CHAPTER IV

RESULTS AND DISCUSSION

4.1 Degree of Deacetylation

The degree of deacetylation is one of the most important chemical characteristics of chitin. In addition, the degree of deacetylation, which determines the content of free amino groups in the polysaccharides, can be employed to differentiate between chitin and chitosan. For instance, chitin with a degree of deacetylation of 50% or above is generally known as chitosan.

The FTIR spectrum of β–chitin, as shown in **Figure 4.1**, exhibited a series of strong characteristic absorption peaks. The absorption peak at 1378 cm^{-1} has been assigned to the CH_3 symmetrical deformation mode of hydroxymethyl groups. Furthermore, the amide I band corresponding to C=O----N-H (intermolecular forces) and C=O----$OHCH_2$ (intramolecular forces) showed the sharp absorption peak at 1655 cm^{-1}. The amide II band corresponding to deformation mode of NHC=O (intramolecular forces) exhibited the absorption peak at 1550 cm^{-1}. Other characteristic peaks of β–chitin which appeared at 3270 and 3450 cm^{-1} corresponding to NH-stretching and OH-stretching, respectively, were also observed.

In this study, the degree of deacetylation of β–chitin was calculated based on the FTIR spectra by using the following Eq. 1 (Sannan *et al.*, 1978; Baxter *et al.*, 1992):

$$\% DD = 100 - [(A_{1655} / A_{3450}) \times 115] \quad (1)$$

where %DD is the degree of deacetylation, A_{1655} is the absorbance at 1655 cm^{-1}, and A_{3450} is the absorbance at 3450 cm^{-1}.

According to **Figure 4.1**, the degree of deacetylation (%DD) of the β-chitin sheet fabricated by the paper-making process using a water-based system is 10.94 ± 2.19 (%).

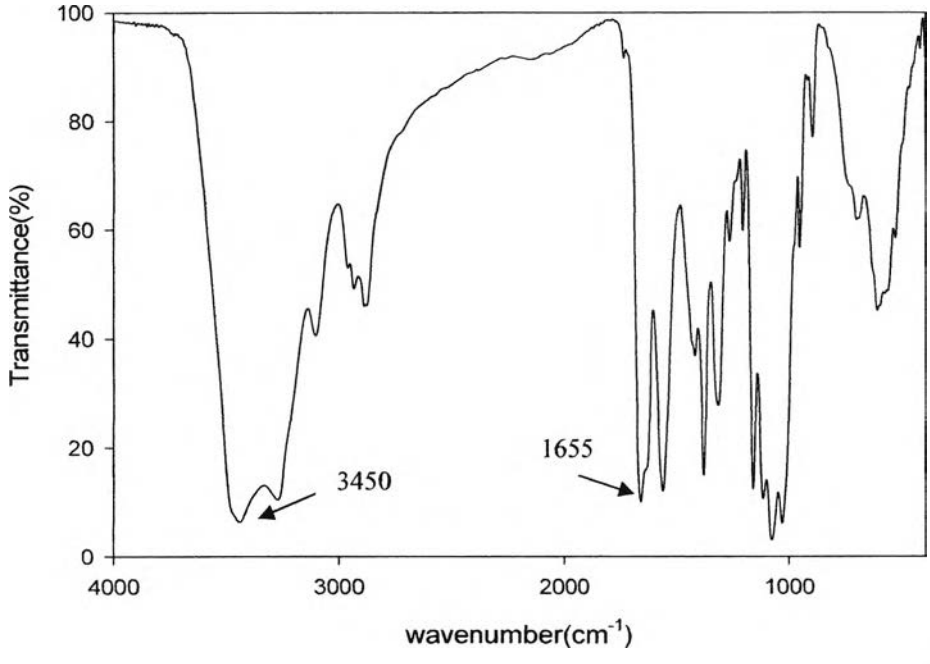

Figure 4.1 FTIR spectra of the β-chitin sheet.

4.2 Morphology Observation of Chitin and Curcumin-Loaded Chitin Sheets

Figure 4.2 showed the surface appearance of the chitin sheet fabricated from chitin suspension by the paper-making process (**Figure 4.2a** and **4.2b**). SEM images revealed that the chitin matrix is formed and had a rough surface consisting of fibrous chitin. In this study, curcumin-loaded chitin sheets were divided into two categories, low-dose (fully-dissolved) and high-dose loading of curcumin, prepared by the different methods. It was found that the contents of curcumin as well as the preparation methods affected to the surface morphology of the chitin sheets. According to the low-dosage of loaded curcumin, before mixing with the chitin suspension, curcumin in the specific content (0.04 wt% based on dry weight of chitin sheet) was fully dissolved in distilled water in the presence of up to 0.5% v/v of Tween 20 based on a total amount of the suspension. The obtained curcumin-loaded chitin sheet had a rough surface consisting of fibrous chitin similar to the unloaded one, as shown in **Figure 4.2c** and **4.2d**. This indicated that all of curcumin powder

was completely solubilized into the chitin sheet matrix during the fabricating process.

On the other hand, various contents of curcumin, 1, 5, 10, and 20 wt% based on dry weight of chitin, were also directly incorporated into the chitin suspension consisting of 0.5% v/v of Tween 20, without pre-solubilization. In this case, the high-dosages of curcumin incorporated in the chitin sheets were obtained. **Figure 4.2e** and **4.2f** indicated the excess amounts of curcumin deposited on the surface of chitin sheet. Increasing in the initial curcumin contents resulted in the higher amounts of embedded curcumin presented on the surface of chitin sheet (**Figure 4.3**). Due to a poor water solubility of curcumin, there was some curcumin powder that could not be solubilized in the presence of 0.5% v/v of Tween 20. However, the concentration of Tween 20 could not be increased to higher than 0.5% v/v, since the obtained chitin sheet was so weak and would tear easily during removal from the mold.

