (IL)-1β, pro-inflammatory cytokine which is produced mainly by blood monocyte and mediates the wide range of reactions involved in the acute phase response. The small amount of IL-1β causes fever, hypertension, and production of cytokines. Interleukin-6 (IL-6) induces the synthesis of hepatic acute-phase proteins and furthermore produces leukocytosis and thrombocytosis by stimulating the synthesis of adhesion molecules in endothelial cells and leukocytes (Ferrero *et al.,* 2006).

**Table 1** Chemical mediators in inflammation **(**Tedgui and Mallat, 2006; Kleemann, Zadellar, and Kooistra, 2008**)**

| Name | Type | Source | Description / Function |
|---|---|---|---|
| **Lysosome granules** | Enzymes | Granulocytes | These cells contain a large variety of enzymes which perform a number of functions. Granules can be classified as either *specific* or *azurophilic* depending upon the contents, and are able to break down a number of substances, some of which may be plasma-derived proteins which allow these enzymes to act as inflammatory mediators. |
| **Histamine** | Vasoactive amine | Mast cells, basophils, platelets | Histamine is stored in preformed granules and released in response to a number of stimuli. It causes arteriole dilation and increased venous permeability. |
| **Interferon gamma (IFN-γ)** | Cytokine | T-cells, NK cells | This interferon was originally called macrophage-activating factor, and is especially important in the maintenance of chronic inflammation. Its effect includes antiviral, immunoregulatory, and anti-tumour properties. |
| **Interleukin-8 (IL-8)** | Chemokine | Primarily macrophages | Activation and chemoattraction of neutrophils, with a weak effect on monocytes and eosinophils. |

**Table 1** (cont.)

| Name | Type | Source | Description / Function |
|---|---|---|---|
| **Leukotriene B4 (LTB4)** | Eicosanoid | Leukocytes | Ability to mediate leukocyte adhesion and activation, allowing them to bind to the endothelium and migrate across it. In neutrophils, it is also a potent chemoattractant, and is able to induce the formation of reactive oxygen species and the release of lysosome enzymes by these cells. |
| **Nitric oxide (NO)** | Soluble gas | Macrophages, endothelial cells, some neurons | Potent vasodilator. Relax smooth muscle, reduce platelet aggregation, aids in leukocyte recruitment, direct antimicrobial activity at high concentrations. |
| **Prostaglandins (PGE)** | Eicosanoid | Mast cells | A group of lipids which can cause vasodilation, fever, and pain. |
| **Tumur necrosis factor-alpha and interleukin-1 (TNF-α and IL-1)** | Cytokines | Primarily macrophages | Both affecting a wide variety of cells to induce many similar inflammatory reactions: fever, production of cytokines, endothelial gene regulation, chemotaxis, leukocyte adherence, activation of fibroblasts. Responsible for the systemic effects of inflammation such as loss of appetite and increased heart rate. |
| **Interleukin-6 IL-6** | Cytokines | T-cells, macrophages | IL-6 is one of the most important mediators of fever and of the acute phase response. It is capable of crossing the blood brain barrier and initiating synthesis of PGE2 in the hypothalamus, thereby changing the body's temperature setpoint. |

The mediator secretion of vasoactive and chemotactic factor such as histamine and nitric oxide results in vasodilation of smooth muscle. Then, blood vessels are increased in permeability and the white blood cells as macrophages are able to go out for scavenging through intercellular space and can be developed themselves into activated macrophage for better scavenging performance. Others cellular mediators are tumor necrotic factor alpha (TNF-α), interferon-γ (IFN-γ), and interleukin-8 (IL-8), is the chemokine involved in the inflammation.

## 2.1 Inflammatory pathways

### 2.1.1 Arachidonic pathway

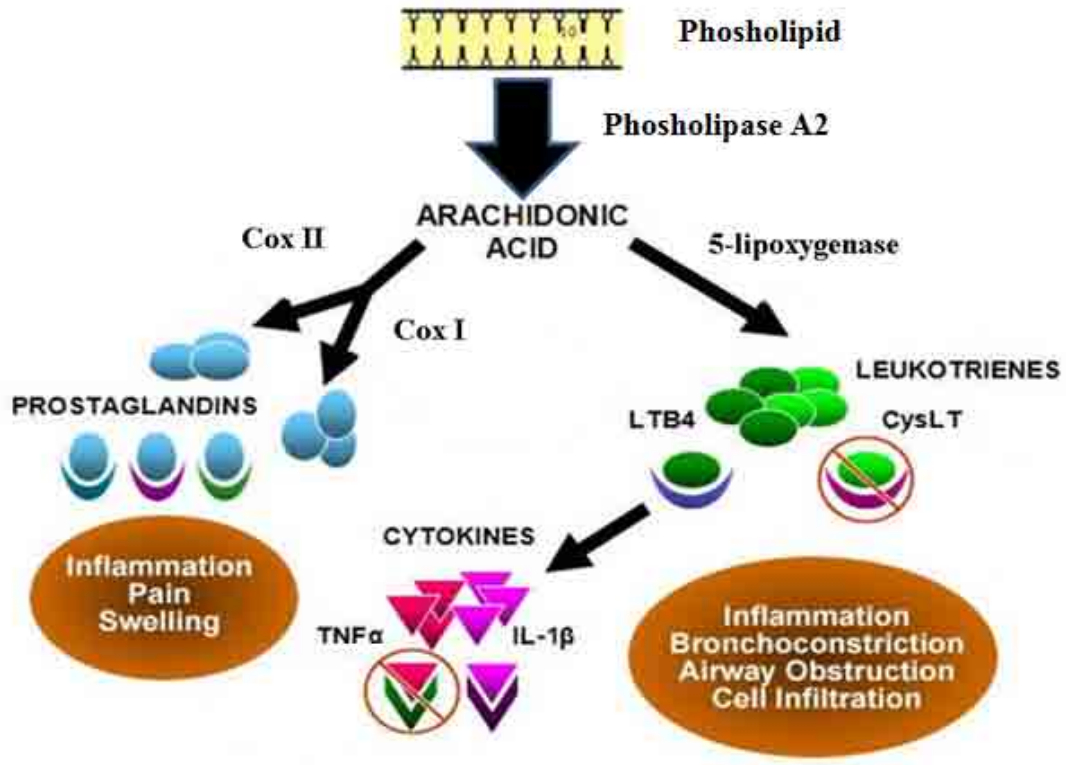



**Figure 7** Arachidonic acid pathway involved in inflammation (Natalia *et al.*, 2005)

The liberation of arachidonic acid (AA) induced by stimuli from its storage site (phospholipid bilayer) appears to be the initial step in the synthesis of inflammatory mediators (Natalia *et al.,* 2005). In fact, there are two major routes for AA deacylation: phospholipase A2 and diacylglycerol lipase. When tissue is damaged, the enzyme phospholipase A2 will digest membrane lipid to AA and free AA may be metabolized by 5-lipoxygenase to leukotrienes (LTB) and then to cytokines. Alternatively, the free AA can be metabolized by an enzyme known as cyclooxygenase (COX I, COX II) to form prostaglandins (PGE) and thromboxanes which drive the process of inflammation (Jesus *et al.,* 1992).

### 2.1.2 Nitric oxide pathway

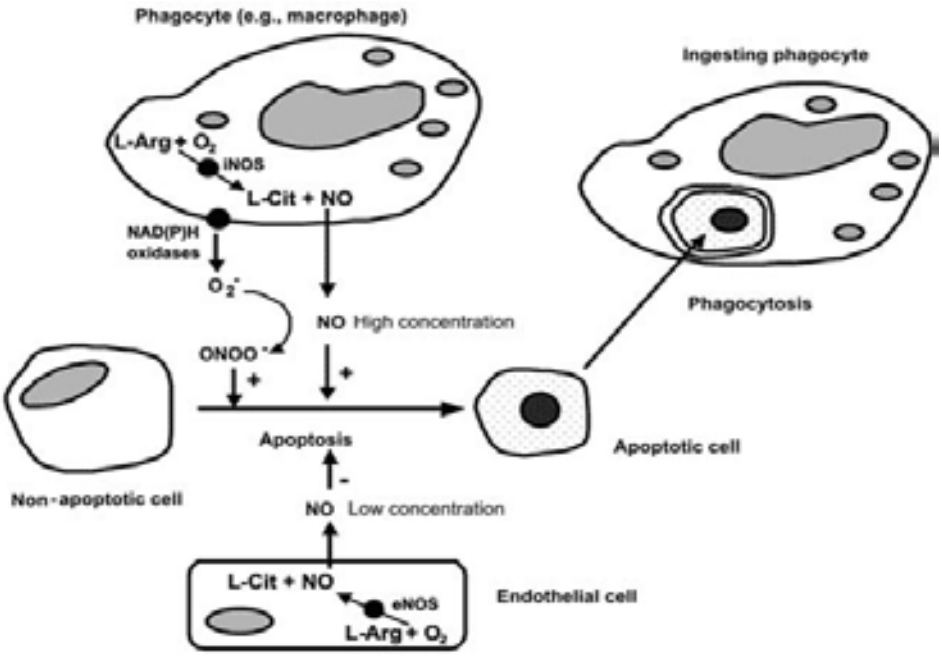



**Figure 8** Nitric oxide pathway involved in inflammation (Catherine *et al.,* 2005)

Nitric oxide (NO) plays an important role in many biological processes including the regulation of inflammatory response (Lisardo *et al.,* 2005). Progression of inflammatory condition depends not only upon the recruitment and activation of inflammatory cells but also upon their subsequent removal from the inflammatory surroundings. Apoptosis or programmed cell death is a fundamental process for regulation of inflammatory cell survival and is critically involved in ensuring the successful resolution of an inflammatory response.

High concentration of NO synthesised by inducible nitric oxide synthase (iNOS) in phagocytes, such as macrophages induces apoptosis in neighbouring cells. In addition, apoptosis can also be induced by ONOO-(highly cytotoxic peroxynitrile) which is generated from $O^-_2$ (superoxide anion) produced by phagocytes reacted with NO. Apoptotic cells are subsequent recognized and ingested by phagocytes, thus aiding the resolution of inflammation. Conversely, low concentration of NO produced constitutively by endothelial isoform of NOS (eNOS) in endothelial cells can inhibit apoptosis. The ability of these activated macrophages to induce apoptosis is greatly reduced in the presence of the NOS inhibitor (Catherine *et al.,* 2005).

## 3 Anti-inflammatory agents

### 3.1 Whey proteins

Whey protein is a by-product of cheese making. It constitutes about 85–90% of the volume of the milk used for transformation to ripened cheese (Rhicha *et al.,* 2007). Recently, Whey proteins are widely attractive owing to their nutritional and

biological properties which are associated with the types of proteins in whey such as α-lactalbumin and β-lactoglobulin (Ana *et al.,* 2007).

### 3.1.1 Whey protein compositions

Whey proteins are globular molecules with a substantial content of α-helix motifs, in which the acidic/basic and hydrophobic/hydrophilic amino acids are distributed in fairly balanced way along their polypeptide chains (Evans, 1982). The pH of whey is equal or higher than 5 depending on type of whey, source of milk, time, type of feed, the stage of lactation and the quality of processing (Pintado, Macedo, and Malcata, 2001). The profile of protein in whey includes β-lactoglobulin (β-LG), α-lactalbumin (α-LA), immunoglobulins (IG), bovine serum albumin (BSA), lactoferrin (LF), and lactoperoxidase (LP), together with other minor components (**Table 2**).

**Table 2** Protein profile and properties found in whey (Brew *et al.*,1970; Eigel *et al.*,1984; Korhonen, 1995; de Wit,1998).

| **Protein** | **Concentration (g/l)** | **Molecular weight (Da)** | **Number of amino acids residues** |
|---|---|---|---|
| β-lactoglobulin | 1.3 | 18,277 | 162 |
| α-lactalbumin | 1.2 | 14,175 | 123 |
| Bovine serum albumin | 0.4 | 66,267 | 58 |
| Immunoglobulins | 0.7 | 25,000 (light chain) +70,000 (heavychain) | - |
| Lactoferrin | 0.1 | 80,000 | 700 |
| Lactoperoxidase | 0.03 | 70,000 | 612 |
| Glycomacropeptide | 1.2 | 6,700 | 64 |

### 3.1.2 Types of whey protein

Whey protein typically comes in three major forms: concentrate (WPC), isolate (WPI), and hydrolysate (WPH). WPC contains 30-89% protein by weight, high carbohydrates in the form of lactose but low level of fat and cholesterol. When the threshold of 90% protein by weight is reached, a WPI is accordingly obtained and both of fat and lactose are not presented. WPH undertakes higher processing than either WPC or WPI. During hydrolysis, proteins are broken down into peptides of different sizes and free amino acids, so WPH is considered more easily absorbed and beneficial for infants intolerant to cow's milk protein (Rhicha *et al.,* 2007).

### 3.1.3 Biological activities of whey protein

The pharmacological activities of whey protein have been reported as following.

#### 3.1.3.1 Anti-inflammation

A sulfur-containing amino acid, cysteine, which is mainly found in whey protein is the initial substance to produce an antioxidant glutathione (GSH). GSH can inhibit the transcription factor NF-κB induced or enhanced by reactive oxygen species (ROS). Because NF-κB is involved in various biological responses, including inflammatory reaction and the induced expression of lymphokines and cytokines (Wulf, 2005). Thus, inhibition of NF-κB should be the prominent inhibition of inflammatory cytokines which are induced by the transcription factor NF-κB.

Interestingly, a novel fermented whey protein-based product described as a gel-like Malleable Protein Matrix (MPM) could exhibit an anti-inflammatory effect

on the atopic contact dermatitis mouse model (ACD) induced by oxazolone hydrocortisone. The result showed that mice fed with MPM had a significant reduction of the ear inflammation as compared to the control (Josee, Claude, and Pierre, 2007).

#### 3.1.3.2 Anticarcinogenicity

Whey proteins have been claimed to prevent several types of cancers including breast cancer, intestinal cancers, colon cancer both *in vivo* and *in vitro* (MacIntosh, *et al.,* 1995; Gill and Cross 2000; Hakkak *et al.,* 2000; Tsuda *et al*. 2000; Badger, Ronis, and Hakkak, 2001).

*In vivo* experiment has also unfolded the anticancer activities ascertained to WPC from their effect on increasing of glutathione (GSH) concentration in relevant tissues and stimulation of immunity via the GSH pathway (**Figure 9**) (Bounous, 2000). On the other hand, published work by Parodi (1998) suggested that WPC depleted tumour cells possessed a higher GSH concentration than the normal cells. Hydrolysed WPI can protect against oxidant-induced cell death in a human prostate epithelial cell line (RWPE-1) (Kent, Harper, and Bomser, 2003). WPI was also used as a coadjuvant with an anticancer drug baicalcin. Drug and WPI in combination were able to induce more apoptosis in the human hepatoma cell line (Hep G2) (Tsai *et al.,* 2000).


**Figure 9** Schematic diagram of glutathione (GSH) synthesis following whey protein intake, and most important functions associated therewith (Ana *et al.*, 2007).

#### 3.1.3.3 Adjuvant in HIV treatment

The Human Immunodeficiency Virus (HIV) infection is characterized by enhanced oxidation stress and a deficiency of the tripeptide glutathione (GSH). Supplementation of WPI can increase GSH levels in HIV-infected individuals and thus scavenging the free radicals (Cotgreave and Gerdes R.G., 1998). The study of Micke *et al.*, (2001) showed that HIV patients aged over 18 years who recieved oral supplementation with whey protein for 2 weeks had increased plasma GSH levels by 44% compared to control group.

