CHAPTER 3

RESULTS AND DISCUSSION

The results and discussion of this study is divided into 7 parts as the followings:

3.1 Development of the modified proper elastic nanovesicular formulations loaded with the model drug (diclofenac diethylammonium; DCFD)

3.1.1 Characteristics of the vesicles

Liposomes with 0–5% ethanol, Tween 61 niosomes with 0–25% ethanol and Span 60 niosomes with 0–20% ethanol gave no sedimentation, no layer separation and unchanged particle sizes at 4, 27 and 45°C within 3 months. The maximum loading of ethanol at 27 ± 2°C for Tween 61 niosomes was higher than DPPC liposomes and Span 60 niosomes which gave 25, 5 and 20%, respectively. Precipitation was observed with high ethanol contents. This may be due to the high hydrophilicity of the polar head group of Tween 61 which is more compatible with ethanol. When the ethanol content was higher than 25%, aggregation of the Tween 61 vesicles was observed. This may be from the salting out effect of the lipid or non-ionic surfactants from ethanol, thereby decreasing the water to lipid or surfactants ratio which was not suitable to form the physical stable vesicles. This result agreed with the previous study that vesicles can not coexist with high concentrations of ethanol (Namdeo and Jain, 1996). Tween 61 niosomes was selected to load DCFD because of its highest ethanol loading contents. The effects of ethanol on the mean vesicle size of the Tween 61 niosomal vesicles investigated by DLS, entrapment efficiency, zeta potential and deformability index were demonstrated in **Table 14**. A

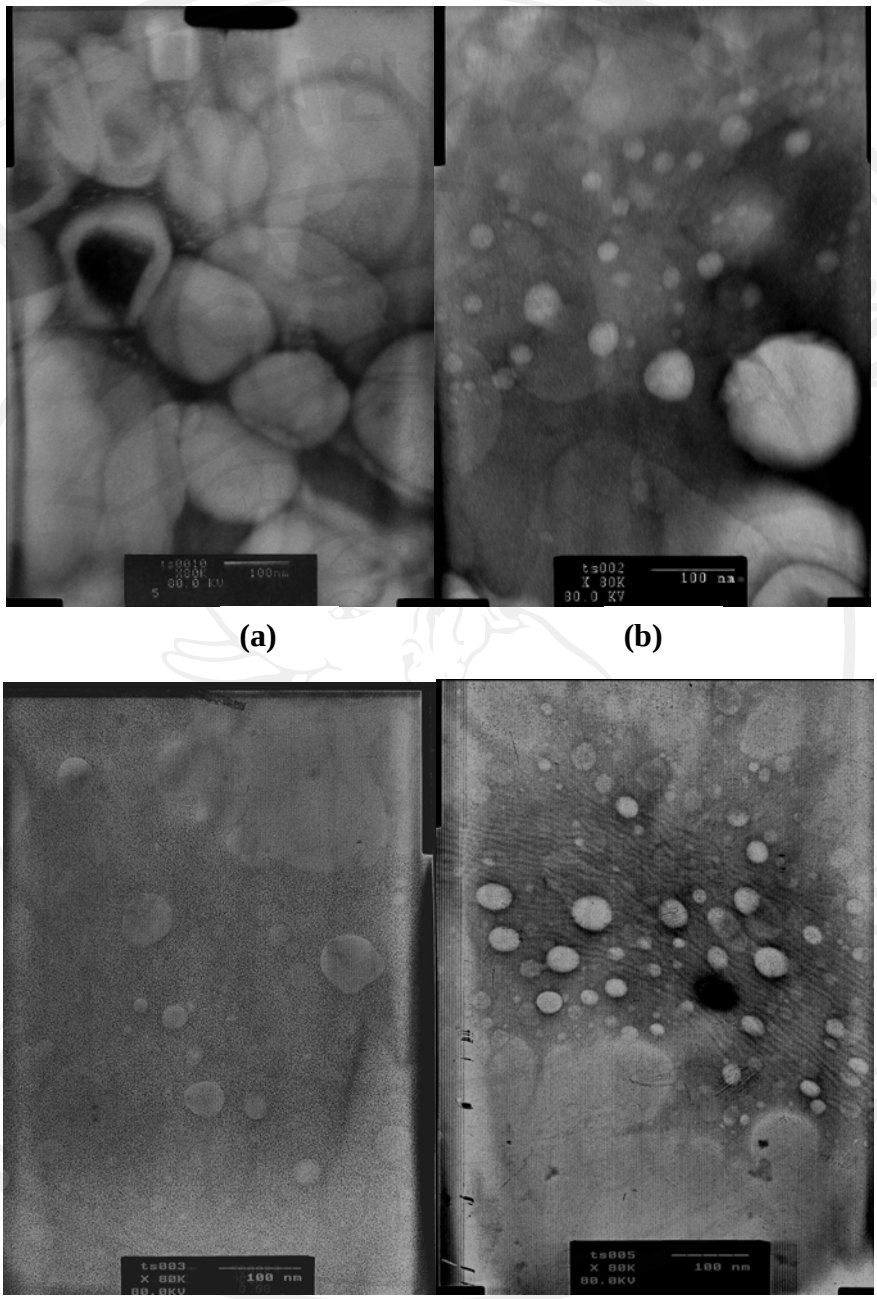
decrease in vesicular size was observed when 25% ethanol was incorporated. It has been reported that the higher ethanol concentration in the vesicles, the lesser membrane thickness was observed owing to the formation of a phase with interpenetrating hydrocarbon chains (Dubey et al., 2007; Barry and Cullis, 1995).

Table 14 Effects of ethanol contents on size, entrapment efficiency, zeta potential and deformability index of niosomes loaded and unloaded with DCFD

Formulations	Initial size (nm)	Size after filtration (nm)	Entrapment efficiency	Zeta potential	Deformability index
Niosomes	395.05±14.38	83.55±11.91	-	-48.57±0.38	1.65±0.46
Elastic niosomes (25% ethanol)	256.20±12.73	185.65±13.18	-	-18.37±1.04	5.67±0.88
DCFD niosomes	327.15±6.72	37.82±3.13	64.98±4.49	-32.17±0.81	0.30±0.05
DCFD elastic niosomes (25% ethanol)	224.45±11.95	207.90±13.44	93.15±2.87	-17.73±0.32	4.13±0.53

Values represent as mean ± SD (n = 3)

Also, ethanol may cause a modification of the net charge of the system resulting in some degree of steric stabilization that may finally lead to a decrease in the mean particle size (Lasic et al., 1998). Both conventional (327.50 ± 6.72 nm) and elastic niosomes (224.45 ± 11.95 nm) loaded with DCFD gave smaller mean vesicle sizes than their corresponding unloaded niosomes (395.05 ± 14.38 and 256.20 ± 12.73 nm, respectively). DCFD which located within the hydrophobic tails of Tween 61 in the vesicles may tighten the bilayer membranes (Friberg and Osborne, 1985). All niosomal formulations exhibited negative charges. The larger the size of the vesicles, the higher the number of charges was observed. Visualized by negative-stain TEM in **Figure 34**, both conventional and elastic niosomes loaded or unloaded with DCFD