4.3 Interaction between Curcumin and Chitin Sheets

The chemical structures of chitin (**Figure 4.4a**), curcumin (**Figure 4.4c**), and curcumin-loaded chitin sheet (**Figure 4.4b**) were determined using FTIR spectroscopy. The characteristic peaks of chitin which appear at 3270, 3400, 1660, and 1558 cm^{-1} corresponding to NH-stretching, OH- stretching, C=O stretching of amide I and amide II, respectively, were observed in FTIR spectra of chitin and curcumin-loaded chitin sheet. The characteristic peaks of curcumin which appear at 1620 and 1512 cm^{-1} corresponding to C=C stretching of alkene and aromatic ring, respectively, were found to present in FTIR spectra of curcumin as well as curcumin-loaded chitin sheet. However, no new peak and peak shift could be observed, indicating that there is no interaction between chitin and curcumin incorporated in chitin sheet. Moreover, the increasing in the intensities of the peaks at 3400 and 1700 cm^{-1} were observed in FTIR spectrum of curcumin-loaded chitin sheet, compared to those of chitin sheet. This is due to the presence of hydroxyl groups (peak at 3400 cm^{-1}) presented in both curcumin and Tween 20 and the presence of C=O stretching (1700 cm^{-1}) of ester group presented in Tween 20 (**Figure 4.4d**).

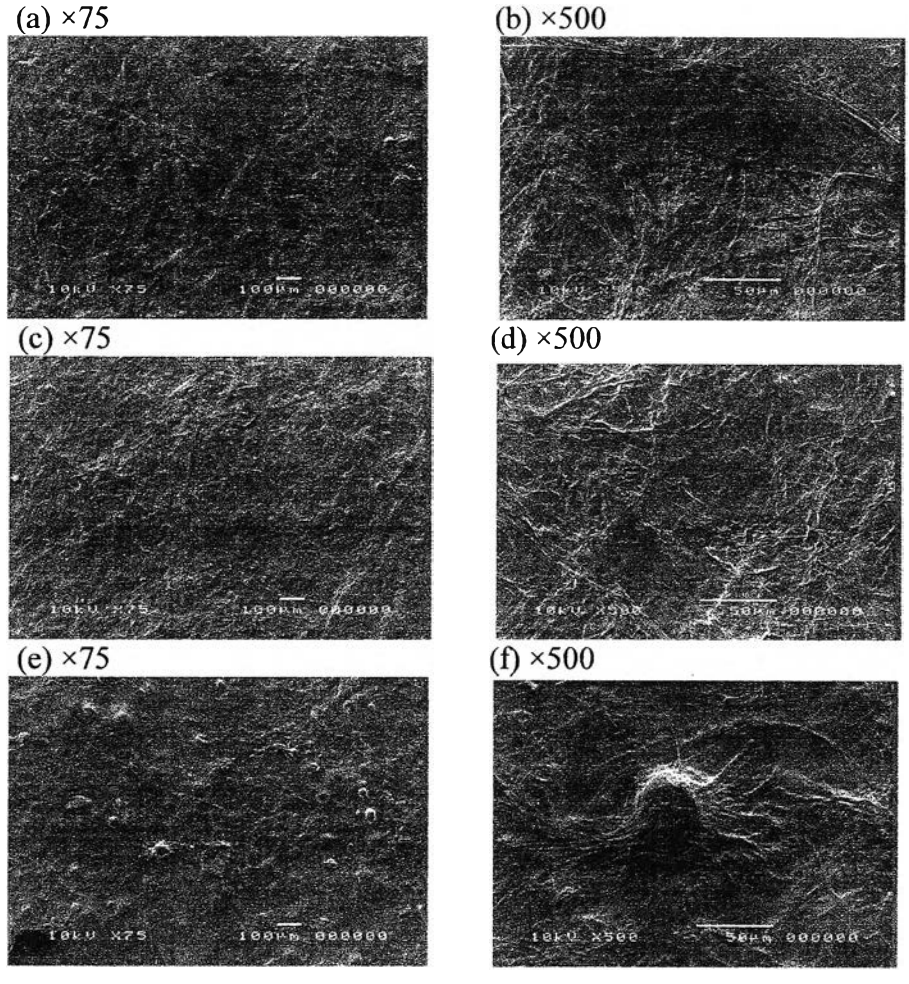

Figure 4.2 SEM images of chitin (a, ×75; b, ×500), (low-dosed) curcumin-loaded chitin sheets (c, ×75; d, ×500), and (high-dosed) curcumin-loaded chitin sheets (e, ×75; f, ×500),respectively.

As described above, the FTIR spectra of curcumin-loaded chitin sheets exhibited the characteristic peaks of curcumin which appear at 1620 and 1512 cm^{-1}. However, the peak at 1512 cm^{-1} corresponding to aromatic ring of curcumin could be rarely observed (shoulder band). It was also found that the various initial contents of loaded curcumin, ranging from 0.04 to 20 wt%, affected to the peak intensity. The higher initial contents resulted in an increasing in the peak intensity, as shown in **Figure 4.5**.

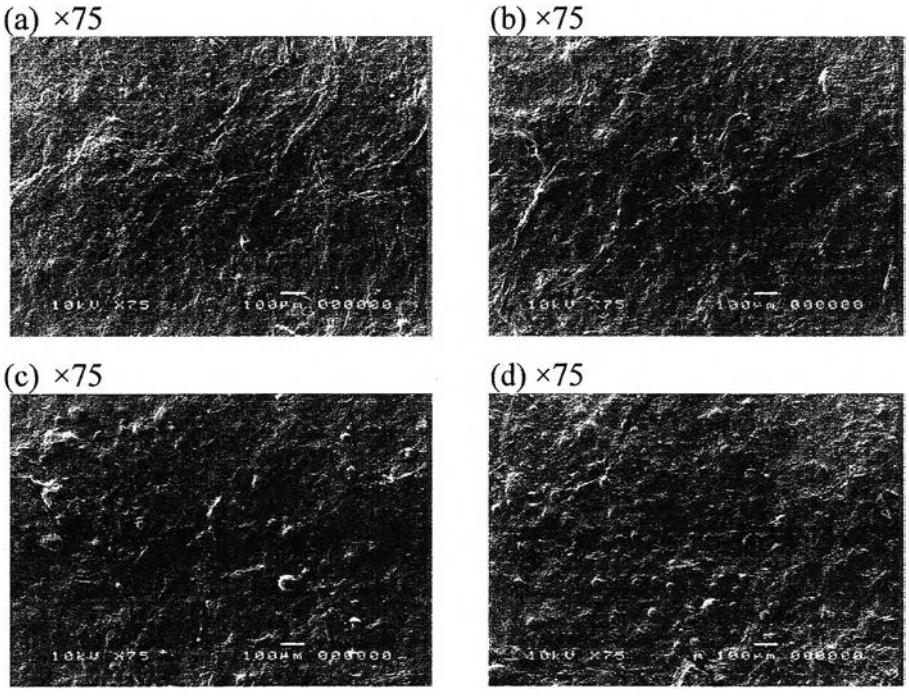

Figure 4.3 SEM images (×75) of high-dosed curcumin deposited on the surface of chitin sheets; (a) 1 wt%, (b) 5 wt%, (c) 10 wt%, and (d) 20 wt% of loaded curcumin, respectively.