#### 3.1.3.4 Other biological effects

Whey proteins are contributed to improve muscle strength and reduce the tendency of bone breaking which are directly related to their amino acid profiles. In fact, their overall amino acid composition is rather similar to that of skeletal muscle; hence, whey protein may be a good anabolic supplement. The advantages of whey proteins in terms of muscle anabolism rely on their fast absorption, coupled with the leucine abundance as well as on their amino acid composition that provides substrates for protein synthesis (Ha and Zemel, 2003).

### 3.2 Omega-3 oil

Omega (ω) 3 polyunsaturated fatty acids (n-3 PUFAs) include α-linolenic acid (18:3, ω=3), eicosapentaenoic acid (EPA) (20:5, ω3) and docosahexaenoic acid (DHA) (22:6, ω3). Recent extensive studies with omega-3 oil demonstrated its beneficial effects that include altered susceptibility to atherosclerotic and atherothrombotic disorders and improvement in autoimmune and inflammatory diseases (You *et al.,* 2006). EPA and DHA are readily incorporated into the cell membrane and alter the membrane phospholipids composition. It can change mediators of inflammation and major secondary messengers that modulate macrophage inflammatory responses**.** For the anti-inflammation, omega-3 oil can inhibit inflammatory signaling pathways (NF-κ B activity) (Chong-Jeh *et al.,* 1999). There was a study on the effect of EPA on inflammatory cytokines of human macrophages and hepatocytes (THLE-3) which were induced to inflammatory condition by using lipopolysaccharide (LPS) or prostaglandin E2 ($PGE_2$). The results showed that EPA effectively reduced LPS-induced or PGE2-induced TNF-α and IL-6 expression, and increased the anti-inflammatory cytokine IL-10 expression significantly when compared with those obtaining arachidonic acid (AA). Hence, there are some suggestions to use ω-3 oil in patients receiving the total parenteral nutrition in order to suppress the inflammatory response (Wei *et al.,* 2010).

The ω-3 oil which is normally consumed is in form of fish oil. There were several reports on ability of fish oil to suppress proinflammatory cytokines and modulate the gene expression of adhesion molecules (Calder, 2003 and You *et al.,* 2006). Fish oil also demonstrated the equivalent effect in reducing arthritic pain to

Non-steroidal anti-inflammatory (NSAID) drugs (Joseph and Jeffrey, 2005). However, the ability of marine fish to convert linolenic (18:3n-3, LNA) and linoleic (18:2n-6, LA) acids to the highly unsaturated fatty acids, 20:5n-3 (eicosapentaenoic, EPA) and 22:6n-3 (docosahexaenoic, DHA) is poor. Moreover, vegetable oils (VO) and excessive consumption of VO and particularly 18:2n-6 has been related to some detrimental effects on health (Montero *et al.,* 2008). While the Echium genus (Boraginaceae) seed oils are relatively rich in LNA and 18:4n-3 (stearidonic acid, SDA) as well as 18:3n-6 (γ-linolenic acid, GLA), and only contain moderate levels of LA compared to other VO (Guerrero *et al.,* 2000). Thus, Echium oil has a good n-3/n-6 PUFA balance as well as an interesting profile with its high content of unusual fatty acids (SDA and GLA) that are of increasing pharmacological interest based on their competitive and inhibitory effects in the production of proinflammatory eicosanoids derived from arachidonic acid (Mercedes et al., 2010).

### 3.2.1 *Echium plantagineum* seed oil

Although source of ω-3 oil is from oily fish, the seed of some plant especially *Echium* spp. shows a potential source of ω-3 oil. *Echium plantagineum* L., is also known as viper's bugloss, blueweed, and snake's flower (Soressa, Peter, and Mahinda 2011). *E. plantagineum* is an annual plant in genus *Echium* (Fam. Boraginaceae) (Gui *et al.,* 2001). Flowers uncurl on an inflorescence called a cyme that has as many as 30 flowers. Flowers are perfect, blue, purple, sometimes white, and pink, trumpet-shaped, and sessile. One to four seeds are produced at the calyx. Seeds are dark brown or grey, small only 3 mm long, and have a triangular shape with three sides. The plant grows to about 70 to 120 cm in height (**Figure 10**) (Berti *et al.,* 2007). Seeds from

Echium species have been recently identified as among the richest source of γ-Linolenic acid (GLA,18:3 ω6). They are also rich in stearidonic acid (SDA,18:4 ω3 or 6,9,12,15 octadecatetraenoic acid). The oil contains 9% to 16% of SDA from 200 to 250 g/kg of echium seed (Clough 1993). SDA is an intermediate in the biosynthesis of eicosapentaenoic acid (EPA, 20:5 ω3) and docosahexaenoic acid (DHA, 22:6 ω3), compared to linolenic acid, since it does not require the first, rate-limiting, Δ6 desaturation step. Echium seed oil has a unique ratio of omega-3 to omega-6 fatty acids not present in any other plant (Coupland and Hebard 2002)**.**

**Table 3** The fatty acid profile of Echium oil (Soressa, Peter, and Mahinda 2011).

| **Fatty Acid** | **Carbon length** | **Content (%)** |
|---|---|---|
| Palmitic acid | 16:0 | 6-8 |
| Stearic acid | 18:0 | 3-5 |
| Oleic acid | 18:1 | 15-19 |
| Linoleic acid | 18:2 n-6 | 14-18 |
| Gamma linolenic acid | 18:3 n-6 | 9-12 |
| Alpha linolenic acid | 18:3 n-3 | 28-33 |
| Stearidonic acid | 18:4 n-3 | 10-14 |

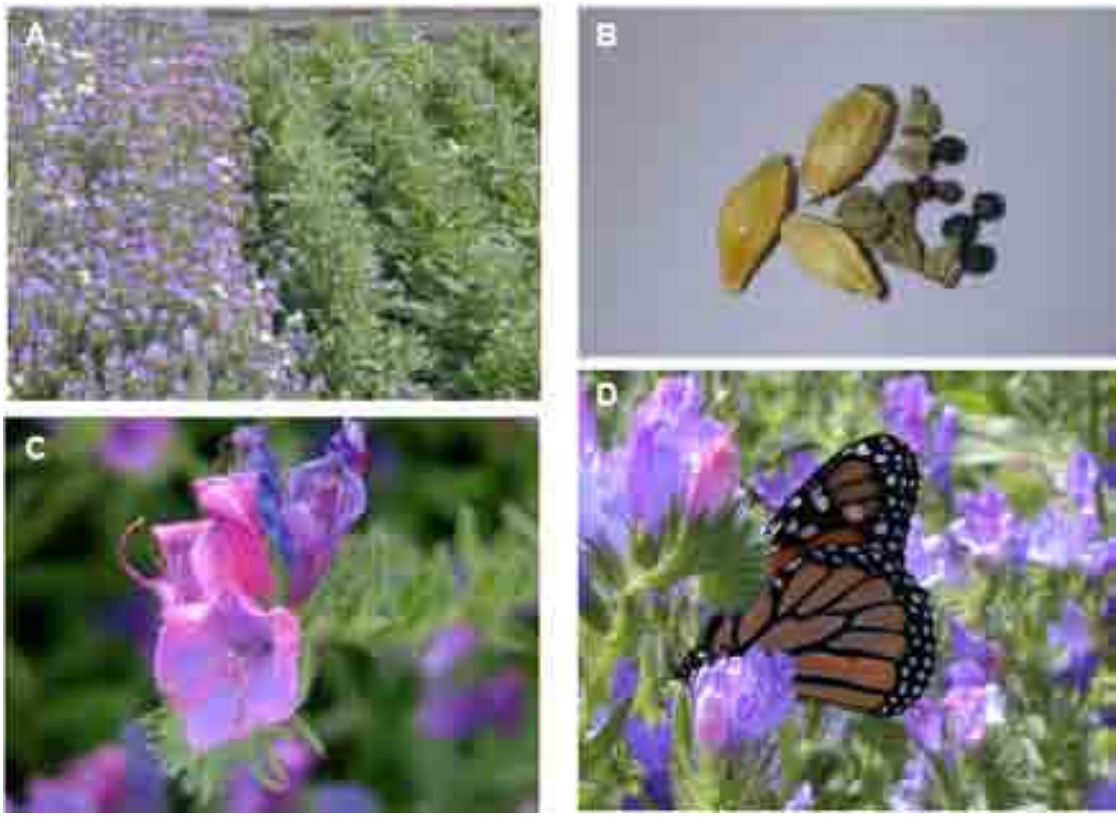



**Figure 10** Schematic illustration of the flowers and seeds of *Echium plantagineum* L. (Berti *et al.*, 2007).

### 3.2.1.1 Biological activities of *Echium plantagineum* seed oil

Echium oil has many potential uses in the pharmaceutical industry for treatment of anti-inflammation, eczema, acne, and other skin disorders and also in the cosmetic products (Soressa, Peter, and Mahinda 2011).

As describe above, Echium oil is very rich in SDA, an essential omega-3 fatty acid known for its anti-inflammatory property. The SDA (c18:4, n-3) effectively inhibits the 5 lipoxygenase emzyme (5-LOX) and thus retards the inflammatory mechanism (**Figure 11**). N-3 Fatty acids are generally less inflammatory than the n-6 fatty acid. However, $PGE_2$ derived from n-6 fatty acid can have an anti-inflammatory

effect by decreasing $LTB_4$ production. The process involves the inhibition of 5-LOX and increasing production of Lipoxin $A_4$ ($LXA_4$) by stimulating 15-lipoxygenase (15-LOX). The equally high proportion of 7% – 12% of γ-linolenic acid in the seed oil is also responsible for the optimum protection of the skin barrier; as unsaturated fatty acids regenerate the intercellular cement of the epidermis (Rahn, 2008). The oil rich in SDA could results in higher amounts of SDA, EPA, DHA and total ω3 fatty acid once uptake into the body (Matthew, Peter and Chris, 2007). In order to reduce the consumption of ω-3 oil from fish and discard fishy odor, *echium* seed oil can be considered to be the source of the oil.

As previously mentioned, both whey protein isolate (WPI) and *E. plantagineum* seed oil have the common bioactivities especially the anti-inflammation. Therefore, this study was aimed to prepare nanoemulsions by using WPI as an emulsifier and used the plant seed oil as an oil-phase. The WPI-stabilized nanoemulsions were determined for their potential on modulation of pro-inflammatory cytokines in the human monocytic leukemia (THP-1) cell line.

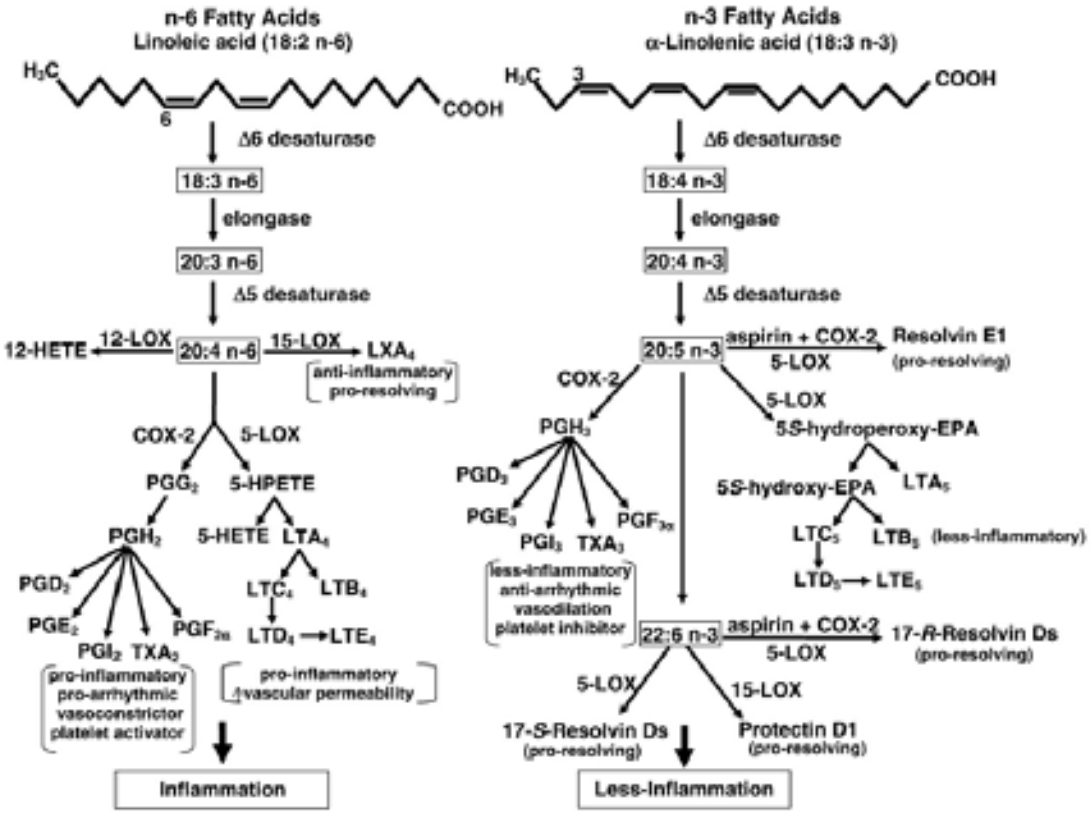



**Figure 11** The metabolism of n-3 and n-6 fatty acid and the biosynthesis of their respective eicosanoid and mediators. Abbreviations: HPETE = hydroperoxyeicosatetraenoic acid; $LTA_n$ = leukotriene $A_n$; $LXA_n$ = lipoxin $A_n$; LOX = lipoxygenase; $PGD_n$ = prostaglandin $D_n$; $LTD_n$ = leukotriene $D_n$; $TXA_n$ = thromboxane $A_n$ (Yuriko and Darshan, 2010).