(a) **(b)**

(c) **(d)**

Figure 34 Negative staining TEM images of conventional niosomes and elastic niosomes loaded with DCFD. (a) conventional Tween61 niosomes (× 80K). (b) elastic Tween61 niosomes (× 80K). (c) conventional Tween61 niosomes loaded with DCFD (× 80K). (d) elastic Tween 61 niosomes loaded with DCFD (×150K).

were in unilamellar structure. In fact, the presence of a hydrophilic surfactant in the bilayer structure has been reported to form unilamellar vesicles (Cevc et al., 1996). The entrapment efficiency of DCFD in the elastic niosomes (93.15 ± 2.87%) was higher than in the conventional niosomes (64.98 ± 4.49%). This may be due to the solubility enhancement of DCFD by ethanol that facilitates the entrapment of DCFD in the vesicular membrane. For the effect of ethanol on vesicular deformability, the elastic niosomes loaded and unloaded with DCFD showed deformability index of 4.13 ± 0.53 and 5.67 ± 0.88 which were higher than the conventional niosomes loaded and unloaded with DCFD (0.30 ± 0.05 and 1.65 ± 0.46) of about 13.76 and 3.44 times, respectively. After 3 months, the percentages of ethanol remained at 4 ± 2, 27 ± 2 and 45 ± 2°C were 97.98 ± 0.92, 97.24 ± 2.19 and 94.70 ± 0.13, respectively. This has indicated the stability and content uniformity of ethanol in the formulation. Ethanol in the elastic niosomes may interact with the surfactant molecules in the polar head group region, resulting in a reduction in the melting point, thereby increasing the fluidity of the vesicles (Touitou et al., 2000; Dayan and Touitou, 2000). The conventional niosomes (without ethanol) still showed some deformability. This can be explained that, when a surfactant is present in an adequate concentration, it can accommodate to particle shape deformation of the bilayer vesicles under stress. The location of a proper amount of surfactant within the lipid bilayer has been shown to provoke a disruption and fluidization of the bilayer itself (Touitou et al., 2000). The elastic niosomes loaded with DCFD in the form of dispersion and those incorporated in a gel base gave good physical stability with no sedimentation, no layer separation and no color change at 4 ± 2, 27 ± 2 and 45 ± 2°C for 3 months. The amounts of DCFD remaining in the elastic niosomal dispersion and those incorporated in the gel

were 76 and 87% when stored at 45 ± 2°C for 3 months, respectively (**Figure 35**). At 4 ± 2°C, no loss of DCFD in both samples was observed.

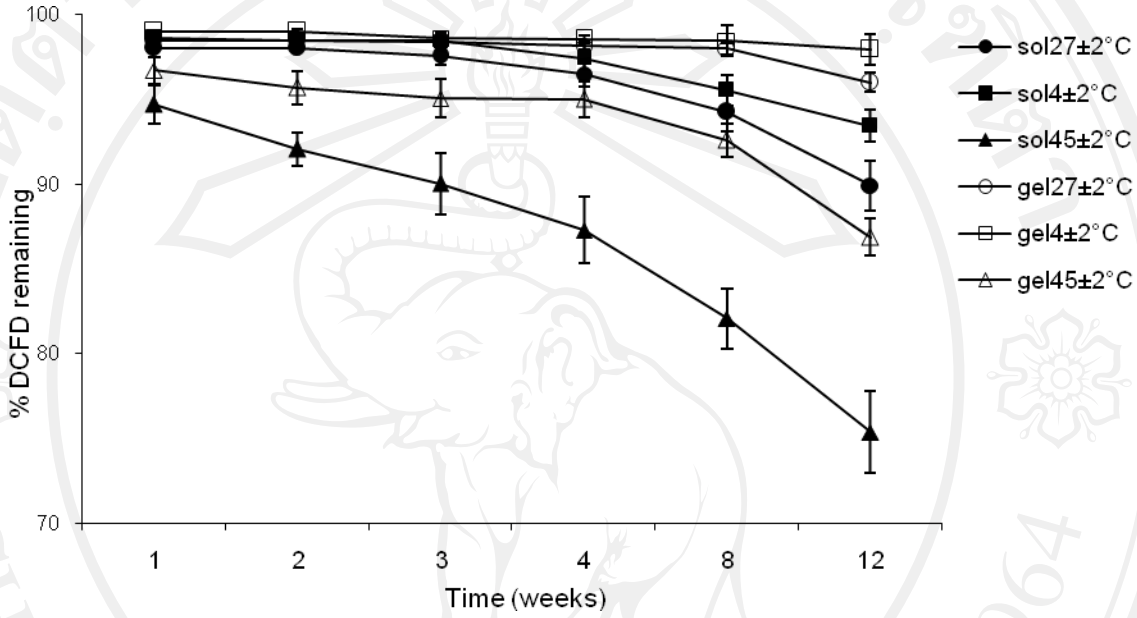


Figure 35 The percentages of DCFD remaining in the elastic niosomal dispersion (sol) and the gel containing DCFD loaded in elastic niosomes (gel) at different storage temperatures (27 ± 2, 4 ± 2 and 4 5 ± 2°C) versus times (weeks)

At elevated temperatures, leakage of the drug from the vesicles was due to the fluidity of the vesicular membrane and the chemical degradation of the drug. DCFD loaded in the vesicles and incorporated in the gel appeared to be more protected against thermal degradation than in the form of vesicular dispersion. The gel structure may retard the leakage of the drug from the vesicles, thereby decreasing the thermal effects on the drugs (Bochot et al., 1998; Ruel-Gariepy et al., 1994; Glavas-Dodov et al., 2002).

3.1.2 *In vitro* transdermal absorption through the excised rat skin of different gel formulations containing DCFD

The cumulative amounts and the fluxes after 6 h of DCFD per area of different gel formulations investigated by vertical Franz diffusion cells were presented (**Table 15** and **Figure 36**). The cumulative amounts (μg/cm^2, n = 3) through skin of all DCFD gel formulations increased with times. The gel containing DCFD loaded in elastic niosomes exhibited the highest amount in SC, VED and the receiving solution in comparing to the commercial emulgel and gel containing the unloaded DCFD and gel containing the DCFD loaded in conventional niosomes. This formulation showed the amounts of DCFD after 6 h in SC, VED and the receiving solution of 1069.39 ± 28.14, 94.81 ± 8.90 and 21.01 ± 7.92 μg/cm^2 which were 3.14, 2.31 and 25.94 times higher than the commercial emulgel which gave 340.18 ± 53.52, 40.96 ± 5.84 and 0.81 ± 0.12 μg/cm^2, respectively. The fluxes (μg/(cm^2 h), n = 3) of all gels containing DCFD decreased with times (**Table 15** and **Figure 37**). The gel containing DCFD loaded in elastic niosomes exhibited the highest flux after 6 h in SC, VED and receiving solution at 191.27 ± 9.52, 16.96 ± 2.77 and 3.76 ± 0.54 μg/(cm^2 h) which were 3, 2.3 and 27 times higher than the commercial emulgel which gave 60.84 ± 13.63, 7.33 ± 1.70 and 0.14 ± 0.01 μg/(cm^2 h), respectively. The fluxes through rat skin in SC and VED after 6 h of DCFD loaded in conventional niosomes or elastic niosomes incorporated in gel were significantly different from the unloaded DCFD in gel and the commercial emulgel ($p < 0.05$). The fluxes of DCFD in the gel containing the unloaded drug were close to that of DCFD in the commercial emulgel. No DCFD was found in the receiving solution of the gel containing unloaded and loaded DCFD in the conventional niosomes. This result has supported that conventional niosomes