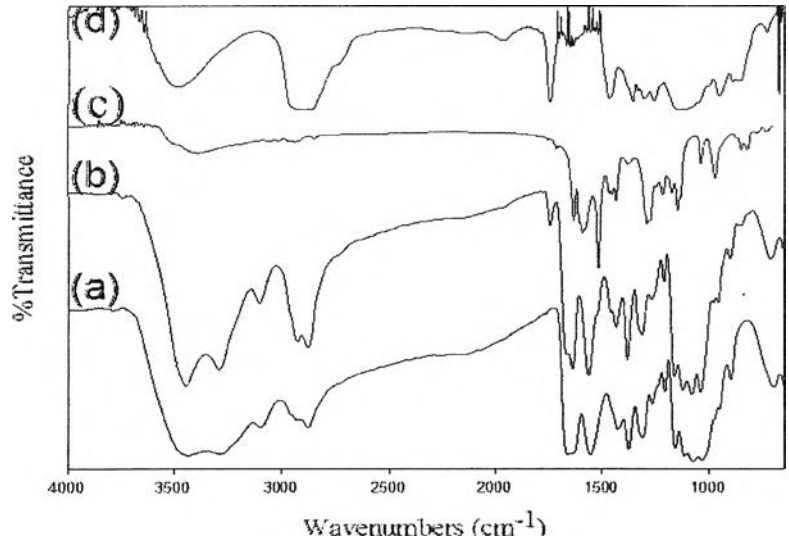

Figure 4.4 FTIR spectra of (a) chitin, (b) chitin incorporated with curcumin, (c) curcumin, and (d) Tween 20, respectively.

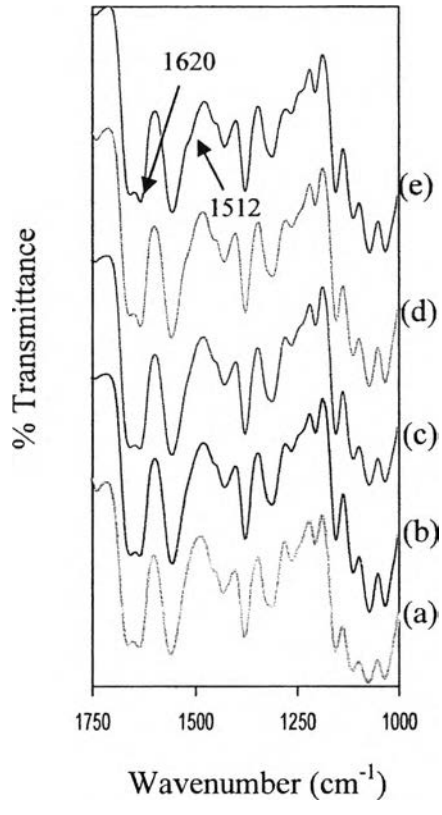

Figure 4.5 FTIR spectra of curcumin-loaded chitn sheets; (a) 0.04 wt%, (b) 1 wt%, (c) 5 wt%, (d) 10 wt%, and (e) 20 wt% of loaded curcumin, respectively.

4.4 Releasing Behaviour of Curcumin from Curcumin-Loaded Chitin Sheets

4.4.1 Releasing Profiles of Fully Dissolved Curcumin (Low Dosage) from Chitin Sheets

Due to the limitation in solubility of curcumin in the acetate buffer solution at pH 5.5, the releasing behaviour of curcumin from chitin sheet could be affected by the presence of the surfactant, Tween 20, in the curcumin-loaded chitin sheets. In this study, the percentage of released curcumin was reported based on the actual amounts of curcumin containing in the samples. **Figure 4.6** showed release profiles of fully dissolved curcumin from curcumin-loaded chitin sheets. Remarkable increase in the releasing amounts of curcumin was observed during the first 16 hours and then the releasing amounts gradually increased until reach 99% releasing in the

fifth day. In the presence of Tween 20, the incorporated curcumin (0.04 wt% based on dry weight of chitin) was pre-solubilized before adding into the chitin suspension during the fabricating process. It was found that almost all of the loaded curcumin released from the chitin sheets to the exterior solution. This indicated that the releasing of curcumin resulted from the solubilized curcumin in the chitin sheets.

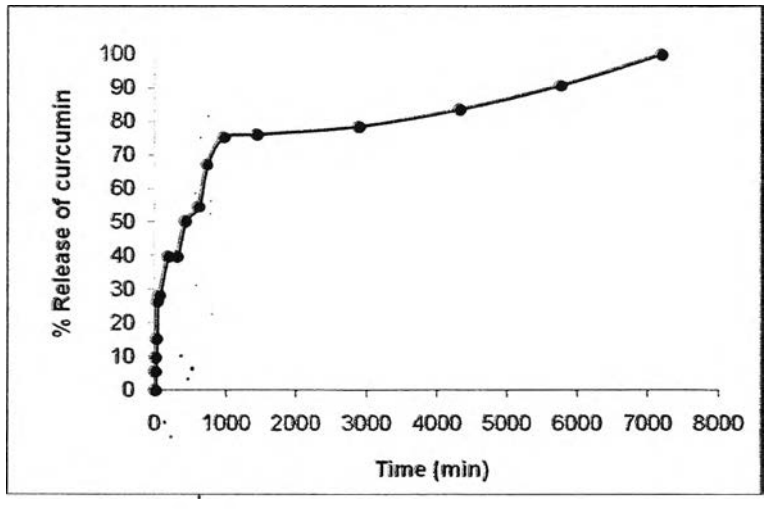

Figure 4.6 Release profiles of 0.04 wt% of loaded curcumin (fully dissolved) from curcumin-loaded chitin sheets.