# CHAPTER III

# MATERIAL AND METHODS

## 1. Material

### 1.1 Equipment

1. 6- well plate (Costar, USA)
2. 24-well plate (Costar, USA)
3. 96-well plate (Costar, USA)
4. De-ionized water (DI water) system (ELGAStat Option 3B) (ELGA, UK)
5. Centrifuge (Hettich Zentrifugen Universal 320R, USA)
6. $CO_2$ incubator (Forma, US)
7. Conical tube 15 ml, 50 ml (Axygen scientific, USA)
8. High intensity ultrasonic processor VCX 750 (Sonics & materials, USA)
9. Laminar air flow (Microflow, UK)
10. Microcentrifuge tube 1.5 ml (Sigma-Aldrich, USA)
11. Micropipettes SL-20 (2-20 µL), SL-200 (20-200 µL) and SL-1000 (100-1,000 µL) (Rainin Instrument, USA)
12. Micropipettes tips (Gibson, France)
13. Microplate reader ($SPECTRA_{max}$ Plus 384, Molecular Devices, USA)
14. Microplate shaker capable of 300-1,100 rpm (TSZ Scientific LLC, USA)

15. Multichannel pipettes (12 channels) 20 and 100 µL (Rainin Instrument, USA)
16. Multichannel tips RT-L250 (250 µL), TR-222-C (200 µL) (Rainin Instrument, USA)
17. Petri dish 100 mm x 20 mm (Corning, USA)
18. Reagent reservoir (Rainin Instrument, USA)
19. Refrigerator (Mitsubishi, Japan)
20. Refrigerated centrifuge (Hitachi, Japan)
21. Spectrophotometer (Beckman coulter DTX800, USA)
22. Sterile filter plate, 96-well (Bio-rad, USA)
23. Timer (Citizen, Japan)
24. Tissue culture flask, T25 (Corning, USA)
25. Ultracentrifuge (Optima L-80, Beckman Coulter, USA)
26. Ultra-purifier water system (Maxima UF, UK)
27. Vortex mixer (Clay Adams, USA)
28. Water bath (Julabo, Germany)
29. Zetasizer NanoZS (ZEN3600, Malvern, UK)

## 1.2 Chemicals

1. Bio-Plex™ cytokine assay kit (Bio-Rad, US) including components as shown in **Table 4**)
2. Bradford's reagent (Bio-Rad, US)
3. Dimethylsulfoxide (DMSO) (Sigma-Aldrich, USA)
4. *Echium Plantagineum* seed oil (RevitElix™, CRODA, France)

5. Ethanol (Sigma-Aldrich, USA)
6. Ethylenglycol-monophenylether (CRODA, France)
7. Fetal bovine serum (Gibco, USA)
8. Glycerin (Sigma-Aldrich, USA)
9. Hydrochloric acid (HCl) (Carlo Erba Reagents, Italy)
10. Lipopolysaccharide (LPS) (Sigma-Aldrich®, USA)
11. Methanol (Sigma-Aldrich, USA)
12. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich, USA)
13. Penicillin-Streptomycin (Pen Strep) (Gibco, USA)
14. Roswell Park Memorial Institute (RPMI) medium 1640 (Invitrogen, USA)
15. Sterile water for injection (Thai Otsuka, Thailand)
16. Triton X-100 (USB, USA)
17. Trypan blue (Sigma-Aldrich, USA)
18. Ultrapure water (18.2 MΩ) (Elga, UK)
19. Whey protein isolate (Glanbia Nutritionals, Ireland)

### 1.3 Cell lines

1. The human monocytic leukemia (THP-1) cell line (The American Type Culture Collection (ATCC), USA)

**Table 4** The components of Bio-Plex™ cytokine assay kit

| Components | Volume/size |
|---|---|
| Bio-Plex assay buffer | 75 ml |
| Bio-Plex wash buffer | 150 ml |
| Bio-Plex detection antibodies<br>- Pro human cytokine: IL-1β, IL-2, IL-4, IL-6, IL-8, GM-CSF, IFN-γ and TNF-α. | 2x320 µl |
| Bio-Plex detection antibody diluent | 5 ml |
| Conjugated magnetic beads<br>- Pro human cytokine: IL-1β, IL-2, IL-4, IL-6, IL-8, GM-CSF, IFN-γ and TNF-α. | 2x600 µl |
| Standard cytokine<br>- IL-1β, IL-2, IL-4, IL-6, IL-8, GM-CSF, IFN-γ and TNF-α. | 1 vial |
| Standard cytokine diluent | 10 ml |
| Streptavidin-PE (100x) | 1 vial |

## 2. Methods

### 2.1 Preparation of nanoemulsions (NEs) stabilized by whey protein isolate

In order to produce 10-ml o/w nanoemulsions by ultrasonicator, the pre-emulsion was initially made. Whey protein isolate (WPI) powder was mixed with a wetting agent, glycerin, to enhance wettability. Then, the sterile water for injection was added to disperse powder. It was noted that sterile water for injection was used when preparing sample for cell experiments. The mixture was stirred until WPI powder was well dissolved. After that *Echium plantagineum* seed oil and ethyleneglycol-monophenylether (preservative) were added into liquid solution, respectively. Then, the mixture was introduced to treatment with ultrasonicator to provide an emulsion with small droplet size (detailed in 2.1.1 and 2.1.2). The preparation was then cooled and stored at room temperature for 24 h, 2 weeks, 1, 2, and 3 months. The steps of sample preparation are shown in **Figure 12**. The effect of ingredients and parameters involved in ultrasonication were studied as followed.

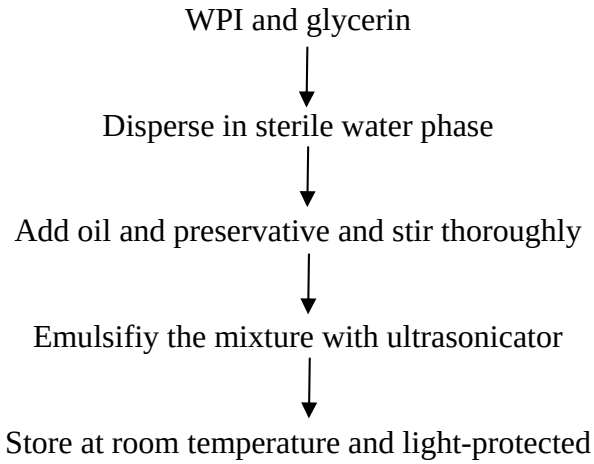



**Figure 12** Preparation of WPI-stabilized nanoemulsions

### 2.1.1 Effect of ingredients on nanoemulsions preparation

The effect of main ingredients, oil and WPI, were studied for ability to form NEs. The oil concentrations of 5% and 10% w/w were used and WPI concentrations were varied from 1 to 20% w/w. For all formulations, glycerin and ethyleneglycol-monophenylether were used at 2.5% and 0.7% w/w, respectively.

### 2.1.2 Ultrasonic emulsification

The 10-ml samples containing various concentrations of WPI and *E. plantagineum* seed oil were prepared and subjected to emulsify by ultrasonication method using 20 kHz high intensity ultrasonicator, equipped with an ultrasonic probe (3-mm diameter). The parameters affecting the cavitation were studied at amplitude in a range of 20-40%, controlled temperature between 30-50$^{o}$C and supply energy of 500-3,000 Joules (J). The process was run with a non-stop pulse mode until the final energy supply was reached to the actual power. The experimental setup is shown in **Figure 13**.

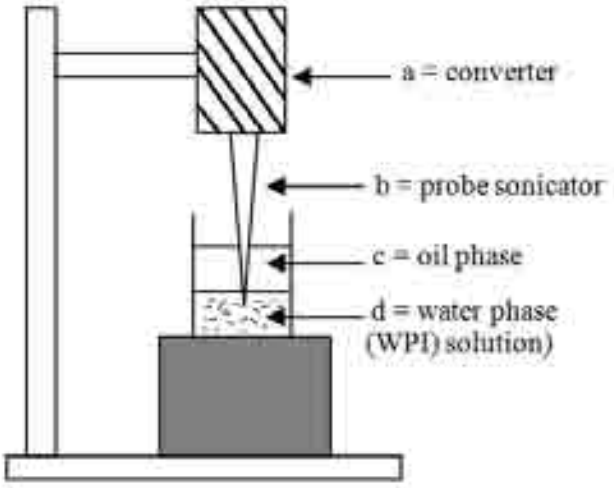



**Figure 13** Experimental setup of ultrasonic emulsification

## 2.2 Characterization of nanoemulsions

The particle size and size distribution of NEs were determined by dynamic light scattering apparatus (DLS) which analyzes fluctuations in the intensity of light scattering due to Brownian movement of the paticles (Vikas, Mushir and Javed, 2010). DLS measurement gives the Z-average hydrodynamic diameter (or Cumulant mean) which is an intensity mean diameter, and the polydispersity index (PDI) which is considered as a rough indication of the broadness of size distribution (Johanna *et al.*, 2011). To avoid multiple scattering effect, all emulsion samples were diluted with deionized water to the final concentration of 1% (Tushar, Aliasgar and Mansoor, 2008; Po and Been, 2011). Dynamic light scattering was performed at 25$^{o}$C and a wavelength of 633 nm (Vikas, Mushir and Javed, 2010). Emulsion droplet sizes were estimated as averages of three measurements.

The stability of NEs was evaluated for any signs of instability (creaming, oil separation, etc.) and the change in size and zeta potential of NEs were determined after storage for 24 h, 14 days, 1, 2 and 3 months at room temperature.

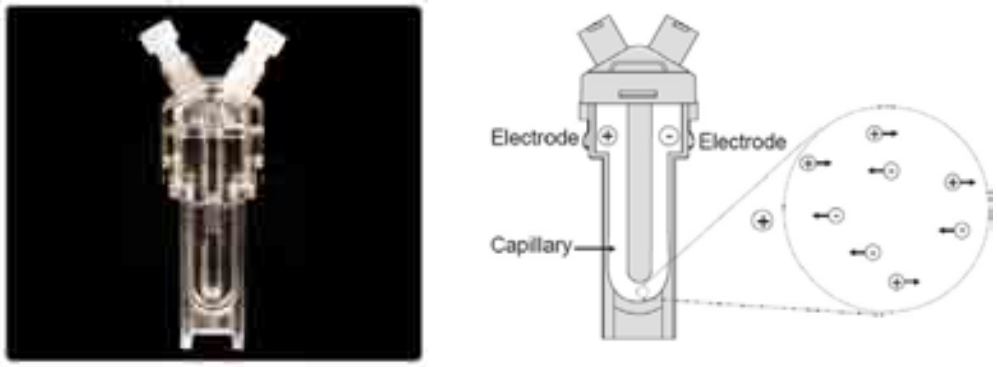



**Figure 14** The disposable capillary cell for zeta potential measurement.

For the zeta potential measurement, samples were added into a clear disposable capillary cell (**Figure 14**). Zeta potential is measured by using laser doppler electrophoresis. The instrument (Zetasizer) was equipped with avalance photodiode detector. Basically, an electric field is applied to the nanoparticles, resulting in the acceleration of the particles (Note *et al.*, 2006). Light, scattered by moving particles, alters frequency. The interference between the scattered light and original beam provides a modulated signal. Then the frequency analysis leads to electrophoretic mobility ($U_E$) and the zeta potential is calculated by using the Henry's equation as following (Sjöblom, 2006):

$$U_E = \frac{2\ \varepsilon\ \zeta\ f(\kappa a)}{3\ \eta} \qquad (1)$$

| | | |
|---|---|---|
| $\zeta$ | = | zeta potential (millivolt; mV) |
| $\varepsilon$ | = | dielectric constant (Farads per meter; F/m) |
| $\eta$ | = | viscosity (Pascal second; Pa s) |
| $f(\kappa a)$ | = | Henry's function |

### 2.3. Determination of cytotoxic effect of nanoemulsions on THP-1 cell line

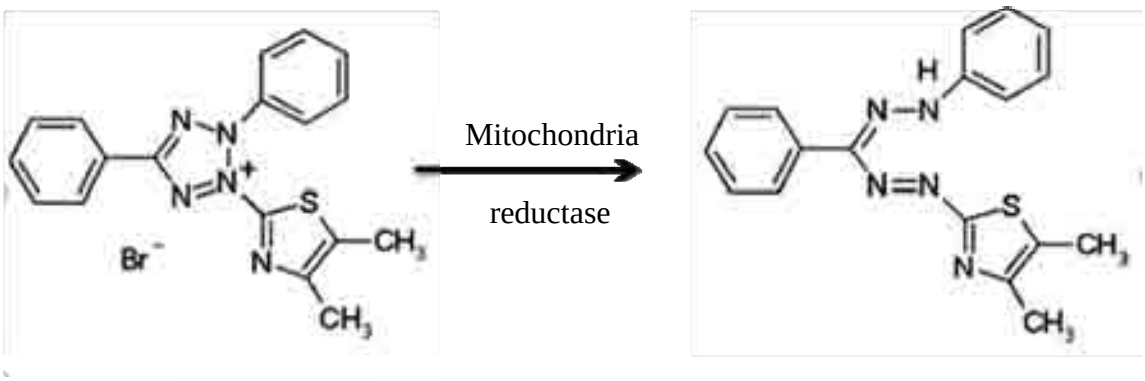
#### 2.3.1 Cell line and cell culture

The human monocytic leukemia cell line, THP-1, obtained from American Type Culture Collection (ATCC) was used in this study. THP-1 cells were grown

routinely in T75 plastic tissue culture flasks in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% v/v fetal bovine serum (FBS), 2 mM L-glutamine and antimycotics-antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). Cultures were maintained in atmosphere of 5% $CO_2$ at 37°C under 95% humidity for 24 h and medium was refreshed every three or four days with subculturing.

### 2.3.2 Cytotoxicity test of nanoemulsions

To obtain the optimal concentration of NEs for further experiment, the toxicity of NEs on THP-1 cells was performed. THP-1 cells were harvested to a final concentration at $5x10^4$ cell per ml. One hundred microlitres of cell suspension were added into each well of 96-well plate and treated with 100 µl of sample contain various concenrations (0.01, 0.1, 1, 10, and 100 mg/ml) of WPI. After incubation time (24, 48, and 72 h), cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells not treated with NEs (containing only medium) were used as a control. The viable cell was detected by the ability to converse the water soluble MTT to an insoluble formazan by mitochondrial reductase (Jian *et al.,* 2007) (**Figure 15**). Briefly, MTT was dissolved in PBS, pH 7.4, at a concentration of 5 mg/mL and added to the cell culture to the final concentration of 0.5 mg/mL. After 2 h of incubation, medium was removed and the remaining insoluble formazan was dissolved in 100 µl dimethyl sulfoxide (DMSO) and the optical density was determined at 570 nm by UV spectrophotometer.




MTT
(yellow)

Formazan
(purple)

**Figure 15** Reduction reaction scheme of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Jian *et al.,* 2007)

Cell viability was then calculated as followed:

$$\% \text{ Cell viability} = \frac{A_{570/620} \text{ of sample}}{A_{570/620} \text{ of control}} \times 100 \quad (2)$$

where, $A_{570/620}$ = Absorbance at 570 nm and reference wavelength at 620 nm.

The results were expressed as percentage of cell viability compared to a control (untreated cells). Dose-response curve was plotted between % cell viability and the concentrations of WPI in NEs. The concentration which is non-toxic (95% cell viability) compared to control group will be used in the following study.

### 2.4 Determination of anti-inflammation of nanoemulsion.

#### 2.4.1 Cell culture treatment and sample preparation

THP-1 cells ($1x10^6$ cells/ml) were 24-h incubated at 37ºC, 5% $CO_2$ with different samples: i) incubated with NEs, ii) with WPI solution, iii) with *E. Plantagineum* seed oil, iv) with medium for 24 h. All treatments were then incubated

with 200 ng/ml of lipopolysaccharide (LPS) for another 4 h. The cell incubated with only medium was used as a control group. It was noted that LPS was added in order to stimulate the cells produce pro-inflammatory cytokines. The method of cell culture and sample preparation for determination of anti-inflammation of the treated sample is shown in **Figure 16**.