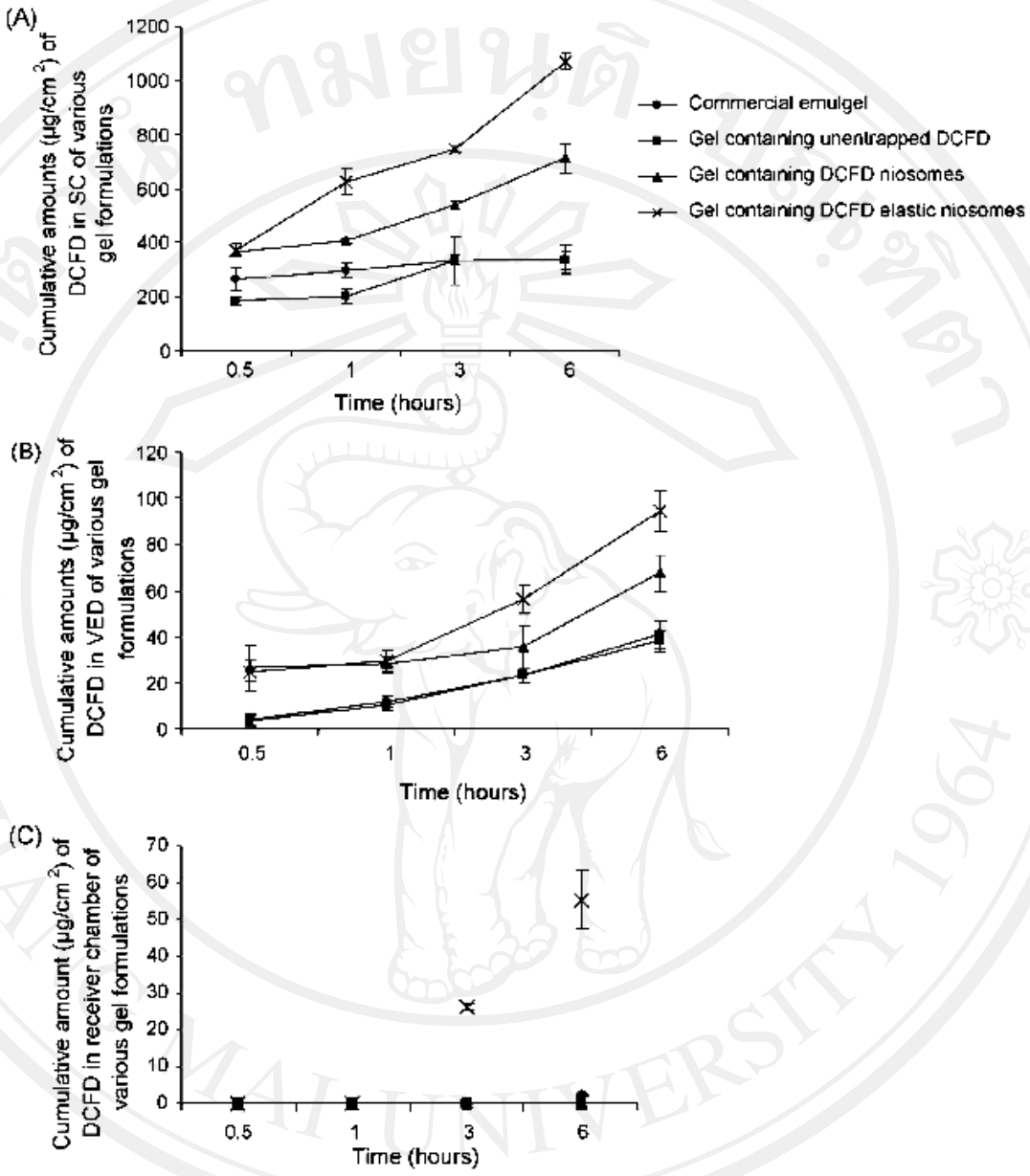


Figure 36 Cumulative amounts (µg/cm^2) of DCFD versus time (hours) in SC (stratum corneum) (A), VED (viable epidermis and dermis) (B) and receiver chamber (C) following transdermal absorption across excised rat skin by Franz diffusion cells from various gel formulations. Each value represents the mean ± SD (n = 3)