4.4.2 Releasing Profiles of Curcumin from Chitin Sheets in case of High-Dosed Loading of Curcumin

Remarkable increases in the amounts of curcumin released from the samples during the first 3 hours were observed for all samples having curcumin contents of 1, 5, 10, and 20 wt% based on dry weight of chitin, as shown in **Figure 4.7**. The fast releasing at the beginning period of the release profile resulted from the presence of solubilized curcumin on the surface of chitin sheet. The incorporated curcumin was solubilized due to the action of Tween 20 added in chitin suspension before fabrication of chitin sheet. However, not all curcumin was solubilized by tween 20 during agitation of chitin suspension, especially at high curcumin content of 10 and 20 wt%. After 7 hr of immersion time, the maximum releasing amounts of curcumin was observed for all samples. The maximum releasing amounts of curcumin from chitin sheet incorporated with 1, 5, 10, and 20 wt% of curcumin were

52.81, 65.12, 94.59, and 72.63%, respectively. The increasing in the initial curcumin contents in chitin sheet resulted in the increasing of the maximum releasing amount of curcumin from the matrix when the initial curcumin content was increased up to 10 wt%. However, at the initial curcumin content of 20 wt%, the maximum releasing amount of curcumin tended to decrease. This might be due to the presence of incompletely solubilized curcumin embedded in chitin sheet. Since the added amount of Tween 20 was limited to 0.5% v/v, some curcumin remained as solid particles in chitin sheet. The solubility of curcumin in acetate buffer solution at pH 5.5 was very low. Therefore, the percentage of maximum releasing amount of curcumin from chitin sheet with 20 wt% curcumin content was lower than that of chitin sheet with 10 wt% curcumin content.

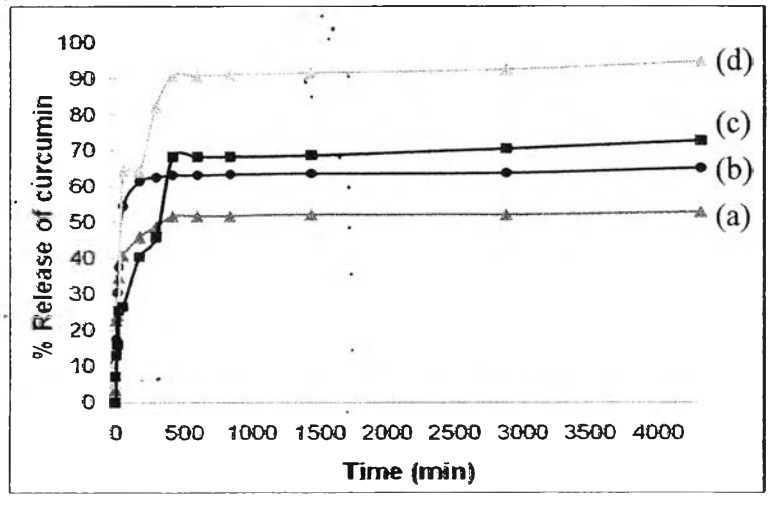

Figure 4.7 Release profiles of high-dosed loading of curcumin from curcumin-loaded chitin sheets; (a) 1 wt%, (b) 5 wt%, (c) 10 wt%, and (d) 20 wt% of loaded curcumin, respectively.

4.4.3 Releasing Profiles of Curcumin from Chitin Sheets: Effect of the Added Tween 20 to the Releasing Buffer Solution

Nevertheless, the higher releasing amount of curcumin from chitin sheet with high loading of curcumin (20 wt%) could be achieved by the addition of Tween 20 in the releasing solution of acetate buffer solution at pH 5.5, as shown in

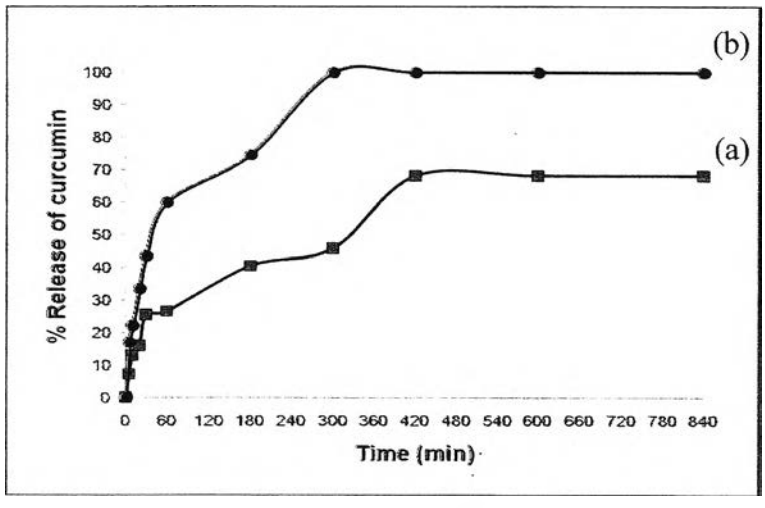

Figure 4.8 Release profiles of high-dosed loading of curcumin (20 wt%) from curcumin-loaded chitin sheets; (a) in the absence of tween 20 and (b) in the presence of Tween 20.

figure 4.8. To elucidate the effect of the surfactant, Tween 20, on the releasing amounts of curcumin from chitin sheet at high loading dosage, 20% v/v of Tween 20 was added to the releasing buffer solution. It was found that the presence of Tween 20 in the releasing solution resulted in higher releasing amounts of curcumin, i.e. increasing from 73% (in the absence of Tween 20) to 100 % releasing of curcumin after 5 hours. It should be noted that the addition of Tween 20 during fabrication of chitin sheet was limited to at 0.5 % (v/v). Otherwise, it will affect sheet forming ability of chitin. This limitation could be affected by the interaction between chitin and Tween 20 indicating by the FTIR spectra. According to **Figure 4.9**, a new peak at 1630 cm^{-1} was observed. When the amount of Tweeen 20 increased, the peak intensity also increased. Focher *et al.* (1992) suggested that the 1621 cm^{-1} band consisting in the FTIR spectra of chitin represented a specific hydrogen bond of C=O with the hydroxymethyl group in the same chitin chain. Hence, it might be indicated that Tween 20 could interrupt this bonding and has a potential to form the hydrogen bond with chitin instead. Based on the previous study, the certain molecular interaction was predicted to exist between the C=O group of chitin and the O-H group of Tween 20 (**Figure 4.10**). With this result, increasing in the amounts of

Tween20 could decrease sheet forming ability of chitin during the fabricating process.