24-h Incubate at 37ºC, 5% $CO_2$ of THP-1 ($1x10^6$ cells/ml) with

i) NEs, ii) WPI solution, iii) *E. Plantagineum* seed oil, iv) medium, v) medium

↓

4-h Incubate with 200 ng/ml of lipopolysaccharide (LPS) except group v (control group)

↓

Centrifuged the THP-1 cell suspension at 3,000 rpm $4^oC$ for 10 minutes

↓

Separate the THP-1 cell and supernatant (kept into micro-centrifuge tube at $-80^oC$ until assay)

[Note: The following steps were done for collecting the cytokines in THP-1 cell]

Resuspend and wash the THP-1 cells with 5 ml of phosphate buffered saline (PBS)

↓

Centrifuge the cell suspension at 3,000 rpm $4^oC$ for 10 minutes and remove the supernatant

↓

Add 1 ml of PBS and transferred into micro-centrifuge tube and centrifuge at 4,000 rpm 4$^{o}$C for 5 minutes and remove the supernatant (2 times)

↓

Resuspend the pellet with 150 µl of PBS and incubate for 24 h at -20$^{o}$C

↓

Lyzed cells with hand homogenizer for 5 minutes

↓

Centrifuge at 10,000 rpm 4$^{o}$C for 10 minutes and collect the supernatant

↓

measure pro-inflammatory cytokines

**Figure 16** Steps of determination of anti-inflammation. The experiment was run in triplicate using different cell generation (passage) (See in Appendix A).

### 2.4.2 Principle of Bio-Plex immunoassay

Cytokines, chemokines, and growth factors are cell signaling proteins, which act as mediation in a wide range of physiological responses, including inflammation and immunity. These molecules are typically measured either by bioassay or immunoassay and the technique involved are Western blot analysis and polymerase chain reaction (PCR). These techniques are considered time consuming and the

analysis is normally performed on only a single target at a time. In this study, the Bio-Plex immunoassay-based technology was used. The Bio-Plex incorporates novel xMAP technology using color-coded beads and permits the simultaneous detection of up to 100 cytokines in a single well of a 96- well microplate, by aid of 100 unique fluorescently dyed beads (**Figure 17**)

**Figure 17** Microscopic beads, each with a different color code, or spectral address, to permit discrimination among multiplex assays.

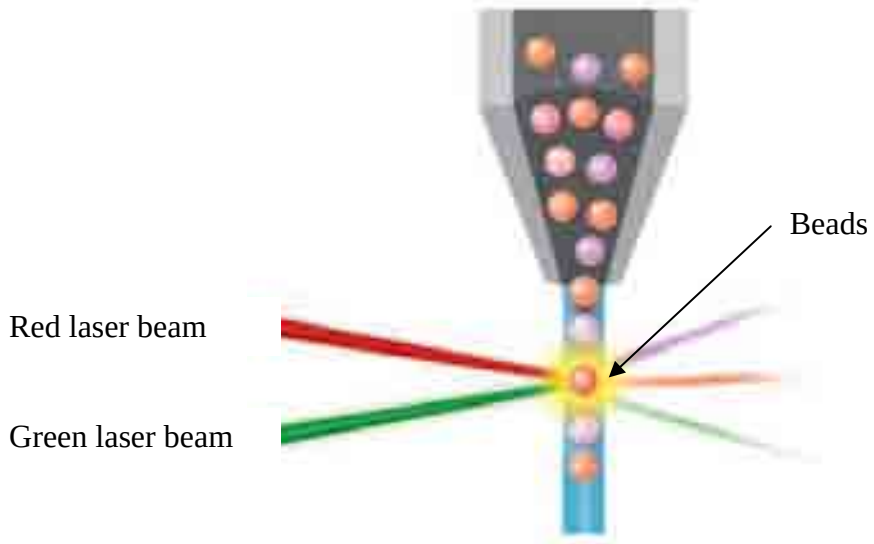



**Figure 18** In the array reader, red classification and green reporter lasers illuminate individual beads to identify each bead's spectral address and associated reporter signal.

Principally, the Bio-Plex xMAP technology is the 96- well plate-formatted, bead-based assay similar to a capture sandwich immunoassay (Fernando *et al.*, 2008). An antibody directed against the desired cytokine, chemokine, or growth factor target is covalently coupled to internally polystyrene dyed beads. The coupled beads are allowed to react with a sample containing the target biomolecules. After a series of washes to remove unbound protein, a biotinylated detection antibody specific to an epitope different from that of the capture antibody is added to the reaction. This results in the formation of a sandwich of antibodies around the cytokine, chemokine, or growth factor target. A streptavidin-phycoerythrin (streptavidin-PE) reporter complex is then added to bind to the biotinylated detection antibodies on the bead surface **Figure 19**.

Data from the reaction are acquired using the Bio-Plex system, a dual-laser (red, λ = 635 nm and green, λ = 532 nm) flow-based microplate reader system. The lasers and associated optics detect the internal fluorescence of the individual dyed beads as well as the fluorescent reporter signal on the bead surface (**Figure 18**). This identifies each assay and reports the level of target protein in the sample. The red classification laser excites the dyes in each bead, identifying the specific bead address. The green reporter laser excites the reporter molecule associated with the bead, allowing quantitation of target molecules (cytokines) in the tested samples. A high-speed digital processor efficiently manages the data output, which is further analyzed and presented as fluorescence intensity (FI) and target concentration on Bio-Plex Manager software.

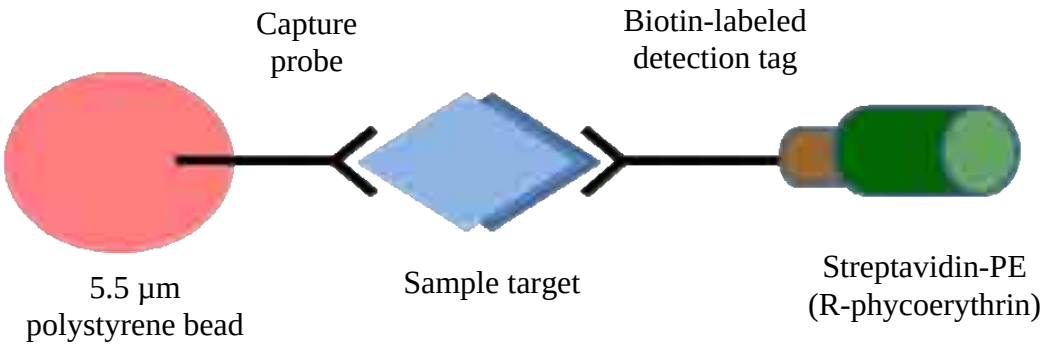



**Figure 19** A capture molecule conjugated to a color-coded bead binds to a target analyte followed by binding with biotinylated detection antibody and a reporter molecule, streptavidin-PE.

### 2.4.3 Determination of pro-inflammatory cytokines in THP-1 cells

Cytokines levels in THP-1 cell lysates from 2.4.1 were quantified by using multiplex bead-based assays designed to quantitate cytokines (IL-1β, Il-2, IL-4, IL-6, IL-8, GM-CSF, IFN-γ, and TNF-α). Moreover, the cell supernatant was also determined for the cytokine level. Briefly, the 96-well sterile filter plate was prewetted with assay buffer at 150 µl/well (Figure 20). Then 50 µl/well of diluted beads coupled to antibodies against target proteins were added and the well-plate was washed 2 times by wash buffer. The test sample (cell lysates) was added (50 µl/well) and incubated for 30 minutes before washing the plate 3 times with wash buffer (100 µl/well). Then the biotin-labeled detection antibodies specific for secondary epitopes for cytokine were added at 25 µl/well. Then, the plate was sealed and shaken at 1,100 rpm for 30 seconds, then 300 rpm for 30 minutes in the dark condition. After that the plate was washed 3 times with 100 µl/well of wash buffer. Fifty µl/well of fluorescently labeled streptavidin (streptavidin-PE (phycoerythrin)) was added for binding to the biotin-labeled detection antibodies and the plate was sealed and shaken at 1,100 rpm for 30 seconds, then 300 rpm for 10 minutes in the dark condition. The plate was washed again with 100 µl/well with wash buffer for 3 times. Lastly, 125 µl/well of assay buffer were added into the well-plate in order to resuspend beads prior to shaking at the 1,100 rpm for 30 seconds. The cytokine levels of the sample were determined using a multiplex array reader. It was noted that the cytokine standards (Master standard) was also measured for calibration and comparing the results. The assay scheme established for microplate (96-well plate) is shown in **Figure 21**. The preparation of Master standard, beads, detection antibodies and streptavidin-PE was described later.

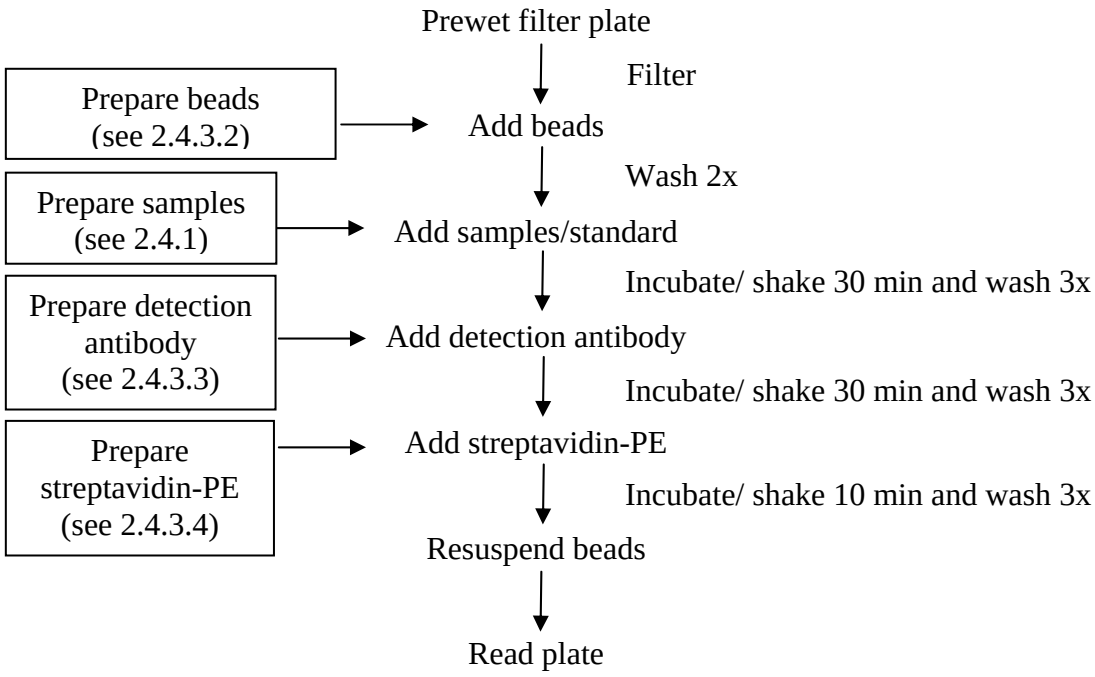



**Figure 20** Cytokine assay workflow

| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | S2 | S6 | B | C3 | L2 | W2 | O2 | N1 | sC1 | sL2 | sW3 | sN1 |
| **B** | S2 | S6 | B | C3 | L3 | W2 | O2 | N2 | sC2 | sL3 | sO1 | sN1 |
| **C** | S3 | S7 | C1 | C3 | L3 | W3 | O2 | N2 | sC2 | sL3 | sO1 | sN2 |
| **D** | S3 | S7 | C1 | L1 | L3 | W3 | O3 | N2 | sC3 | sW1 | sO2 | sN2 |
| **E** | S4 | S8 | C1 | L1 | W1 | W3 | O3 | N3 | sC3 | sW1 | sO2 | sN2 |
| **F** | S4 | S8 | C2 | L1 | W1 | O1 | O3 | N3 | sL1 | sW2 | sO3 | sN3 |
| **G** | S5 | S9 | C2 | L2 | W1 | O1 | N1 | N3 | sL1 | sW2 | sO3 | sN3 |
| **H** | S5 | S9 | C2 | L2 | W2 | O1 | N1 | sC1 | sL2 | sW3 | sN1 | sN3 |

**Figure 21** Schematic of microplate organization for BioPlex assay kit protocol.

S2-9; Cytokine standards

C1-3; Control (supernatant of cell lysate)

L1-3; Supernatant of cell lysate with LPS

W1-3; Supernatant of cell lysate with LPS and whey protein isolate

O1-3; Supernatant of cell lysate with LPS and *E*. Plantagineum seed oil

N1-3; Supernatant of cell lysate with LPS and nanoemulsions

sC1-3; Control (cell supernatant)

sL1-3; Cell supernatant with LPS

sW1-3; Cell supernatant with LPS and whey protein isolate

sO1-3; Cell supernatant with LPS and *E*. Plantagineum seed oil

sN1-3; Cell supernatant with LPS and nanoemulsions

#### 2.4.3.1 Master standard preparation

The multiplex standard dilutions (Master standard) were prepared for use as an internal control for optimal sensitivity and accuracy of the assay. The lyophilized cytokine standard was reconstituted with 200 µl of RPMI medium (standard diluent) and the mixture was gently vortexed for 3 second and incubated on ice for 30 minutes. After incubation, master standard stock was done by pipetting 192 µl from the reconstituted standards into a single 1.5 ml tube containing 183 µl of the medium (final volume = 375 µl). The master stock (S1) was serially diluted by pipetting 50 µl from the previous dilution into 1.5 ml tube with 150 ml of RPMI medium. It was noted that the master stock (375 µl) contained cytokines at 32,000 pg/ml (**Figure 22**).

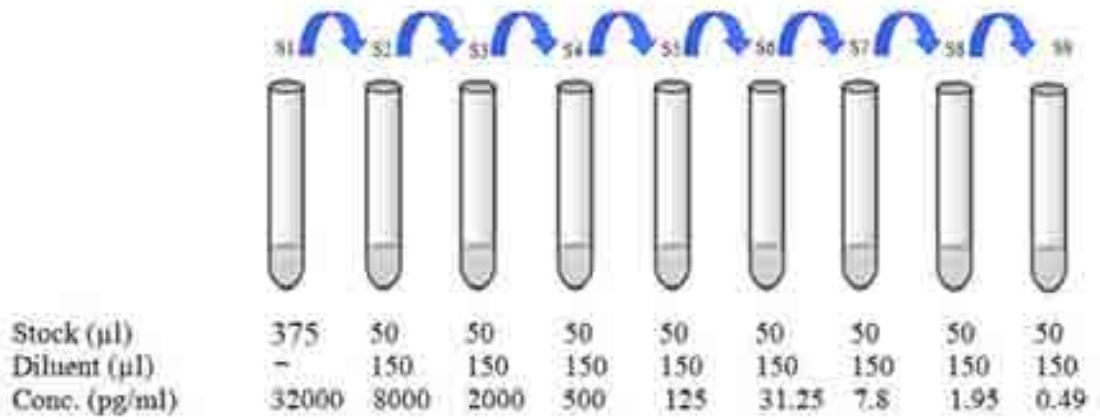



**Figure 22** Schematic of master standard dilutions started with a concentration at 32,000 ρg/ml (S1). * Each standard is a 4-fold dilution of preceding one. The stocks were diluted with media culture, RPMI-1640, to final volume of 200 µl.

#### 2.4.3.2 Bead preparation

The vial of anti-cytokine conjugated beads (5x) was vortexed at medium speed for 20 seconds. The multiplex bead stock was prepared by adding buffer at the volume specified the number of wells per experiment (**Table 5**). After that, all tubes were kept on ice and protected the beads from light until used.