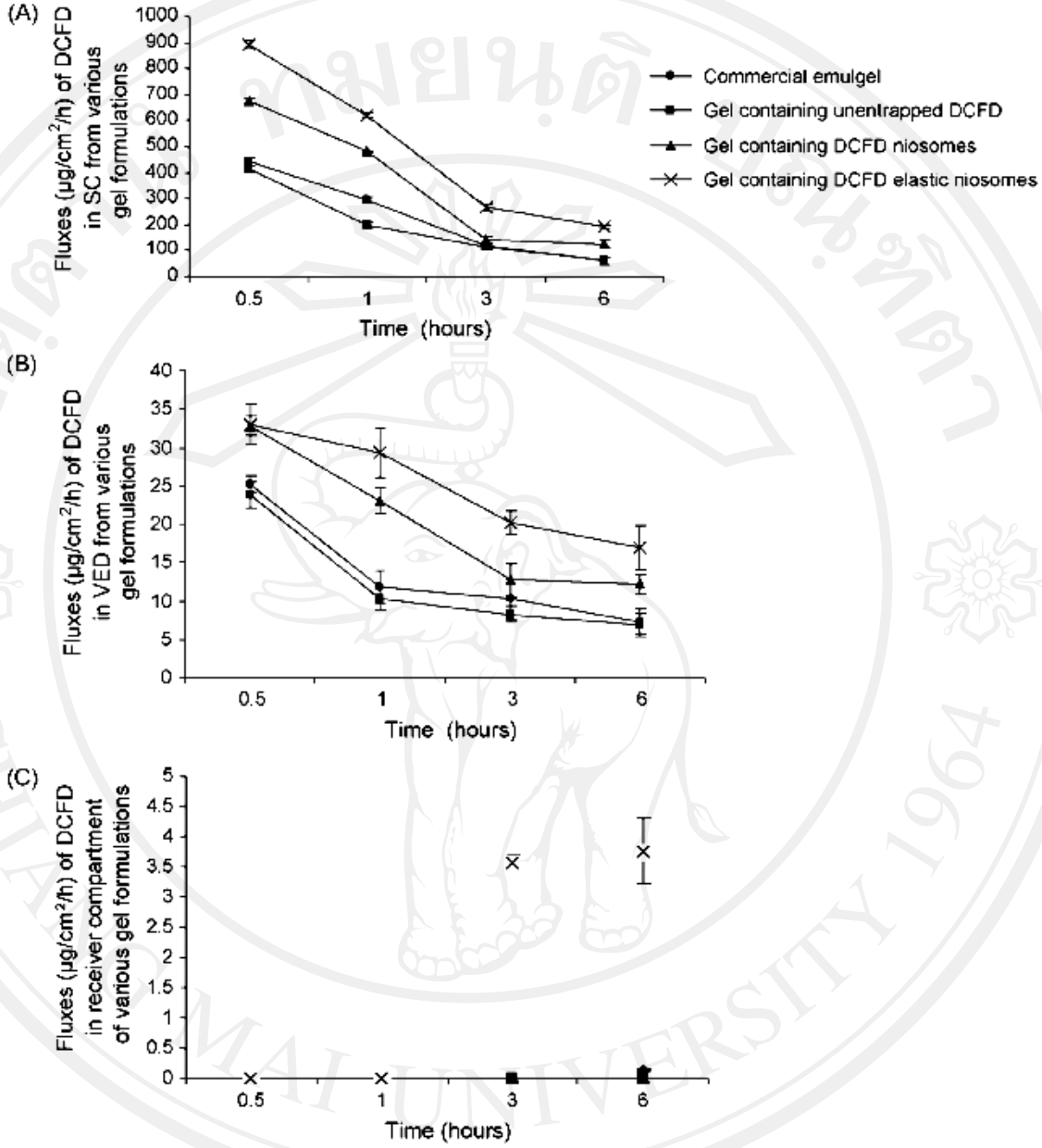


Figure 37 The fluxes (µg/cm^2 /h) of DCFD in SC (stratum corneum) (A), VED (viable epidermis and dermis) (B) and receiver chamber (C) versus times (hours) following transdermal absorption across excised rat skin by Franz diffusion cells from various gel formulations. Each value represents the mean ± SD (n = 3)

Table 15 The cumulative amounts (ug/cm2) and fluxes (μg/cm2 /h) in SC (stratum corneum), VED (viable epidermis and dermis) and receiver chamber following transdermal absorption across excised rat skin by Franz diffusion cells from various gel formulations. Each value represents the mean ± SD (n = 3)

Formulation	amount of DCFD (ug/cm^2)			Flux (μg/cm^2 /h)		
	SC	VED	receiver chamber	SC	VED	receiver chamber
Commercial emulgel	340.18±53.52	40.96±5.84	0.81=0.12	60.84=13.63	7.33±1.7	0.14±0.01
Gel containing the unentrapped DCFD	333.62±31.37	38.24±4.54	0	59.67=14.32	6.84=1.46	0
Gel containing the entrapped DCFD in conventional niosomes	711.22±55.35	67.84±7.86	0	127.21±13.75	12.13±1.21	0
Gel containing the entrapped DCFD in elastic niosomes	1069.39±28.14	94.81±8.90	21.01±7.92	191.27±9.52	16.96±2.77	3.76±0.54

are usually not efficient to transdermally delivery across the skin, because they do not deeply penetrate the skin, but rather remain on the upper layer of SC and in the skin (VED). This has also indicated that transdermal absorption through rat skin of DCFD was enhanced by loading in elastic niosomes, which may be due to not only the suitable polarity for transdermal absorption of the drug modified by the arrangement of the DCFD molecule in the elastic vesicular membrane and in the inner hydrophilic core of the bilayer vesicles, but also the deformability of the vesicles as well. Gel containing DCFD loaded in the conventional niosomes and elastic niosomes showed 1.77 and 2.45 times higher fluxes in VED, respectively, than the gel containing the unloaded DCFD. A synergistic mechanism between ethanol, vesicles and the skin lipids was suggested (Touitou et al., 2000). Ethanol may provide with soft flexible characteristics which allow the vesicles to easily penetrate into deeper layers of the skin. It was also proposed that ethanol may penetrate into the skin and influence the bilayer structure of SC leading to the enhancement of drug penetration (Kirjavainen et al., 1999).

3.1.3 *In vivo* anti-inflammatory activity of different gel formulations containing DCFD

The developed gel containing DCFD loaded in elastic niosomes showed % inhibition of rat ear edema after 1 h of application higher than the commercial emulgel, gel containing DCFD loaded in conventional niosomes and gel containing the unloaded drug of 16.22, 10.81 and 24.33%, respectively, but lower than phenylbutazone of 5.4% (**Figure 38** and **Table 16**). This result was in the same trends as the fluxes of DCFD in this formulation observed in VED (**Table 15**). This has indicated the *in vivo* anti-inflammatory activity enhancement of DCFD when

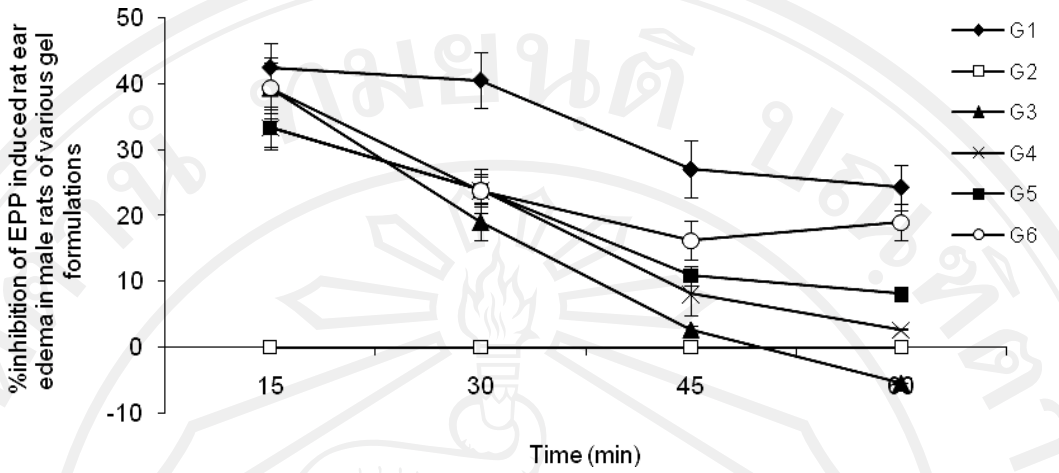


Figure 38 The plot of the % inhibition of EPP induced rat ear edema of phenylbutazone (G1), gel base (G2), gel containing the unloaded DCFD (G3), commercial emulgel (G4), gel containing conventional niosomal vesicles loaded with DCFD (G5) and gel containing elastic niosomal vesicles loaded with DCFD (G6)

Table 16 Effects of various gel formulations containing DCFD on the % inhibition of EPP-induced ear edema at various time intervals