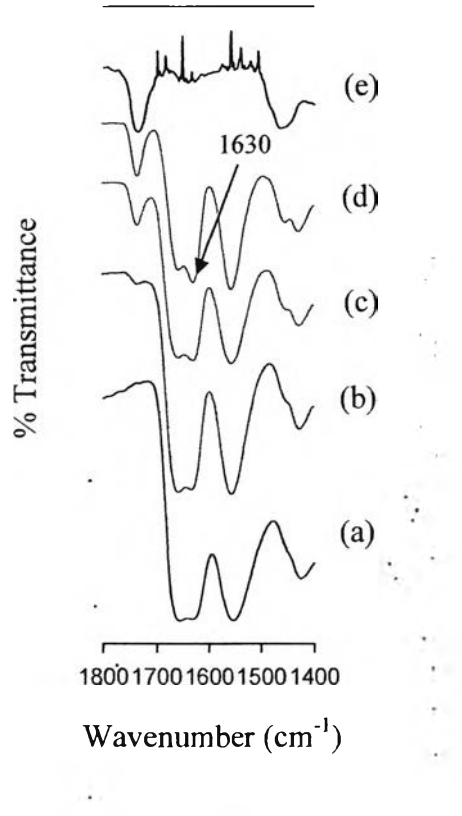

Figure 4.9 FTIR spectra of (a) chitin sheets, (b) chitin sheets with 0.1%, (c) 0.5%, (d) 1% (v/v) of tween 20 based on total chitin suspension, and (e) Tween20, respectively.

Another evidence indicated the interaction between chitin and Tween 20 is the disintegration of chitin fibrous observed by the TEM image (**Figure 4.11**). The addition of Tween 20 in acetate buffer solution resulted in the disintegration of chitin fibrous out of the chitin matrix. This might be due to the hydrogen bonding between chitin and Tween 20, as described above. The width of chitin fibrous, 9.31±4.32, was measured. However, it is difficult to observe the length of chitin fibrous, since they were woven together, and could not be separated individually even in a high dilution of the chitin suspension. Base on the previous study, it was founded that chitin can be degraded by lysozyme presented in human body, and subsequently generates

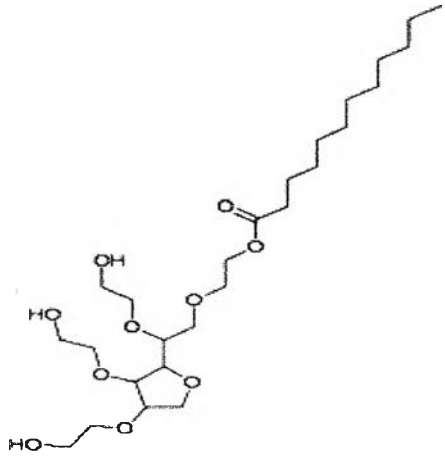

Figure 4.10 The chemical structure of Tween 20, a non-ionic surfactant.

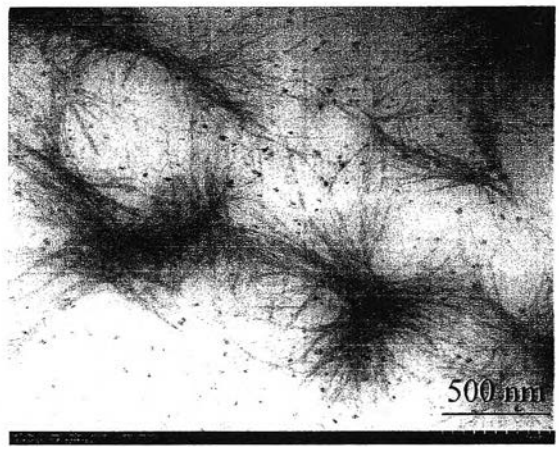

Figure 4.11 The TEM image of chitin fibrous presented in acetate buffer with the addition of Tween 20.

harmless products (Kurita *et al.*, 2000). Although the investigation of enzymatic degradation was not included in this study, it should be noted that the disintegration of chitin fibrous resulted in a higher surface area of the chitin sheet compared to the integrated one. An increasing in the surface area could accelerate the enzymatic degradation. Therefore, the chitin sheet, in the presence of Tween 20, was probably more suitable to be used as a biosorbable product for wound healing applications due to the enhancement of the enzymatic degradation.

4.5 Stability of Curcumin at the Neutral Buffer Solution

In this study, phosphate buffer (pH 7.4) was chosen as a neutral medium stimulating the physiological condition at 37°C.

4.5.1 Stability of Ethanol-Soluble Curcumin in Phosphate Buffer, pH 7.2

Curcumin is a naturally yellowish pigment that exhibits a maximum absorption at 424 nm in ethanol solution based on the spectrophotometric measurement (**Figure 4.12**). When ethanol-soluble curcumin was added to phosphate buffer at 37°C, the change in the absorption peak was observed. It was found that λ_{max} represented to curcumin shifted from 424 to 431 nm, as shown in **Figure 4.13**. This could be caused by the dipole-dipole interaction between dissolved curcumin and the buffer solution. A similar result was also reported by Gopinath et al. (2004), which indicated that λ_{max} of ethanol-soluble curcumin shifted from 420 to 429 nm. The change in the absorption shift stabilized, and the intensity of peak increased with increasing curcumin concentration suggested the effect of hydrophobic environment on curcumin. However, further investigation was conducted in this study. As prolonging the incubation times, the peak intensity decreased and disappeared after 7 hours. This indicated the loss in stability of curcumin under the physiological condition. In addition, **figure 4.13** showed the higher intense in absorption spectra at 360, 260, and 210 nm compared to those of ethanol-soluble curcumin spectra. According to many studies, it was also found that curcumin decomposed rapidly in buffer solutions at 37°C, neutral-basic pH conditions (Oetari *et al.*, 1996; Wang *et al.*, 1997). It is unstable at basic pH, and degrades within 30 min to trans-6-(4'-hydroxy-3'-methoxyphenyl)-2,4-dioxo-5-hexenal, ferulic acid, feruloyl methane and vanillin (Lin *et al.*, 2000). Based on chemical structures of the degradation products (**Figure 4.14**), the broad absorption band at 360 nm as well as the strong

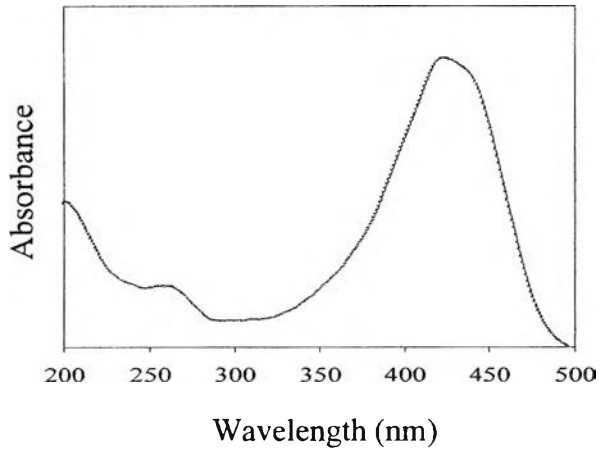

Figure 4.12 UV-vis spectra of ethanol-soluble curcumin at the maximum absorption, 424 nm.