**Table 5** Compositions of multiplex bead stock solution**.**

| Number of wells | Volume of stock beads (µl) | Bio-plex assay buffer (µl) | Total volume (µl) |
|---|---|---|---|
| 96 | 1,150 | 4,600 | 5,750 |
| 88 | 1,054 | 4,217 | 5,271 |
| 80 | 959 | 3,833 | 4,792 |
| 72 | 862 | 3,450 | 4,312 |
| 64 | 766 | 3,067 | 3,833 |
| 56 | 671 | 2,683 | 3,354 |
| 48 | 575 | 2,300 | 2,875 |

#### 2.4.3.3 Preparation of detection antibodes

Two vials of detection antibody (5x) were vortexed at medium speed for 20 seconds and diluted with assay buffer (diluent) to obtained the antibody solutions (1x) at the volume depended on the number of wells per experiment (**Table 6**). The stock solution was stored in the dark at room temperature and used within 4 h.

**Table 6** Compositions of detecting antibodies stock solution**.**

| **Number of wells** | **detection antibody (µl)** | **Bio-plex assay buffer (µl)** | **Total volume (µl)** |
|---|---|---|---|
| 96 | 600 | 2,400 | 3,000 |
| 88 | 550 | 2,200 | 2,750 |
| 80 | 500 | 2,000 | 2,500 |
| 72 | 450 | 1,800 | 2,250 |
| 64 | 400 | 1,600 | 2,000 |
| 56 | 350 | 1,400 | 1,750 |
| 48 | 300 | 1,200 | 1,500 |

#### 2.4.3.4 Preparation of streptavidin-PE

The vial of streptavidin-PE (100x) was centrifuged for 30 seconds before making a dilution by using assay buffer to an assay concentration of 1x. The working volume for preparation is in **Table 7**. The streptavidin-PE stock solution was stored in the dark at room temperature and used within 4 h.

**Table 7** Compositions of streptavidin-PE stock solution.

| Number of wells | Streptavidin-PE (µl) | Bio-plex assay buffer (µl) | Total volume (µl) |
|---|---|---|---|
| 96 | 60 | 5,940 | 6,000 |
| 88 | 55 | 5,445 | 5,500 |
| 80 | 50 | 4,950 | 5,000 |
| 72 | 45 | 4,455 | 4,500 |
| 64 | 40 | 3,960 | 4,000 |
| 56 | 35 | 3,465 | 3,500 |
| 48 | 30 | 2,970 | 3,000 |

**3. Statistical analysis**

The measurement values were presented in mean ± S.D. and for mean ± SEM was used only for determination of pro-inflammation cytokines in THP-1 cell. One-way repeated Analysis of Variance (ANOVA) was used to statistically determine differences between treatment groups. *p* value less than 0.05 indicated statistical significance.

# CHAPTER IV

# RESULTS AND DISCUSSION

## 1. Preparation and characterization of nanoemulsions stabilized by whey protein isolate

The size of an emulsion droplet formed is controlled by the emulsifier properties which interplays between droplet breakup and droplet coalescence (Tadros *et al.*, 2004). Droplet break up is controlled by the type and amount of shear applied to droplets as well as the droplets resistance to deformation (Laplace pressure). The rate of droplet coalescence is determined by ability of the emulsifier to rapidly adsorb to the surface of newly formed droplets. In this study, the effect of composition and emulsification process parameter on nanoemulsions size and size distribution was determined.

### 1.1 Effect of ingredient concentration on nanoemulsions preparation

Nanoemulsions (NEs) were prepared using various concentrations of WPI and *E. Plantagineum* seed oil. The process was carried out at preset with 20% ultrasonic amplitude, 1,250 Joules of total energy input and the temperature was controlled at 45 $^{o}$C. From the results, the average oil droplet diameters of NEs were in the range of 264-1,248 nm (**Table 8**). It was found that NEs containing either 5% or 10 % (w/w) oil formed with higher concentration of WPI 20% (w/w) provided a good result in term of size and size distribution (smaller size and PdI value). It was owing to sufficient amount of WPI to stabilize the newly formed droplets (Morr and Ha, 1993).

The theory used to calculate the size distribution assumes that the particles are isolated homogeneous spheres (Keowmaneechai and McClements, 2002). The size distribution of these NEs showed a single peak with narrow width and no tail was observed (**Figure 23** for 10% NEs and **Figure 24** for 5% NEs) probably suggesting the monodispersed spherical droplet. It was also observed that the acid-base (pH) value of NEs was around neutral (pH=6.5). The values of zeta potential were in range of -31.10 to -46.00 mV, suggesting the negative charges of WPI that stabilized the NEs (Jorge, Angeles and Alfonso, 2001).

**Table 8** Droplet size, polydispersity index (PdI) and zeta potential of nanoemulsions prepared using WPI (emulsifier) and *E. Plantagineum* seed oil. obtained from Zetasizer.

| Formulation | Properties of nanoemulsions | | |
|---|---|---|---|
| Oil : WPI (%w/w) | size diameter (nm) | PdI | Zeta potential (mV) |
| 10 : 1 | 769.20 ± 35.03 | 0.86 ± 0.11 | -44.90 ± 2.23 |
| 10 : 5 | 613.10 ± 29.91 | 0.73 ± 0.10 | -46.00 ± 0.69 |
| 10 : 10 | 398.30 ± 4.62 | 0.60 ± 0.04 | -38.80 ± 0.76 |
| 10 : 15 | 426.60 ± 4.14 | 0.39 ± 0.02 | -38.40 ± 0.58 |
| 10 : 20 | 326.60 ± 1.79 | 0.26 ± 0.02 | -34.30 ± 0.94 |
| 5 : 1 | 1,248.00 ± 81.64 | 0.88 ± 0.05 | -44.40 ± 0.50 |
| 5 : 5 | 576.50 ± 16.18 | 0.59 ± 0.02 | -44.70 ± 1.79 |
| 5 : 10 | 389.10 ± 31.39 | 0.52 ± 0.05 | -37.30 ± 1.11 |
| 5 : 15 | 349.70 ± 9.15 | 0.37 ± 0.01 | -38.60 ± 0.12 |
| 5 : 20 | 263.80 ± 1.90 | 0.23 ± 0.01 | -31.10 ± 2.81 |

Values are means ± S.D. (n=3).

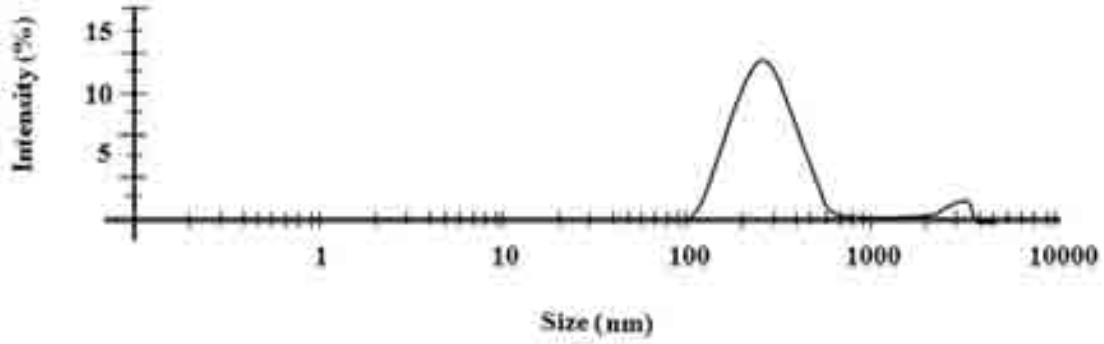



**Figure 23** Size distribution graph by intensity of o/w nanoemulsions prepared from 10% oil and 20% WPI. Ultrasonic condition of 1,250 Joules, 20% amplitude and 45$^{o}$C.

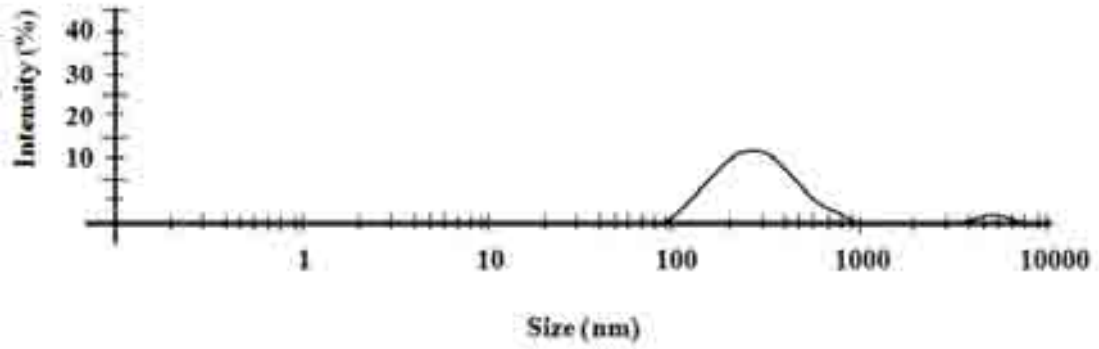



**Figure 24** Size distribution graph by intensity of o/w nanoemulsions prepared from 5% oil and 20% WPI. Ultrasonic condition of 1,250 Joules, 20% amplitude and 45$^{o}$C.

### 1.2 Ultrasonic emulsification

From the results of the previous section, NEs prepared at a weight ratio of 5:20 oil:WPI gave the smallest size and narrow size distribution and would be used to study the parameters (input energy, % amplitude, and temperature) which involved in ultrasonic emulsification.

### 1.2.1 Effect of input energy

The effect of applied input energy had been studied using at constant 20% amplitude and 45°C. The input energy was varied from 500 to 3,000 Joules. The results are shown in **Table 9** and **Figure 25**.

**Table 9** Effect of input energy on size, polydispersity index (PdI) and zeta potential of nanoemulsions containing 5% oil and 20% WPI.

| Input energy (Joules, J) | Properties of nanoemulsions | | |
|---|---|---|---|
| | size diameter (nm) | PdI | Zeta potential (mV) |
| 500 | 553.90 ± 22.59 | 0.56 ± 0.02 | -35.10 ± 1.93 |
| 1,000 | 386.80 ± 5.35 | 0.38 ± 0.01 | -33.70 ± 1.03 |
| 1,250 | 263.80 ± 1.90 | 0.23 ± 0.01 | -31.10 ± 2.81 |
| 1,500 | 285.80 ± 5.18 | 0.26 ± 0.02 | -35.00 ± 0.15 |
| 1,750 | 225.83 ± 1.52 | 0.24 ± 0.01 | -31.50 ± 0.20 |
| 2,000 | 318.70 ± 3.52 | 0.31 ± 0.02 | -38.90 ± 2.14 |
| 2,250 | 299.70 ± 4.47 | 0.25 ± 0.01 | -34.80 ± 2.17 |
| 2,500 | 251.90 ± 3.32 | 0.21 ± 0.01 | -31.20 ± 0.47 |
| 3,000 | 235.00 ± 1.62 | 0.16 ± 0.00 | -35.40 ± 2.25 |

Values are means ± S.D. (n=3).

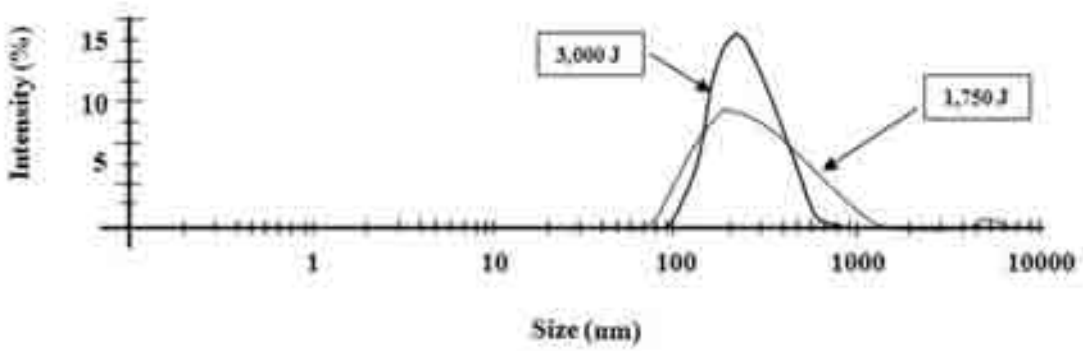



**Figure 25** Size distribution by intensity of o/w nanoemulsions prepared with 5% oil and 20% WPI. Ultrasonic condition of different energy input, 20% amplitude and 45°C.

The formulation emulsified at a low energy input (500, 1000 and 1250 J) showed more than one peak of intensity (data not showed) while, higher energy input, caused a decrease in NEs droplets size and only one peak was observed. NEs emulsified with 1,750 and 3,000 J provided droplet size under 250 nm with narrow size distribution. However, the lower input energy (1,750 J) would be used for further study. The zeta potential values of all NEs were in the range of -31.10 to -38.90 mV.

### 1.2.2 Effect of ultrasonic amplitude

The effect of amplitude was studied at constant energy of 1,750 J and 45$^{o}$C. From the ultrasonicator instruction, the %amplitude was limited to the highest value of 40% in order to avoid the damage of microtip sonotrode during ultrasonic emulsification. In this study, the percentages of amplitude were varied from 20% to 40% amplitude. The results are shown in Table 10 and the graph of size distribution is shown in **Figure 26**.

**Table 10** Effect of amplitude on size, polydispersity index (PdI) and zeta potential of the nanoemulsions containing 5% oil and 20% WPI.

| Amplitude (%) | Properties of nanoemulsions | | |
|---|---|---|---|
| | size diameter (nm) | PdI | Zeta potential (mV) |
| 20 | 225.83 ± 1.52 | 0.24 ± 0.01 | -31.50 ± 0.20 |
| 25 | 423.50 ± 8.56 | 0.46 ± 0.02 | -40.00 ± 1.59 |
| 30 | 434.30 ± 5.47 | 0.63 ± 0.02 | -36.70 ± 1.25 |
| 35 | 486.30 ± 16.80 | 0.46 ± 0.03 | -38.90 ± 0.03 |
| 40 | 501.10 ± 8.73 | 0.47 ± 0.03 | -39.90 ± 0.62 |

Values are means ± S.D. (n=3).

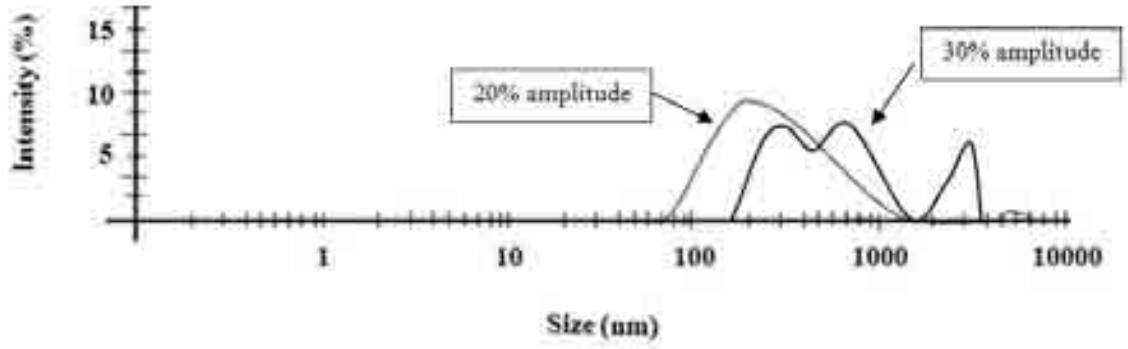



**Figure 26** Size distribution by intensity of o/w nanoemulsions prepared with 5% oil and 20% WPI. The ultrasonic condition of different amplitudes, 1,750 J, and 45$^{o}$C.