Formulations	Time after topical application of EPP							
	15 min		30 min		45 min		60 min	
	Tt (mm)	PI(%)	Tt (mm)	PI(%)	Tt (mm)	PI(%)	Tt (mm)	PI(%)
G1	0.19±0.02	42.42	0.25±0.06	40.48	0.27±0.02	27.03	0.28±0.10	24.32
G2	0.33±0.04	-	0.42±0.03	-	0.37±0.03	-	0.34±0.08	-
G3	0.20±0.05	39.39	0.34±0.03	19.05	0.36±0.07	2.70	0.39±0.04	-5.41
G4	0.22±0.03	33.33	0.32±0.05	23.81	0.34±0.03	8.11	0.36±0.02	2.70
G5	0.22±0.01	33.33	0.32±0.11	23.81	0.33±0.01	10.81	0.34±0.07	8.11
G6	0.20±0.01	39.39	0.32±0.02	23.81	0.31±0.01	16.22	0.30±0.01	18.92

Values are mean ± SD. (n=3), Tt: edema thickness; PI: percent inhibition; G1: phenylbutazone; G2: gel base; G3: gel containing the unloaded DCFD; G4: Commercial emulgel; G5: gel containing niosomal vesicles loaded with DCFD and G6: gel containing elastic niosomal vesicles loaded with DCFD.

loaded in niosomes, especially the elastic niosomes. The commercial emulgel gave a little superior rat skin transdermal flux and % ear edema inhibition than the gel containing the unloaded drug, since the commercial emulgel which did not contain any bilayer vesicles, may have some transdermal absorption enhancers.

3.2 *In vitro* anti-aging activities of *Terminalia chebula* gall extract and Thai Lanna medicinal plant extracts

3.2.1 Percentage yields of the plant extracts prepared by different processes

The percentage yields of the 60 extracts prepared by four different extraction processes (CM, HM, CW and HW) of the 15 selected Thai Lanna medicinal plants were presented in **Table 17**. Aqueous extracts of most plants gave higher percentage yield than the methanol extracts. The plants may contain more water soluble than water insoluble constituents. The highest percentage yields were from *T. chebula* gall by CM, CW and HW at 59.02, 49.85 and 60.00% respectively. For HM, *P. parkinsonii* flower gave the highest percentage yield at 29.25% while *T. chebula* gall gave 23.28%. HW of all plants gave higher yields than CW, while CM showed higher yields than HM. Most plants may contain heat labile water insoluble components. Thus, the hot process appeared to be the superior process for water soluble components, whereas the cold process was advantageous for water insoluble substances.

3.2.2 Phytochemical tests of the extracts

Table 18 showed the phytochemical constituents of the 60 extracts. Alkaloid was a basic secondary metabolite in all extracts, while glycosides and tannin were in most extracts. Few extracts contained anthraquinones. Interestingly, the extracts of

Table 17 Comparison of percentage yields of the 60 extracts from the 15 selected Thai Lanna plants including *T. chebula* gall prepared by aqueous and methanol cold and hot processes

Scientific name (Family)	% yield			
	CM	**HM**	**CW**	**HW**
Acorus gramineus L. (Araceae)	7.10	7.05	10.45	14.85
Cassia fistula L. (Fabaceae)	15.45	15.10	25.39	27.54
Cyperus rotundus L. (Cyperaceae)	5.20	6.00	13.03	21.71
Dregea volubilis (L.f.) Benth. Ex Hook.f. (Asclepiadaceae)	11.30	6.16	15.00	25.26
Eclipta prostrate L. (Asteraceae)	6.95	2.64	8.40	17.70
Myristica fragrans Houtt. (Myristicaceae)	4.87	3.94	1.20	5.07
Nigella sativa L. (Ranunculaceae)	12.10	12.61	1.20	12.86
Plumbago indica L. (Plumbaginaceae)	17.38	16.13	14.02	29.30
Piper nigrum L.(Piperaceae)	4.46	3.08	7.54	9.12
Pellacalyx parkinsonii Fisch. ST (Rhizophoraceae)	31.41	29.25	22.70	36.70
Piper sarmentosum Roxb. (Piperaceae)	7.81	4.10	22.70	25.03
Plumbago zeylanica L. (Plumbaginaceae)	5.58	2.13	8.30	11.95
Terminalia chebula Retz. (Combretaceae)	59.02	23.28	49.85	60.00
Tinospora crispa L. (Menispermaceae)	8.95	6.80	15.87	18.76
Zingiber officinale Roscoe (Zingiberaceae)	12.30	12.60	15.74	22.98

Note: CM = cold methanol process; HM = hot methanol process; CW = cold aqueous process; HW = hot aqueous process

C. fistula fruit prepared by all processes (CM, HM, CW and HW) contained all phytochemical compounds including alkaloids, anthraquinones, flavonoids, glycosides, saponins, tannins and xanthones. All extracts by all extraction processes contained same phytochemicals, except for antraquinones in *M. fragrans*, *P. indica* and *P. zeylanica* which were found in HM and CM, but not found in HW and CW. Saponins in *P. nigrum* and tannins in *A. gramineus* were found in CW and HW; CM

Table 18 Qualitative determination of constituents by phytochemical tests in 60 extracts from the 15 selected Thai Lanna plants including *T. chebula* gall prepared by various extraction processes

	Alkaloids				Anthraquinones				Flavonoids				Glycosides				Saponins				Tannins				Xanthones			
Extracts of plants	CM	HM	CW	HW	CM	HM	CW	HW	CM	HM	CW	HW	CM	HM	CW	HW	CM	HM	CW	HW	CM	HM	CW	HW	CM	HM	CW	HW
A. gramineus	+	+	+	+	–	–	–	–	–	–	–	–	+	+	+	+	–	–	–	–	+	+	–	–	–	–	–	–
C. fistula	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C. rotundus	+	+	+	+	–	–	–	–	–	–	–	–	+	+	+	+	–	–	–	–	+	+	+	+	–	–	–	–
D. volubilis	+	+	+	+	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–
E. prostrata	+	+	+	+	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
M. fragrans	+	+	+	+	+	+	–	–	–	–	–	–	+	+	+	+	–	–	–	–	+	+	+	+	–	–	–	–
N. sativa	+	+	+	+	–	–	–	–	+	+	+	+	+	+	+	+	–	–	–	–	+	+	+	+	–	–	–	–
P. indica	+	+	+	+	+	+	–	–	–	–	–	–	+	+	+	+	–	–	–	–	+	+	+	+	–	–	–	–
P. nigrum	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	–	–	–	–
P. parkinsonii	+	+	+	+	–	–	–	–	+	+	+	+	+	+	+	+	–	–	–	–	+	+	+	+	+	+	+	+
P. sarmentosum	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P. zeylanica	+	+	+	+	+	+	–	–	–	–	–	–	+	+	+	+	–	–	–	–	+	+	+	+	–	–	–	–
T. chebula	+	+	+	+	–	–	–	–	+	+	+	+	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+
T. crispa	+	+	+	+	–	–	–	–	–	–	–	–	+	+	+	+	–	–	–	–	+	+	+	+	–	–	–	–
Z. officinale	+	+	+	+	–	–	–	–	–	–	–	–	+	+	+	+	–	–	–	–	+	+	+	+	–	–	–	–

Note: CM, cold methanol process; HM, hot methanol process; CW, cold aqueous process; HW, hot aqueous process; + presence; – absence.

and HM, but not found in CM and HM; CW and HW, respectively. For all *T. chebula* gall extracts, they contained alkaloids, flavonoids, saponins, tannins and xanthones but no anthraquinones and glycosides. Phytochemicals in the extracts appeared to depend on types of plants more than the extraction conditions. Generally, solvents and temperatures are important parameters to obtain high yields. Temperature has a positive effect on the extraction yields and rates by enhancing the solubility of the compounds. However, some heat labile phytochemicals may be degraded by high temperature as CW gave higher yield than HW of most plants.