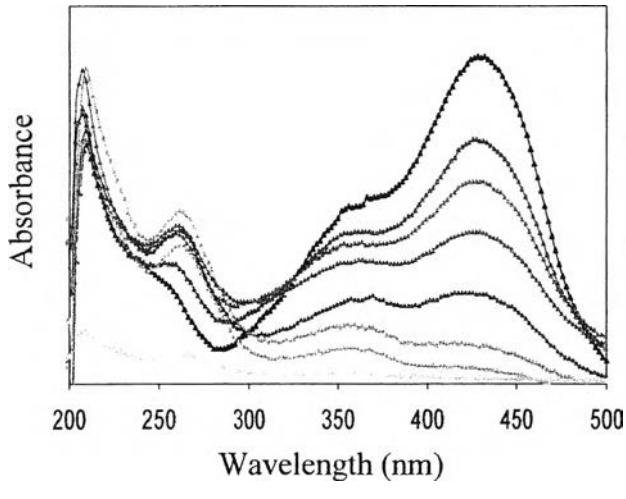

Figure 4.13 UV-vis spectra of ethanol-soluble curcumin in phosphate buffer, pH 7.4, at 37°C as a function of incubation times.

absorption peak at 260 nm could be related to C=C-C=O group presented in trans-6-(4'-hydroxy-3'-methoxyphenyl)-2,4-dioxo-5-hexenal, ferulic acid, and feruloyl methane. Theoretically, C=C-C=O group exhibits absorption peak at 300-350 and 210-250 nm (Silver Stein, 1991; Koenig, 1992; Pimpan, 2005). In addition, trans-6-(4'-hydroxy-3'-methoxyphenyl)-2,4-dioxo-5-hexenal, ferulic acid, and vanillin also compose of -CHO (aldehyde) as well as -COOH (carboxyl) group. Both of them

exhibit absorption peak at 200-210 nm that could also affect to the alteration in absorption spectra of ethanol-soluble curcumin added into phosphate buffer at 210 nm as well.

Several attempts have been done to increase the stability of curcumin. Incorporation curcumin into micelles is one of the preliminary techniques that have been determined. Micelles are aggregates of amphiphilic molecules consisting in a surfactant that form at a concentration referred to as critical micelle concentration (CMC). One of the unique and very useful properties of micelles is their capacity to solubilize solute molecules that are otherwise insoluble in aqueous solutions (Iwunze, 2004). In this study, it was found that Tween 20, a non-ionic surfactant, is a key factor to get a high releasing amount of curcumin under acidic condition (pH5.5). Hence, it is interesting to investigate the role of this surfactant to the stability of curcumin released from the chitin sheet under the physiological condition, phosphate buffer (pH 7.4) at 37°C.

4.5.2 Stability of Curcumin Released from Chitin Sheets in Phosphate Buffer, pH 7.2

Figure 4.15 showed the UV-vis spectra of curcumin released from the chitin sheet in phosphate buffer, pH7.4, as a function of incubation times. The exterior solution was investigated after 3 days of incubation times. It was found that curcumin exhibited the maximum absorption peak at 420 nm indicated the stability of curcumin in the presence of Tween 20 (**Figure 4.15a**). As compared to ethanol-soluble curcumin, λ_{max} represented to curcumin released from the chitin sheet shifted to a lower wavelength, from 424 to 420 nm. In addition, the peak intensity decreased and disappeared after 7 days, as shown in **Figure 4.15b**, of incubation suggested the loss in stability of curcumin. Due to the solubility limitation in aqueous solution of curcumin, it should be proposed that curcumin released from chitin sheets was in a solubilized form due to the action of Tween 20 added in the chitin suspension during the fabrication process of chitin sheets. Based on the previous work, Kunwar *et al.* (2006) also reported the absorption spectrum of curcumin shifted from 425 nm to 420 nm in presence of liposomes, suggesting that curcumin in liposomes experiences nonpolar environment probably by binding to the hydrophobic regions of liposomes.

Hence, in this study, it might be explained that the stability of curcumin could be achieved by binding of curcumin to the hydrophobic regions of Tween 20.

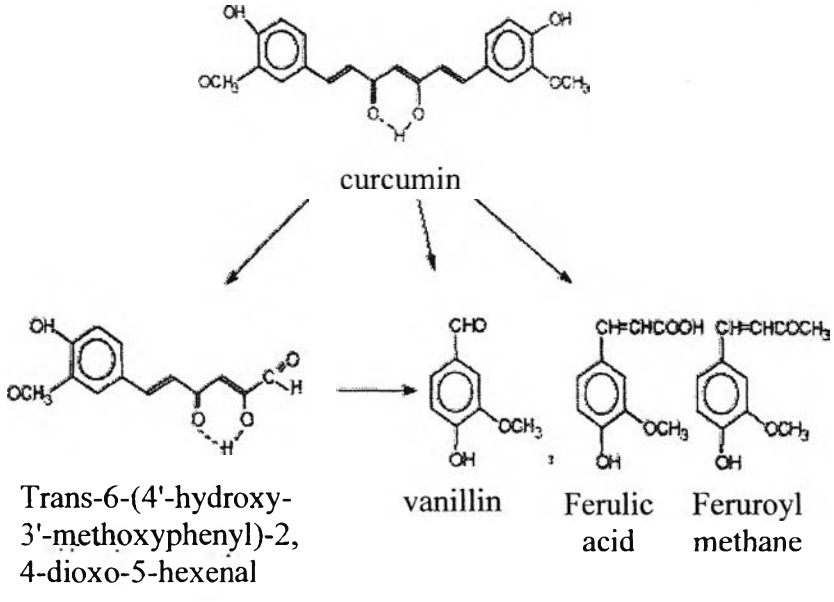

Figure 4.14 Chemical structures of products obtained from the degradation of curcumin in phosphate buffer, pH 7.2, at 37°C.