In this present case, higher % amplitude caused the bigger size and very board in size distribution. It would be expected that the higher amplitude results in more effective creation of cavitation (Canselier *et al.,* 2002). However, at higher % amplitude, time for emulsification process was less than those at lower % amplitude to reach the experimental energy. The time of sonication affected the quality of formulation. The previous study of Shashank *et al.,* (2008) supported that the increase in the irradiation time decreased the droplet size. Hence, the result was in agreement with the previous report and the optimum % amplitude of 20% was used for the next experiment. The zeta potential of the NEs showed in the values in range of -31.50 to -40.00 mV.

### 1.2.3 Effect of temperature

The results shown in **Table 11** and **Figure 27** indicated that the droplet size of NEs became smaller when the temperature increased until 45$^{o}$C. An increase in temperature allows more adsorbed protein at the interface and a concomitant decrease of the amount of whey protein in the aqueous phase (Sliwinski *et al.,* 2003). The

temperature of 45°C was the optimal level since increasing temperature to 50°C could not anymore decrease in sizes of NEs. Upon heating, the dimer-monomer equilibrium of β-lactoglobulin (the main protein in WPI) shifts to the monomeric. Further heating the monomers become denature and aggregate *via* disulfide bonding and non-covalent interactions, making the irreversible process of aggregation (Jon *et al.*, 2011; Le Bon, Nicolai, and Durand, 1999). Hence, when the temperature was reached to 50°C, the WPI might denature and have less emulsifying properties, thus increasing size of the NEs.

**Table 11** Effect of temperature on size, polydispersity index (PdI) and zeta potential of the nanoemulsions containing 5% oil and 20% WPI. Values are means ± S.D. (n=3).

| Temperature (°C) | Properties of nanoemulsions | | |
|---|---|---|---|
| | size diameter (nm) | PdI | Zeta potential (mV) |
| 30 | 417.50 ± 6.39 | 0.42 ± 0.03 | -39.80 ± 1.45 |
| 35 | 409.40 ± 4.10 | 0.43 ± 0.07 | -39.60 ± 0.56 |
| 40 | 253.40 ± 1.32 | 0.33 ± 0.02 | -34.53 ± 0.99 |
| 45 | 225.83 ± 1.52 | 0.24 ± 0.01 | -31.50 ± 0.20 |
| 50 | 335.77 ± 7.00 | 0.41 ± 0.04 | -34.03 ± 1.23 |

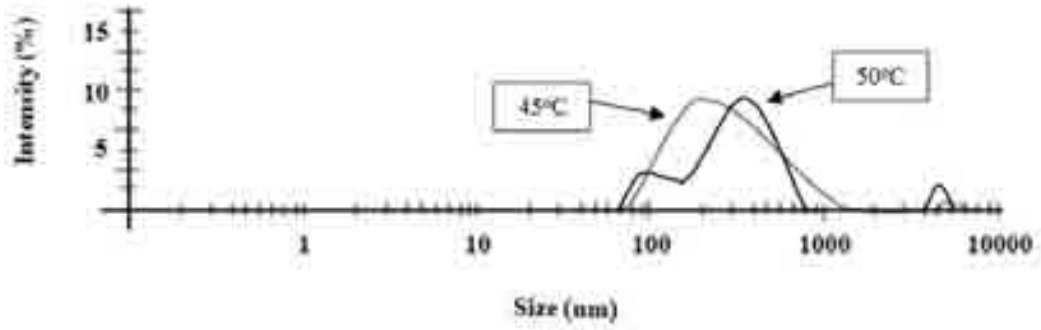



**Figure 27** Size distribution by intensity of o/w nanoemulsions prepared with 5% oil and 20% WPI. Ultrasonic condition of different temperatures, 1,750 J, 20% amplitude.

**2. Storage stability**

The optimized compositions (20% WPI with 5% or 10% oil) and parameters (20% amplitude, 1,750 J, 45$^{o}$C) which provided smaller emulsion droplet size and narrow size distribution were determined further for the stability of the prepared NEs. The stability of NEs was evaluated for any sign of instability (creaming, oil separation, etc.) and the changes in size and size distribution and zeta potential, after storage for 24 h, 14 days, 1, 2 and 3 months at room temperature by using light scattering apparatus (Zetasizer NanoZS).

**Table 12** Stability testing of nanoemulsion containing 5% and 10% oil stabilized by 20% WPI and prepared using the optimized parameters. Values are means ± S.D. (n=3).

| Time (Days) | Properties of nanoemulsions | | |
|---|---|---|---|
| | size diameter (nm) | PdI | Zeta potential (mV) |
| 5% oil nanoemulsions | | | |
| 0 | 225.83 ± 1.52 | 0.24 ± 0.01 | -31.50 ± 0.20 |
| 1 | 223.10 ± 0.52 | 0.17 ± 0.01 | -38.90 ± 2.19 |
| 14 | 224.00 ± 0.72 | 0.19 ± 0.01 | -37.00 ± 0.31 |
| 30 | 277.20 ± 3.78 | 0.26 ± 0.01 | -38.20 ± 2.03 |
| 60 | 407.90 ± 11.10 | 0.48 ± 0.02 | -32.90 ± 0.31 |
| 90 | 1487.00 ± 41.78 | 0.93 ± 0.08 | -26.00 ± 0.85 |
| 10% oil nanoemulsions | | | |
| 0 | 271.30 ± 2.49 | 0.47 ± 0.04 | -34.60 ± 0.53 |
| 1 | 258.40 ± 2.21 | 0.36 ± 0.05 | -35.40 ± 0.88 |
| 14 | 332.50 ± 2.87 | 0.23 ± 0.00 | -32.50 ± 0.10 |
| 30 | 325.90 ± 2.14 | 0.34 ± 0.01 | -30.00 ± 0.97 |
| 60 | 403.70 ± 0.98 | 0.49 ± 0.08 | -32.90 ± 0.31 |
| 90 | ND | ND | ND |

ND = not determined (oil separation)

The findings showed that nanoemulsions containing either 5% or 10% oil had the droplet size below 500 nm when kept until 60 days with had no any sign of creaming or oil separation (**Table 12**). The droplet size of NEs slightly increased with storage time. After 90 days, the sizes of 5% oil NEs dramatically increased from 225.83±1.52 nm (at day 0) to 1,487.00±41.78 nm and PdI increased from 0.24±0.01 (at day 0) to 0.93±0.08 indicating the polydispersity of the stored NEs. In fact the value of PdI should be less than 0.7 for monodisperse particle (Vladimir *et al.*, 2003). On the other hand, 10% oil NEs showed the instability which was creaming and oil separation after 90 days, so the size and zeta potential were unable to be determined.

The zeta potential measurements supported the result of stability test in that the zeta potential of less than -30 mV, -26.00±0.85 mV, was observed after 90 day storage. While all of the samples kept until 60 days showed the zeta potential during -30 to -39 mV. Theoretically, the value of zeta potential should be in between -30 to -50 mV (Jorge, Angeles and Alfonso, 2001). The surface charge of NEs was negative indicating that particles were stabilized by negatively charged molecules of WPI which were mostly beta-lactoglobulin, alpha-lactalbumin, bovine serum albumin and immunoglobulins (Voav 2010)**.** The negative charges stabilize NEs by electrostatic repulsive force (Wei *et al.*, 2011).

From the overall results, these can be conferred that ultrasound processing is a very efficient emulsification technique to produce stable NEs since the quick droplet rupture due to cavitation. This phenomenon is said to favor the formation of electric charges able to absorb onto the interface which play a role in emulsion stabilization. Due to their capacity to induce physical and chemical changes in foods of high-

intensity ultrasound (20–100 kHz), the study of Ibrahim *et al.*, (2007) had resolved that mechanical, thermal and chemical effects of ultrasonication resulted in structural changes in aqueous bovine serum albumin (BSA) since the particle size increased up to 3.4 times after 90 min of sonication because the amount of free sulfhydryl groups in BSA after 90 min of sonication decreased. The increased particle size and decreased number of free sylfhydryl groups may be attributed to formation of protein aggregates. However, some denaturation can be useful in certain proteins, such as whey proteins, due to the changes in their structure that probably expose some antioxidant amino acid group (Laura, Angel and Olga, 2012). The WPI-stabilized nanoemulsions will be determined for their anti-inflammation study.

## 3. Determination of anti-inflammatory effect of nanoemulsions in THP-1 cell line

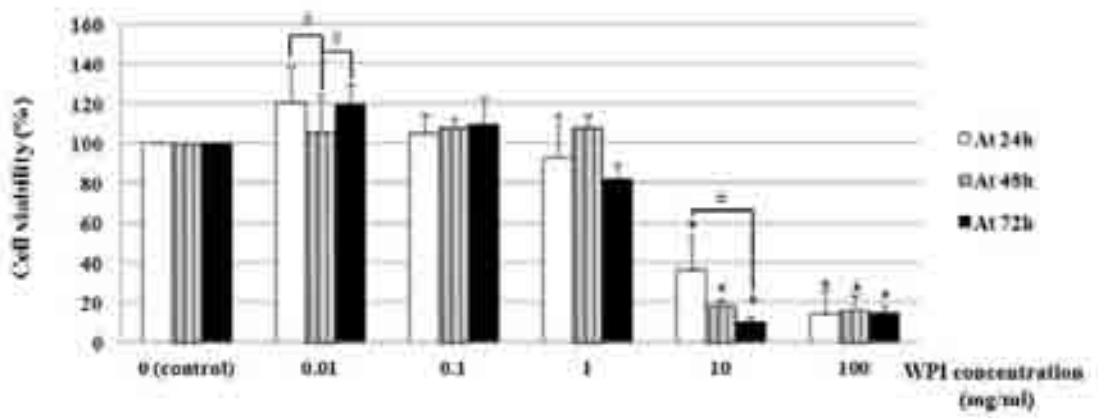
### 3.1 Cytotoxic test of WPI-stabilized nanoemulsions in THP-1 cell line

MTT assay was used to investigate the optimal concentration of WPI in NEs which provided the viable of cells more than 95% cell viability. In this study, $5x10^4$ cells of THP-1 were allowed to suspend into 96-well plate overnight before the experiments and furthered incubated with the NEs for 24, 48 and 72 h. According to Yang *et al.*, (2006) who suggested the dose of whey protein concentrate in human of 10 mg/ml, the concentrations in the range of 0.01, 0.1, 1, 10 and 100 mg/ml of WPI were used in the experiment.

According to the results, the cytotoxic effect of NEs was in a dose-dependent and time-dependent manners (**Figure 28A** and **B**). The treatment time of 72 h resulted in more reduction of cell viability compared to 24-hour treatment. In comparison, the

THP-1 cells treated with 10% oil NEs significantly resulted in more cell death than 5% oil NEs, compared at the same concentration of WPI and time period. The results indicated that no-significant difference in % cell viability of THP-1 treated with NEs containing 0.1 mg/ml of WPI compared to the control. The significant decrease in viability with incubation time was found in 5% oil NEs containing 10 and 100 mg/ml of WPI treated for 24, 48 and 72 h (**Figure 28A**). The similar trend was found in NEs made with 10% oil; however, system containing 1 mg/ml WPI presented significant reduction in cell viability after incubated for 24 and 72 h (**Figure 28B**).

(A)




(B)

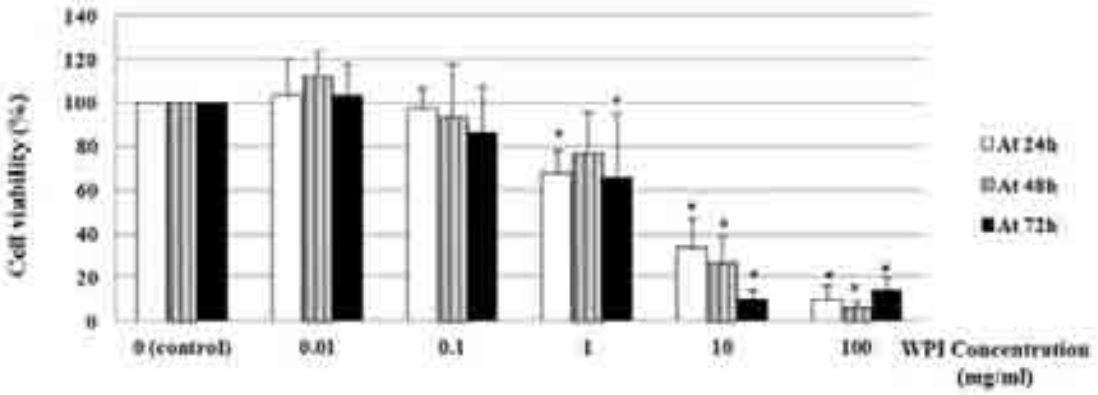



**Figure 28** Percent viability of THP-1 cells after treated 5% oil nanoemulsions (A) and 10% oil nanoemulsions (B) at various concentrations of WPI. Each bar represents

average ± S.D. (n=3). * significant difference ($p \leq 0.05$) compared to control and # significant difference ($p \leq 0.05$) compared among the incubation time.

Since WPI is generally found in milk and there were no previous report on cytotoxic effect. In this study, however, 5% oil NEs stabilized by WPI had no toxic effect (~80% cell viability) on THP-1 cells incubated with 1 mg/ml WPI for 24, 48 or 72 h. NEs containing 10% oil NEs were found more toxic effect to the cells at 1 mg/ml of WPI. So, the oil might affect the viability of THP-1 cell line. From the experiment, 5% oil NEs at the highest WPI concentration of 1 mg/ml could provide more than 80% of cell viability after incubated for 24, 48 and 72 h and were selected for use in the further experiment.

### 3.2 Cytokine detection by multiplex microbead immunoassay

From the results of cytotoxic test of WPI-stabilized NEs in THP-1 cell line, NEs (5% oil and 20% WPI) was determined for inflammatory modulation effect in THP-1 cell by using the optimum concentration at 1 mg/ml of WPI and incubated for 24 h. The composition of NEs namely, WPI solution and oil were also treated for the cytokine modulation effect. The efficacy of the samples for inhibiting LPS-induced pro-inflammatory cytokines was demonstrated by multiplex microbead immunoassay (Bio-plex). THP-1 cell lines were pre-incubated with the sample for 24 hour prior to stimulated with LPS (200 ng/ml) for another 4 h. The supernatants collected from the cell and cell lysate were determined to the concentration of the pro-inflammatory cytokines of IL-1b, IL-4, IL-6, IL-8, GM-CSF, IFN-γ, and TNF-α. In addition, the toxicity of LPS on THP-1 cell was also confirmed using MTT assay (**Figure 29**) (data shown in Appendix C).