3.2.3 DPPH radical scavenging activity

Table 19 showed the IC_{50} values of the DPPH radical scavenging assay of the 60 extracts. **Figure 39** demonstrated the percentages of DPPH radical scavenging activity of the 60 extracts at 0.1 mg/ml. The standard antioxidants (ascorbic acid, α-tocopherol and BHT at 0.1 mg/ml) gave the scavenging activity of 96.50 ± 0.10, 35.74 ± 0.20 and 27.43 ± 0.10% with the IC_{50} values of 0.014 ± 0.001, 0.038 ± 0.002 and 0.049 ± 0.002 mg/ml, respectively. The methanol extract of most plants exhibited higher activity than the aqueous extracts. Alcoholic solvents have been commonly employed to extract phenolic compounds from plants, because of obtaining high yield although they were not highly selective for phenols (Spigno and Faveri, 2007). The phenolic compounds which have been reported to scavenge DPPH include flavonoids, anthraquinones, anthocyanidins, xanthones, and tannins. They also scavenged superoxide and hydroxyl radical by the single electron transfer (Ho et al., 1999; Choi et al., 2002). Extracts from *T. chebula* gall prepared by all processes exhibited higher DPPH radical scavenging activity than those of other plants.

Table 19 The IC_{50} values of the 60 extracts from the 15 selected Thai Lanna plants including *T. chebula* gall determined by the DPPH radical scavenging, chelating and tyrosinase inhibition assays

Extracts of plants	IC_{50} of DPPH radical scavenging assay (mg/mL)				IC_{50} of chelating assay (mg/mL)				IC_{50} of tyrosinase inhibition assay (mg/mL)			
	CM	HM	CW	HW	CM	HM	CW	HW	CM	HM	CW	HW
A. gramineus	0.065±0.008	0.065±0.006	0.159±0.016	0.076±0.009	0.152±0.002	0.138±0.006	4.148±0.149	0.598±0.002	0.132±0.002	0.184±0.002	1.251±0.003	0.598±0.002
C. fistula	0.044±0.001	0.045±0.006	0.057±0.003	0.054±0.003	0.476±0.001	0.375±0.002	1.571±0.088	0.15±0.008	0.076±0.001	0.084±0.002	2.507±0.019	0.15±0.008
C. rotundus	0.035±0.002	0.028±0.001	0.066±0.005	0.033±0.001	0.104±0.001	0.094±0.001	2.125±0.006	1.117±0.001	0.104±0.001	0.129±0.005	3.192±0.007	1.117±0.001
D. volubilis	0.085±0.021	0.102±0.011	0.052±0.002	0.127±0.002	0.487±0.008	0.355±0.001	1.05±0.029	0.436±0.001	0.087±0.008	0.156±0.004	0.696±0.001	0.436±0.001
E. prostrate	0.04±0.011	0.060±0.002	0.057±0.019	0.066±0.001	0.642±0.036	0.548±0.030	0.727±0.01	0.634±0.048	0.142±0.036	0.174±0.007	0.385±0.017	0.334±0.048
M. fragrans	0.042±0.001	0.042±0.001	0.064±0.003	0.041±0.009	0.233±0.002	0.121±0.024	0.669±0.001	1.573±0.132	0.093±0.002	0.106±0.006	2.166±0.068	1.573±0.132
N. sativa	0.085±0.003	0.053±0.003	0.062±0.008	0.074±0.006	0.161±0.010	0.15±0.023	1.18±0.155	3.04±0.039	0.161±0.01	0.189±0.004	3.353±0.018	3.04±0.039
P. indica	0.036±0.002	0.033±0.003	0.06±0.01	0.047±0.007	0.175±0.004	0.165±0.034	2.578±0.105	0.179±0.021	0.145±0.004	0.175±0.002	0.524±0.027	0.179±0.021
P. nigrum	0.058±0.029	0.079±0.003	0.034±0.004	0.054±0.004	6.376±0.031	6.36±0.255	6.742±0.027	1.485±0.064	1.076±0.031	1.137±0.015	1.64±0.004	1.485±0.064
P. parkinsonii	0.026±0.003	0.027±0.003	0.027±0.009	0.036±0.003	0.306±0.008	0.163±0.009	0.886±0.015	0.443±0.012	0.106±0.008	0.129±0.002	0.541±0.03	0.443±0.012
P. sarmentosum	0.028±0.003	0.032±0.001	0.118±0.005	0.07±0.001	0.324±0.003	0.266±0.019	1.743±0.033	1.06±0.005	0.124±0.003	0.14±0.005	2.26±0.198	1.060±0.005
P. zeylanica	0.045±0.002	0.049±0.004	0.063±0.003	0.04±0.005	0.436±0.001	0.125±0.003	2.335±0.449	0.795±0.063	0.096±0.001	0.129±0.003	1.473±0.161	0.795±0.063
T. chebula	0.021±0.004	0.017±0.001	0.016±0.001	0.018±0.007	0.282±0.002	0.217±0.002	2.287±0.275	1.151±0.006	0.082±0.002	0.088±0.001	0.393±0.008	0.151±0.006
T. crispa	0.142±0.016	0.09±0.015	0.171±0.018	0.15±0.01	0.332±0.022	0.124±0.005	0.775±0.005	1.645±0.134	0.152±0.022	0.23±0.016	1.484±0.187	1.645±0.134
Z. officinale	0.035±0.004	0.036±0.002	0.044±0.001	0.087±0.003	0.52±0.001	0.352±0.004	0.865±0.004	1.664±0.013	0.12±0.001	0.174±0.006	2.969±0.022	1.664±0.013
Standards	IC_{50} of DPPH radical scavenging assay (mg/mL)				IC_{50} of chelating assay (mg/mL)				IC_{50} of tyrosinase inhibition assay (mg/mL)			
Ascorbic acid	0.014±0.001				–				0.046±0.001			
α-Tocopherol	0.038±0.002				–				–			
BHT	0.049±0.002				–				–			
EDTA	–				0.079±0.001				–			
Kojic acid	–				–				0.03±0.001			

Note: CM, cold methanol process; HM, hot methanol process; CW, cold aqueous process; HW, hot aqueous process.