4.5.3 Stability of Curcumin Incorporated with Chitin Sheets in Phosphate Buffer, pH 7.2

After 7 days of incubation in phosphate buffer, curcumin was extracted from curcumin-loaded chitin sheets using the T/E solution. According to **Figure 4.16a**, it was found that incorporated curcumin exhibited the maximum absorption peak at 427 nm. A shift in the maximum absorption peak to a higher wavelength might be contributed to the dipole-dipole interaction between curcumin and the buffer solution during incubation that suggested the effect of hydrophobic environment on curcumin. In addition, there are no changes in the absorption spectra of extracted curcumin, which indicated that the stability of curcumin could be maintained after 7 days in the T/E solution, as shown in **Figure 4.16b**.

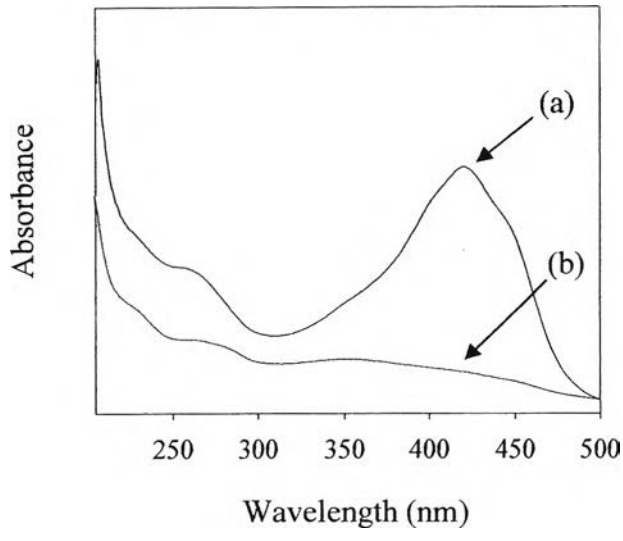

Figure 4.15 UV-vis spectra of released curcumin in phosphate buffer, pH 7.4, at 37°C after (a) 3 days and (b) 7 days of incubation times, respectively.

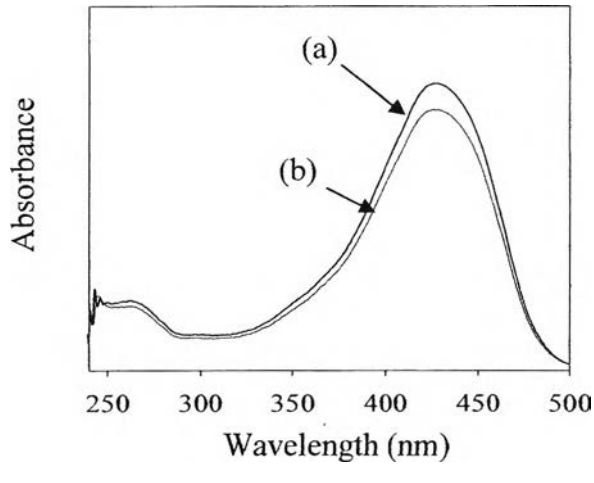

Figure 4.16 UV-vis spectra of curcumin presented in chitin sheets (a) after extraction and (b) after 7 days of extraction in the T/E solution, respectively.

4.6 Direct Cytotoxicity Test

The neutral red (NR) cytotoxicity assay procedure is a cell survival/viability chemosensitivity assay based on the ability of viable cells to incorporate and bind to

neutral red, a supravital dye. **Figure 4.17** showed the chemical structure of NR, a weak cationic dye, which readily penetrated cell membranes by non-ionic diffusion. This phenomenon resulted in accumulating intracellularly in lysosomes, where it bound with anionic sites in the lysosomal matrix. Alterations of the cell surface or the sensitive lysosomal membrane led to lysosomal fragility and other changes that gradually became irreversible. Such changes resulted in a decreased uptake and binding of NR. Hence, it is possible to distinguish between viable and damaged/dead cells.

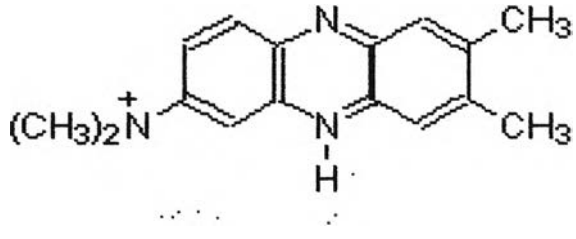

Figure 4.17 The chemical structure of neutral red, a weak cationic dye.

Figure 4.18 showed the microscopic images of L929 (mouse connective tissue) cells stained with NR. In the presence of the chitin sheet (**Figure 4.18b**), the same density of the cells was observed compared to the control cells without sample (**Figure 4.18a**). This indicated that the chitin sheet was not cytotoxic to the L929 cells. However, it was found that chitin incorporated with Tween 20 (0.5% v/v) as well as curcumin (20 wt%) were cytotoxic to the cells showed by the dead and un-staining cells, as shown in **Figure 4.18c** and **4.18d**, respectively.

Similarly, **figure 4.19b** indicated that the chitin sheet was not cytotoxic to the human dermal fibroblast cells. It was determined by the same density of the cells compared to the control (**Figure 4.19a**) under microscopic observation. In addition, the presence of Tween 20 and curcumin, as shown in **Figure 4.19c** and **4.19d**, respectively, resulted in the dead and un-staining cells indicated that they were also cytotoxic to the human dermal fibroblast cells. It was found that chitin sheets in the presence of curcumin and Tween 20 were cytotoxic to both L929 and human dermal fibroblast cells.