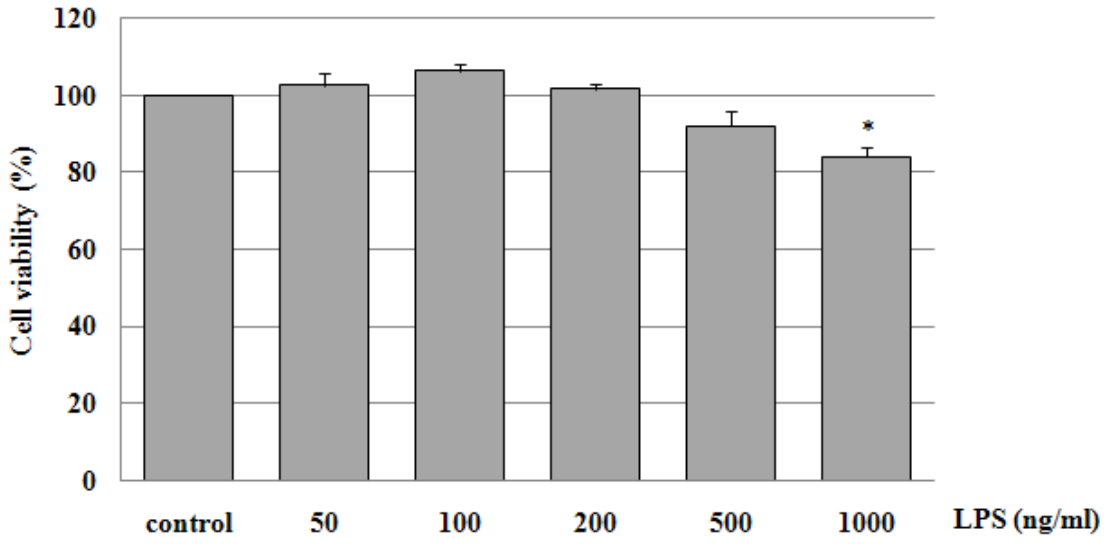



**Figure 29** Cell viability of THP-1 cell line after 4-hour exposure to LPS. Data represent the mean ± ±SEM (n=3) *significant difference ($p \leq 0.05$) compared to the control).

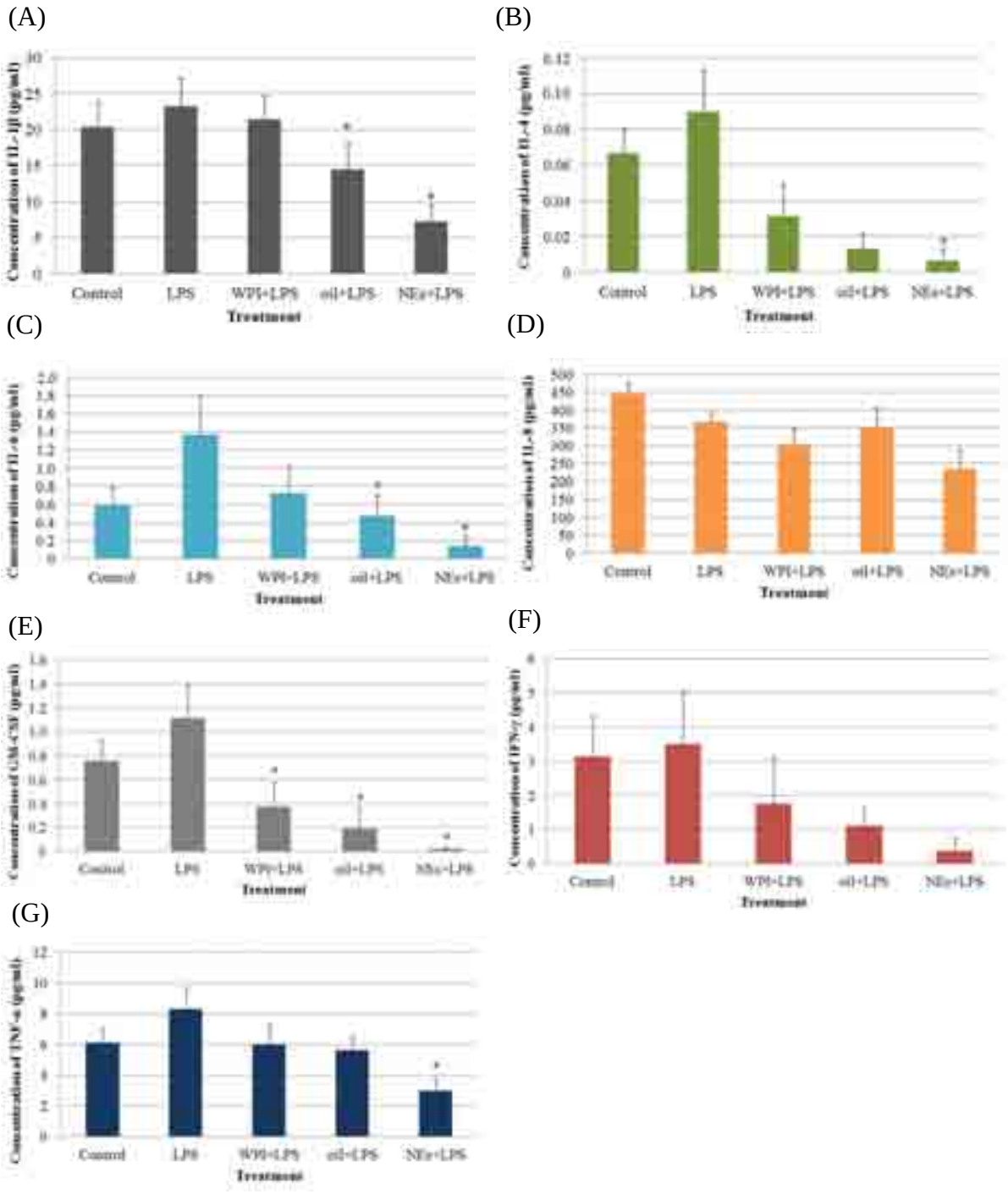



**Figure 30** The concentration (ρg/ml) of pro-inflammatory cytokines of IL-1β (A), IL-4 (B), IL-6 (C), IL-8 (D), GM-CSF (E), IFN-γ (F), and TNF-α (G) from THP-1 cells treated with WPI (1 mg/ml), *E*. Plantagineum seed oil (0.25 mg/ml), and nanoemulsion (NEs) containing 1 mg/ml WPI and stimulated by LPS. Each bar

represents average ±SEM (n=3). Significant differences from LPS of $p \leq 0.05$ are expressed as *. Abbreviations: IL-1β = interleukin-1 β, IL-4 = interleukin-4, IL-6 = interleukin-6, IL-8 = interleukin-8, GM-CSF = Granulocyte-macrophage colony-stimulating factor, IFN-γ = Interferon- γ, and TNF-α = tumor necrosis factor-α.

For the pro-inflammatory cytokine, IL-1β, IL-4, IL-6, IL-8 and IFN-γ are responsible for amplification of the inflammatory reaction. These cytokines produced by activated cells in response to diverse stimuli such as lymphocytes, monocytes and neutrophils (Robert, 1998). The amount of IL-1 β, IL-6 and IL-4 were significantly less than the LPS for the cells, the values 14.39±3.84 ρg/ml (IL-1β) and 0.48±0.22 pg/ml (IL-6) for *E*. plantagineum seed oil-treated cells and 7.29±2.26 pg/ml (IL-1β), 0.14±0.12 pg/ml (IL-6) and 0.01±0.01 pg/ml (IL-4) for NE-treated cells (**Figure 30** and **Appendix D**). Again, for IL-8, a significant decrease in the concentration level of IL-8 (233.77±54.07 pg/ml) was observed when the cells were treated with NEs but not statistically different.

Pro-inflammatory cytokine, GM-CSF which is implicated in the generation of granulocytes and monocytes or macrophages showed the significantly decreased values of 0.37±0.29 pg/ml, 0.19±0.17 pg/ml and 0.02±0.02 pg/ml when the cells were treated with WPI, *E*. Plantagineum seed oil, and NEs, respectively. TNF-α is protein which has a hormone-like function and leads to production of chemokines by endothelial cells, fibroblasts and macrophages and the TNF- α level is associated with the development of inflammation (Greten *et al.,* 2004). From the result, it showed that LPS could induce insignificantly greater concentration of TNF- α than baseline (control) while the significantly lower level at 2.99±0.80 pg/ml was obtained when the THP-1 cell were treated with NEs. Notably, the pro-inflammatory cytokines in the

outer cellular of THP-1 (cell supernatant sample) were also determined but the concentrations of these cytokines were undetectable.

In fact, it has been reported that WPI was able to decrease the concentration of inflammatory cytokines in patients (Mantovani *et al.,* 2004). Moreover, anti-inflammatory effect of omega-3 fatty acids as a competitive inhibition of the pro-inflammatory interleukins (IL-1β, IL-6, and IL-12), TNF-α, and inflammatory prostaglandin E2 was reported (Joseph and Jeffrey, 2006). Hence, the results of the cells incubated either oil which contained omega-3-fatty acids or WPI could lead to a reduction in cytokine mostly significant. Surprisingly, when WPI-stabilized NEs were used, there seemed to have a synergist effect between oil and WPI to inhibit pro-inflammatory response since the cytokine levels of IL-1β, IL-4, IL-8, GM-CSF and TNF-α were significantly decreased. The pro-inflammatory cytokines of IFN-γ was also reduced but not statistically significant. According to the study of Mohammed *et al.,* (2011), the 5-fold increase in bioavailability of atorvastatin (AT) nanoemulsion was found as compared to AT suspension. Additionally, the acidic degradation of AT was significantly reduced. From the results it was proposed that a very small droplets (215.3±14.2 nm) could enhance the bioavailability and stability of the encapsulated drug. In addition, for oral administration of nano formulation can possibly minimize the severity of drug-related side effects including gastrointestinal side effect and thus maintains the plasma drug level for longer period of time. The study of Siah *et al.* (2012) showed the oral administration of NEs (o/w) containing aspirin 60 mg/kg generated using ultrasound cavitation techniques significantly reduced paw edema induced by λ-carrageenan injection and decreased the number of abdominal constriction in acetic acid-induced writhing model and NEs also demonstrated an

enhanced anti-inflammatory and analgesic effects compared to reference suspension. Thus, NEs may be considered as potential nanocarriers for drug particularly NSAIDs for the treatment of inflammatory disorders and alleviating pains.

# CHAPTER V

# CONCLUSION

Nanoemulsions (NEs) are attractive to be employed in many applications because they offer many advantages including stability, simple preparation. Their smaller sizes give enhancement of drug efficacy and increasing the delivery of drugs to the target sites. In general, the preparation of NEs involves synthetic chemical substances and organic solvents as emulsifying or stabilizer agents which might cause toxicity. Therefore, preparation of NEs using natural compounds such as WPI would provide the better formulation.

This study successfully prepared NEs stabilized by WPI and contained *E. plantagineum* seed oil by using high intensity ultrasonic method. The emulsion droplets sizes were in the range of nanometer sizes. WPI-stabilized NEs containing 5% oil (or 10% oil) and 20% WPI were the appropriate compositions to form NEs. The ultrasonic parameters were investigated for successfully generating nano size of emulsion droplet. The experiment was done using 5% oil NEs stabilized by 20% WPI, the results showed that the optimized condition were 20% amplitude, 1750 Joules of energy and the temperature at 45$^{o}$C. The characterization of the NEs revealed that zeta potentials of the obtained NEs were -31.50 ± 0.20 mV and -34.60 ± 0.53 mV, respectively. NEs containing 5% oil showed the mono-dispersed particle size with an average size of 225.83 ± 1.52 nm and PdI of 0.24 ± 0.01. For 10% NEs, the average size of NEs was 271.30 ± 2.49 nm; PdI of 0.47 ± 0.04.

Prior to study a modulation effect of NEs on the inflammatory cytokine in THP-1 cells, the cytotoxic test was performed. The findings demonstrated that no toxic effect occurred when the cells were incubated with NEs containing 1 mg/ml of WPI for 24 and 48 h. The 10% oil NEs had the toxic effect observed on the cells when being incubated for 24 h at the same concentration of WPI studied.

From the anti-inflammatory results, a significant decrease in the pro-inflammatory cytokines, IL-1β, IL-4, IL-8, GM-CSF and TNF-alpha were observed on the THP-1 cells treated with WPI-stabilized NEs containing 5% oil compared to those treated with either oil or WPI alone. This was possibly due to a enchaning effect from a combination of WPI and oil and the better uptake of NEs into the cells.

Conclusively, WPI-stabilized NEs containing omega-3 oil from *E. plantagineum* have an ability to lesser the pro-inflammatory cytokines from the LPS-induced cytokines. In the future, the anti-inflammatory mechanisms of WPI-stabilized NEs should be further investigated on *in vivo* study to find out whether the WPI-stabilized NEs could be applied in the real situation.

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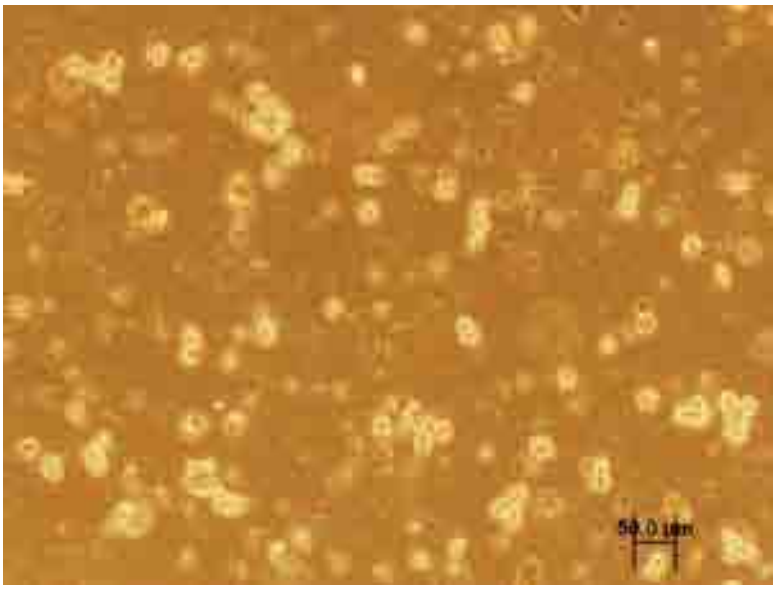
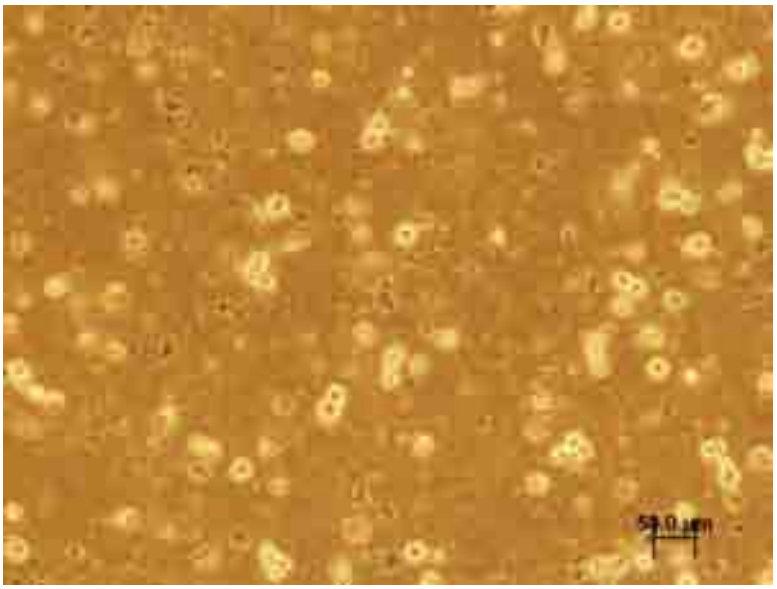
# APPENDICES