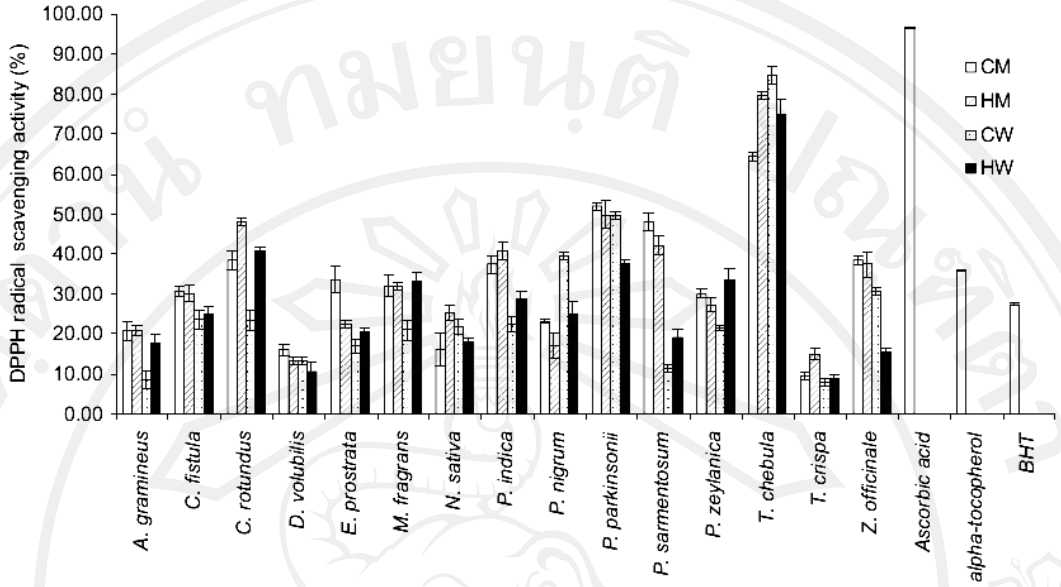


Figure 39 Comparison of the percentages of DPPH radical scavenging activity of the 60 extracts at 0.1 mg/ml from the 15 selected Thai Lanna plants including *T. chebula* gall and the standard antioxidants (ascorbic acid, α-tocopherol and BHT at 0.1 mg/ml) (CM = cold methanol process; HM = hot methanol process; CW = cold aqueous process; HW = hot aqueous process)

The highest scavenging activity of *T.chebula* gall extract at 84.64 ± 2.22% was found (at 0.1 mg/ml) by CW process with the IC_{50} value of 0.016 ± 0.001 mg/ml, which were 2.37 and 3.09 times more potent than *α*-tocopherol (IC_{50} value of 0.038 ± 0.002 mg/ml) and BHT (IC_{50} value of 0.049 ± 0.002 mg/ml), respectively. The phytochemicals which were found in this extract including alkaloids, flavonoids, saponins, tannins and xanthones (**Table 18**) may be synergistic and responsible for this activity. *T. chebula* and its galls have been used traditionally in the combination with other Thai Lanna medicinal plants in many recipes for promoting longevity. Thus, this result has supported the folklore wisdom application of *T. chebula* gall in Thai Lanna medicines.

3.2.4 Chelating activity

Table 19 showed the IC_{50} values of chelating activity assay of the 60 extracts. **Figure 40** presented the percentages of the chelating effect of 60 extracts at 0.1 mg/ml. None of the extracts indicated better chelating activity than EDTA at 0.1 mg/ml which gave 87.74 ± 0.10% and the IC_{50} value of 0.079 ± 0.001 mg/ml. The CM, HM, CW and HW extracts of *T. chebula* gall gave the IC_{50} values of 0.282 ± 0.002, 0.217 ± 0.002, 2.287 ± 0.275 and 1.151 ± 0.006, respectively. The highest chelating activity of 79.49 ± 1.10% was found in HM of *C. rotundus* root with the IC_{50} value of 0.094 ± 0.001 mg/ml. In fact, *C. rotundus* has been reported to contain many phenolic compounds, such as gallic acid, *p*-coumaric acid and epicatechin (Proestos et al., 2005) which are potent antioxidants by ferric reducing antioxidant power and Trolox equivalent antioxidant capacity assays along with metal chelating properties. From the phytochemical test, *C. rotundus* extract by HM contained alkaloids, glycosides and tannins **(Table 18)**.

3.2.5 Tyrosinase inhibition activity

Table 19 showed the IC_{50} values of the tyrosinase inhibition activity of 60 extracts. **Figure 41** demonstrated the percentages of tyrosinase inhibition of 60 extracts at 0.1 mg/ml. The CM, HM, CW and HW extracts of *T. chebula* gall gave the IC_{50} values of 0.082 ± 0.002, 0.088 ± 0.001, 0.393 ± 0.008 and 0.151 ± 0.006 respectively. The CM extract of *C. fistula* fruit gave the highest tyrosinase inhibition activity of 63.55 ± 0.16% with the IC_{50} value of 0.076 ± 0.001 mg/ml, but lower than ascorbic acid and kojic acid (at 0.1 mg/ml) which gave 71.53 ± 0.20 and 88.63 ± 0.10% with the IC_{50} values of 0.046 ± 0.001 and 0.030 ± 0.001 mg/ml, respectively. From **Table 19**

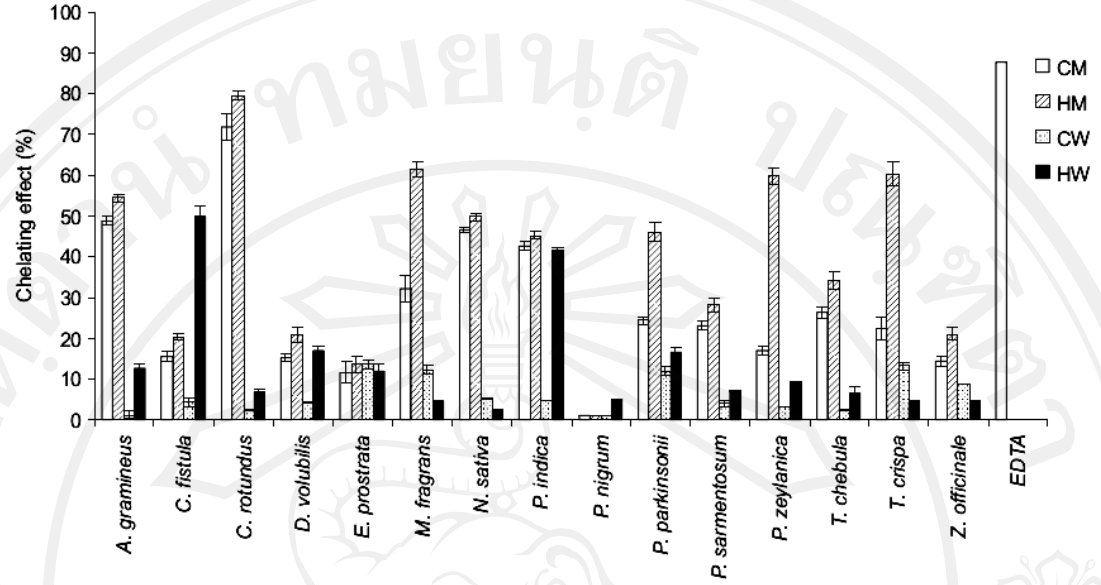


Figure 40 Comparison of the percentages of the chelating effect (%) by the ferrous iron–ferrozine complex method of the 60 extracts at 0.1 mg/ml from the 15 selected Thai Lanna plants including *T. chebula* gall and the standard chelating agent (EDTA at 0.1 mg/ml)