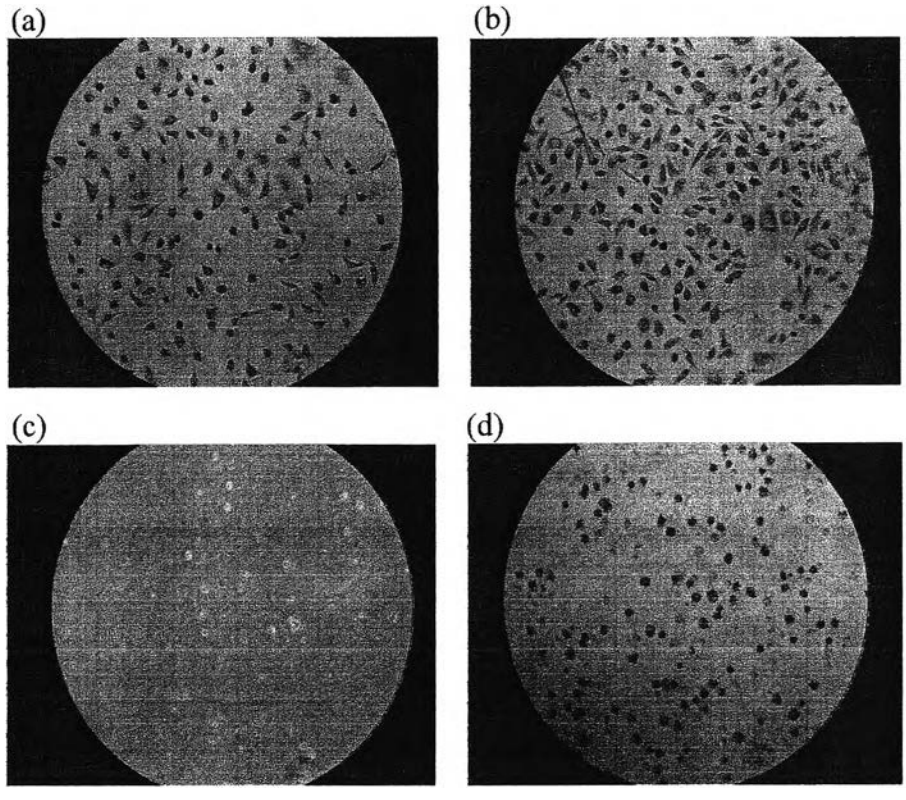

Figure 4.18 The L929 (mouse connective tissue) cells staining with neutral red after 24-hours exposure to the samples; (a) control cells without samples, (b) in the presence of the chitin sheet, (c) chitin incorporated with 0.5% v/v of Tween 20, and (d) chitin incorporated with 20 wt% of curcumin, respectively.

This could be suggested that awareness on curcumin and Tween 20 dosage should be concerned since they could have adverse effects to the cell viability. In case of curcumin, since it is a phenolic compound, highly concentrated application may result in toxic response (Gaspar *et al.*, 1994). However, several studies in a variety of cell lines grown *in vitro* have demonstrated the cytotoxicity of curcumin. In three different malignant colon cell lines, curcumin inhibited proliferation, induced apoptosis and caused accumulation of cells in the G2/M phase of the cell cycle (Hanif *et al.*, 1997; Chen *et al.*, 1999; Mori *et al.*, 2001).

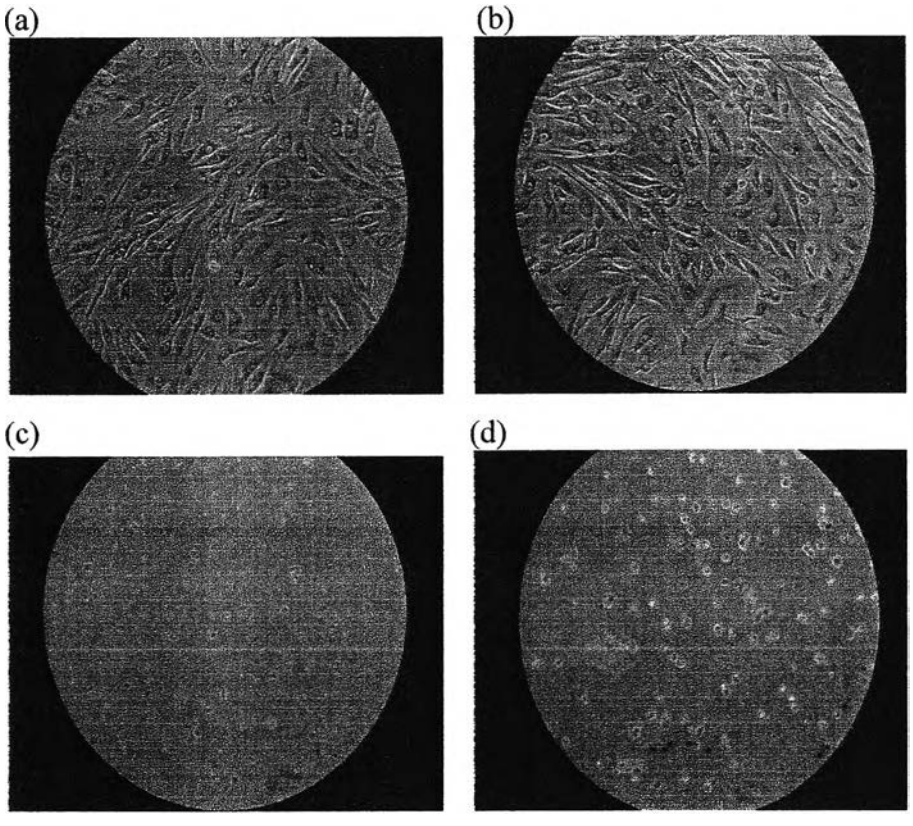

Figure 4.19 The human dermal fibroblast cells staining with neutral red after 24-hours exposure to the samples; (a) control cells without samples, (b) in the presence of the chitin sheet, (c) chitin incorporated with 0.5% v/v of Tween 20, and (d) chitin incorporated with 20 wt% of curcumin, respectively.

Similar results have been observed in breast, kidney, hepatocellular, lymphoid, myeloid, melanoma, oral epithelial and prostate cell lines derived from malignant tumours (Jiang *et al.*, 1996; Simon *et al.*, 1998; Gautam *et al.*, 1998; Han *et al.*, 1999; Bhaumik *et al.*, 2000; Plummer *et al.*, 2000; Aggarwal *et al.*, 2003; Yan *et al.*, 2005). In addition, curcumin has shown growth inhibitory effects *in vitro* in cancer cell lines derived from human prostate, large intestine, bone and leukaemia (Kuo *et al.*, 1996; Dorai *et al.*, 2000; Ozaki *et al.*, 2000; Chen *et al.*, 2005). Simon *et al.* (1998) suggested the presence of the diketone moiety may be a prerequisite for demonstration of antiproliferative activity. Moreover, it was found that the inhibition of cell proliferation may be non-selective with regard to cell lines *in vitro* (Gautam *et al.*, 1998). Regarding to Tween 20, Wilhelm *et al.* (2001) found that Tween 20 was

problematic, as their cytotoxicity was overestimated by the *in vitro* assay. This non-conformity of these high molecular weight (> 1000) compounds was expected, and can be largely attributed to the epidermal permeability barrier.

With these results, it should be noted that although the *in vitro* cytotoxicity model is a useful screening tool, but data should be interpreted critically and require confirmation by appropriate *in vivo* studies. This is due to the epidermal barrier, which greatly limits the percutaneous penetration of xenobiotics *in vivo*, does not exist in cell culture models.