**Appendix A Photographs of THP-1 ($1x10^6$ cells/ml) cultured in RPMI-1640 medium.**

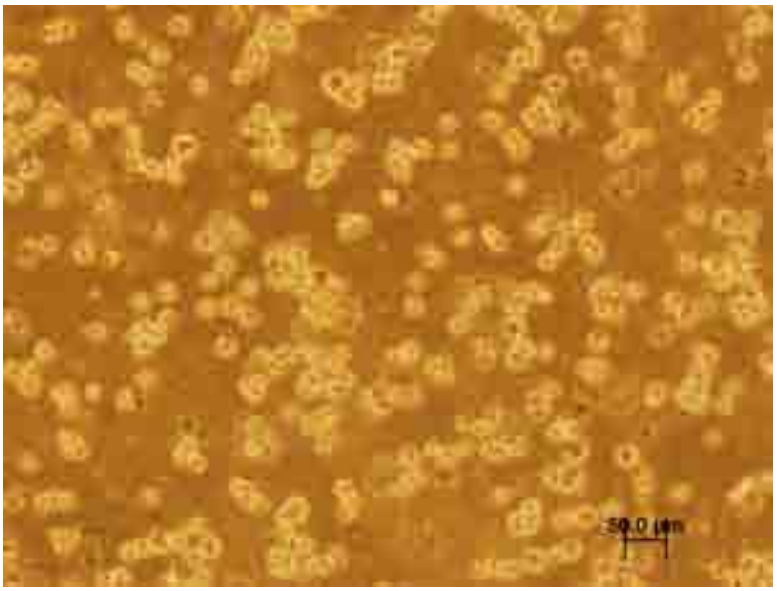
**(A)**



**(B)**



**(C)**




**(D)**

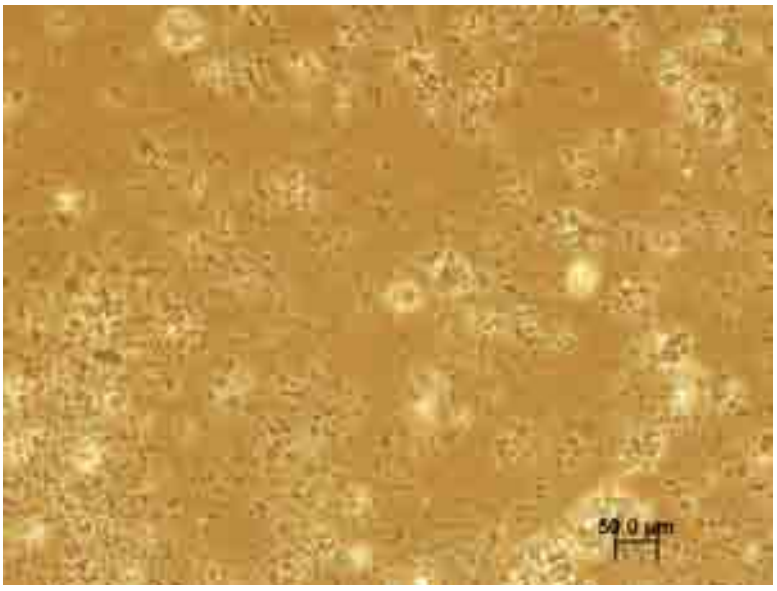



**Figure A1** The electron photographs of THP-1 cells at passage 9th **(A),** 11th, **(B)** 13th, **(C)** and THP-1 cells after lysed by hand homogenizer **(D).**

**Appendix B: Percentage of THP-1 cell viability after treatment with nanoemulsions containing various concentrations of WPI and at different incubation times.**

Table B1 Percentage of viability of THP-1 cells after 24-hour incubation.

| Samples | WPI (mg/ml) | Viability (%) | | | | Average %viability | S.D. |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | | |
| **5% oil 20% WPI** | 0 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 |
| | 0.01 | 103.12 | 123.34 | 143.93 | 114.07 | 121.12 | 17.31 |
| | 0.1 | 101.52 | 98.17 | 120.07 | 100.75 | 105.13 | 10.06 |
| | 1 | 70.45 | 95.15 | 120.76 | 84.79 | 92.79 | 21.22 |
| | 10 | 24.40 | 23.74 | 35.57 | 61.03 | 36.19 | 17.43 |
| | 100 | 10.41 | 5.12 | 31.22 | 9.40 | 14.04 | 11.68 |
| **10% oil 20% WPI** | 0 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 |
| | 0.01 | 99.97 | 106.29 | 123.85 | 83.72 | 103.46 | 16.59 |
| | 0.1 | 86.26 | 110.77 | 97.48 | 93.41 | 96.98 | 10.30 |
| | 1 | 57.21 | 70.77 | 81.12 | 62.70 | 67.84 | 10.40 |
| | 10 | 16.15 | 32.43 | 34.67 | 49.66 | 33.23 | 13.71 |
| | 100 | 9.96 | 3.53 | 18.86 | 6.62 | 9.74 | 6.62 |

Table B2 Percentage of viability of THP-1 cells after 48-hour incubation.

| Samples | WPI (mg/ml) | Viability (%) | | | Average %viability | S.D. |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | | |
| **5% oil 20% WPI** | 0 | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 |
| | 0.01 | 85.43 | 109.28 | 123.25 | 105.99 | 10.12 |
| | 0.1 | 106.52 | 113.65 | 103.11 | 107.76 | 5.38 |
| | 1 | 112.20 | 101.05 | 110.66 | 107.97 | 6.04 |
| | 10 | 16.36 | 21.12 | 17.78 | 18.42 | 2.44 |
| | 100 | 18.18 | 22.57 | 7.47 | 16.07 | 7.76 |
| **10% oil 20% WPI** | 0 | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 |
| | 0.01 | 101.95 | 112.33 | 123.25 | 112.55 | 10.71 |
| | 0.1 | 64.89 | 106.31 | 108.82 | 93.34 | 24.67 |
| | 1 | 57.08 | 80.33 | 94.15 | 77.19 | 18.73 |
| | 10 | 30.58 | 14.06 | 36.47 | 27.04 | 11.62 |
| | 100 | 9.08 | 3.72 | 6.37 | 6.39 | 2.68 |

Table B3 Percentage of viability of THP-1 cells after 72-hour incubation.

| Samples | WPI (mg/ml) | Viability (%) | | | Average %viability | S.D. 1 |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | | |
| **5% oil 20% WPI** | 0 | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 |
| | 0.01 | 109.57 | 122.58 | 128.66 | 120.27 | 9.75 |
| | 0.1 | 124.73 | 103.62 | 100.16 | 109.50 | 13.30 |
| | 1 | 89.71 | 81.21 | 75.42 | 82.16 | 7.19 |
| | 10 | 8.28 | 13.21 | 8.70 | 10.06 | 2.73 |
| | 100 | 16.53 | 16.79 | 10.24 | 14.52 | 3.71 |
| **10% oil 20% WPI** | 0 | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 |
| | 0.01 | 117.48 | 104.22 | 88.33 | 103.34 | 14.60 |
| | 0.1 | 111.07 | 72.46 | 75.19 | 86.24 | 21.54 |
| | 1 | 95.29 | 64.60 | 36.44 | 65.44 | 29.43 |
| | 10 | 13.52 | 10.56 | 4.95 | 9.68 | 4.36 |
| | 100 | 19.44 | 14.48 | 7.10 | 13.67 | 6.21 |

## Appendix C: Percentage of THP-1 cell viability after 4-hour treatment with various concentrations of LPS.

Table C1 Percentage of THP-1 cell viability after 4-hour treatment with various concentrations LPS.

| Concentration (ng/ml) | Viability (%) | | | Average %viability | SEM |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | | |
| 0 | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 |
| 50 | 105.58 | 95.72 | 106.39 | 102.56 | 3.43 |
| 100 | 109.45 | 103.47 | 106.59 | 106.50 | 1.73 |
| 200 | 103.56 | 99.23 | 101.78 | 101.52 | 1.26 |
| 500 | 90.52 | 99.34 | 86.31 | 92.05 | 3.84 |
| 1,000 | 80.33 | 89.21 | 82.83 | 84.12 | 2.64 |

**Appendix D: Concentrations of pro-inflammatory cytokines produced by THP-1 cell line after incubation with LPS, whey protein isolate (WPI) and LPS, *E. plantagineum* seed oil and LPS, nanoemulsion (NEs) and LPS. The cells in only medium were used as control.**

Table D1 Concentrations (ρg/ml) of interleukin-1β (IL-1β) of the cells treated with different samples.

| | **IL-1β concentration (ρg/ml)** | | | | |
|---|---|---|---|---|---|
| | **Medium** | **LPS** | **WPI+LPS** | **Oil+LPS** | **NEs+LPS** |
| | 28.72 | 36.87 | 32.39 | 25.27 | 7.38 |
| | 24.82 | 31.68 | 26.15 | 21.43 | 15.12 |
| | 29.79 | 23.94 | 26.04 | 21.86 | 12.23 |
| | 12.64 | 11.58 | 11.3 | 4.19 | 1.91 |
| | 13.41 | 17.34 | 13.99 | 6.76 | 1.19 |
| | 13.06 | 18.25 | 18.82 | 6.8 | 5.88 |
| **Average** | 20.41 | 23.28 | 21.45 | 14.39 | 7.29 |
| **±SEM (n=3)** | 3.37 | 3.89 | 3.31 | 3.84 | 2.26 |

Table D2 Concentrations (ρg/ml) of interleukin-4 (IL-4) of the cells treated with different samples.

| | **IL-4 concentration (ρg/ml)** | | | | |
|---|---|---|---|---|---|
| | **Medium** | **LPS** | **WPI+LPS** | **Oil+LPS** | **NEs+LPS** |
| | 0.08 | 0.16 | 0.04 | 0.04 | 0.00 |
| | 0.12 | 0.04 | 0.04 | 0.04 | 0.04 |
| | 0.04 | 0.06 | 0.00 | 0.00 | 0.00 |
| | 0.08 | 0.04 | 0.11 | 0.00 | 0.00 |
| | 0.04 | 0.08 | 0.00 | 0.00 | 0.00 |
| | 0.04 | 0.16 | 0.00 | 0.00 | 0.00 |
| **Average** | 0.07 | 0.09 | 0.03 | 0.01 | 0.01 |
| **±SEM (n=3)** | 0.01 | 0.02 | 0.02 | 0.01 | 0.01 |

Table D3 Concentrations (ρg/ml) of interleukin-6 (IL-6) of the cells treated with different samples.

| | IL-6 concentration (ρg/ml) | | | | |
|---|---|---|---|---|---|
| | **Medium** | **LPS** | **WPI+LPS** | **Oil+LPS** | **NEs+LPS** |
| | 0.98 | 2.92 | 2.03 | 1.16 | 0.75 |
| | 1.27 | 2.25 | 0.75 | 0.98 | 0.09 |
| | 0.75 | 1.55 | 0.75 | 0.75 | 0.00 |
| | 0.37 | 0.37 | 0.09 | 0.00 | 0.00 |
| | 0.09 | 0.37 | 0.37 | 0.00 | 0.00 |
| | 0.09 | 0.75 | 0.37 | 0.00 | 0.00 |
| **Average** | 0.59 | 1.37 | 0.73 | 0.48 | 0.14 |
| **±SEM (n=3)** | 0.20 | 0.43 | 0.28 | 0.22 | 0.12 |

Table D4 Concentrations (ρg/ml) of interleukin-8 (IL-8) of the cells treated with different samples.

| | IL-8 concentration (ρg/ml) | | | | |
|---|---|---|---|---|---|
| | **Medium** | **LPS** | **WPI+LPS** | **Oil+LPS** | **NEs+LPS** |
| | 434.14 | 370.83 | 297.68 | 318.10 | 185.82 |
| | 388.44 | 458.40 | 391.71 | 299.22 | 285.04 |
| | 341.33 | 414.92 | 447.94 | 627.68 | 470.43 |
| | 486.63 | 303.80 | 173.57 | 265.42 | 126.81 |
| | 506.81 | 317.13 | 202.00 | 297.75 | 110.19 |
| | 521.51 | 332.54 | 308.96 | 295.61 | 224.30 |
| **Average** | 446.48 | 366.27 | 303.64 | 350.63 | 233.77 |
| **±SEM (n=3)** | 29.13 | 24.71 | 43.15 | 55.83 | 54.07 |

Table D5 Concentrations (ρg/ml) of granulocyte-macrophage colony-stimulation factor (GM-CSF) of the cells treated with different samples.

| | **GM-CSF concentration (ρg/ml)** | | | | |
|---|---|---|---|---|---|
| | **Medium** | **LPS** | **WPI+LPS** | **Oil+LPS** | **NEs+LPS** |
| | 1.29 | 1.62 | 1.21 | 0.12 | 0.00 |
| | 1.21 | 1.21 | 0.69 | 1.04 | 0.12 |
| | 0.32 | 1.21 | 0.32 | 0.00 | 0.00 |
| | 0.87 | 0.32 | 0.00 | 0.00 | 0.00 |
| | 0.32 | 0.32 | 0.00 | 0.00 | 0.00 |
| | 0.51 | 2.02 | 0.00 | 0.00 | 0.00 |
| **Average** | 0.75 | 1.12 | 0.37 | 0.19 | 0.02 |
| **±SEM (n=3)** | 0.18 | 0.28 | 0.20 | 0.17 | 0.02 |

Table D6 Concentrations (ρg/ml) of interferon-gramma (IFN-γ) of the cells treated with different samples.

| | **IFN-γ concentration (ρg/ml)** | | | | |
|---|---|---|---|---|---|
| | **Medium** | **LPS** | **WPI+LPS** | **Oil+LPS** | **NEs+LPS** |
| | 1.00 | 8.28 | 0.00 | 2.22 | 0.00 |
| | 1.00 | 0.00 | 2.22 | 2.22 | 2.22 |
| | 1.00 | 8.28 | 0.00 | 0.00 | 0.00 |
| | 2.22 | 0.00 | 8.28 | 0.00 | 0.00 |
| | 8.28 | 2.22 | 0.00 | 0.00 | 0.00 |
| | 5.34 | 2.22 | 0.00 | 2.22 | 0.00 |
| **Average** | 3.14 | 3.5 | 1.75 | 1.11 | 0.37 |
| **±SEM (n=3)** | 1.24 | 1.56 | 1.36 | 0.50 | 0.37 |

Table D7 Concentrations (ρg/ml) of tumor necrosis factor-α (TNF-α) of the cells treated with different samples.

| | **TNF-α concentration (ρg/ml)** | | | | |
|---|---|---|---|---|---|
| | **Medium** | **LPS** | **WPI+LPS** | **Oil+LPS** | **NEs+LPS** |
| | 7.90 | 11.40 | 9.67 | 5.41 | 6.59 |
| | 7.90 | 13.27 | 8.23 | 6.25 | 3.14 |
| | 5.07 | 3.86 | 5.92 | 9.51 | 3.50 |
| | 6.92 | 7.74 | 7.58 | 3.86 | 1.64 |
| | 6.92 | 6.92 | 2.78 | 3.86 | 1.45 |
| | 2.03 | 6.59 | 2.03 | 4.90 | 1.64 |
| **Average** | 6.12 | 8.30 | 6.04 | 5.63 | 2.99 |
| **±SEM (n=3)** | 0.92 | 1.40 | 1.25 | 0.86 | 0.80 |

# VITA

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