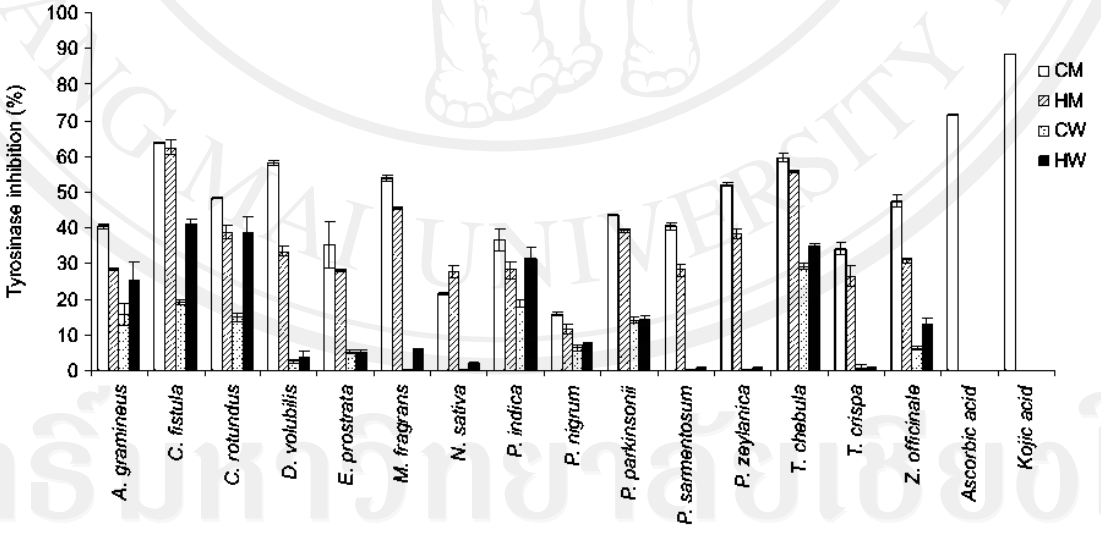


Figure 41 Comparison of the percentages of tyrosinase inhibition of the 60 extracts at 0.1 mg/ml from the 15 selected Thai Lanna plants including *T. chebula* gall and the standard whitening agents (ascorbic acid and kojic acid at 0.1 mg/ml)

the CM extract of *C. fistula* contained anthraquinones, flavonoids, glycosides, saponins and tannins. The methanol extract of *C. fistula* has been evaluated in *in vitro* models in protecting free radical-induced lipid peroxidation in model membranes (Sunil and Muller, 1998). Flavonoids in this plant have also been reported to inhibit tyrosinase due to their ability to chelate copper in the active site (Maity et al., 1998; Cuellar et al., 2001). The fruit tissue of this plant was found to be a rich source of potassium, calcium, iron and manganese (Barthakur et al., 1995) which may be competitive inhibitors of the tyrosinase enzyme. Proanthocyanidins including flavan-3-ol (epiafzelechin and epicatechin) units with an abnormal 2*S*-configuration together with the common flavan-3-ols and proanthocyanidins like catechin, epicatechin, procyanidin B-2 and epiafzelechin which are strong tyrosinase inhibitors have also been found in the pods of *C. fistula* (Kashiwada et al., 1990).

3.2.6 Proliferation of normal human skin fibroblasts by the SRB assay

From the DPPH radical scavenging, chelating and tyrosinase inhibition assays, three plants including *T. chebula* gall, *C. rotundus* root and *C. fistula* fruit were selected to investigate for human skin fibroblasts proliferative assay. The stimulation index (SI) of the extracts at 0.1 mg/ml on normal human skin fibroblasts (15^{th} passage) was shown in **Table 20**. Extracts of all three plants by HW and CW showed higher SI than by HM and CM. The methanol extracts appeared to be more toxic to the cells than the aqueous extracts. The CW extract of *T. chebula* gall gave the highest cell stimulative effect with the SI value of 1.441 ± 0.084 which was more potent than ascorbic acid (at 0.1 mg/ml) that gave the SI value of 1.210 ± 0.033. The higher cell stimulative effect on fibroblasts of the aqueous extracts in comparing to the methanol extracts might be due to the presence of more polar compounds in the aqueous extracts

(CW and HW) which are usually more bioactive and less toxic to cells. This result can anticipate the effect of the extract on collagen synthesis from the stimulation of human fibroblasts proliferation.

Table 20 Comparison of the stimulation index (SI) of the 12 extracts at 0.1 mg/ml of the 3 selected Thai Lanna plants including *T. chebula* gall on normal human skin fibroblasts (15^{th} passage)

Extracts	SI at 0.1 mg/ml			
	CM	**HM**	**CW**	**HW**
C. fistula	1.289 ± 0.181	1.292 ± 0.026	1.366 ± 0.110	1.340 ± 0.123
C. rotundus	1.158 ± 0.070	1.172 ± 0.150	1.250 ± 0.199	1.233 ± 0.167
T. chebula	1.364 ± 0.057	1.411 ± 0.188	1.441 ± 0.084	1.393 ± 0.104
Ascorbic acid	1.210±0.033			

Note: CM = cold methanol process; HM = hot methanol process; CW = cold aqueous process; HW = hot aqueous process. SI is the ratio calculated between the percentages of cell growth of the systems treated and not treated (control) with the extract.

3.2.7 Gelatinolytic activity on MMP-2 inhibition of the plant extracts (Zymography)

Although many studies have reported on the biological activities of *T.chebula* (Lee et al., 1995; Burapadaja and Bunchoo, 1995; Saleem et al., 2002; Chen et al., 2003), there was no report on the effects of the extract of this plant on MMP-2 expression in human dermal fibroblasts. **Figure 42** showed the comparison of the inhibition of MMP-2 between *T. chebula* gall extracts (CW, CM, HW and HM) and ascorbic acid by zymography. All extracts of *T. chebula* gall as well as ascorbic acid inhibited the MMP-2 expression. The CW extract of *T. chebula* gall at 0.1 mg/ml showed the inhibition of MMP-2 at 89.94% of the control which was about 1.37 times more potent than ascorbic acid that gave 65.79% of the control.

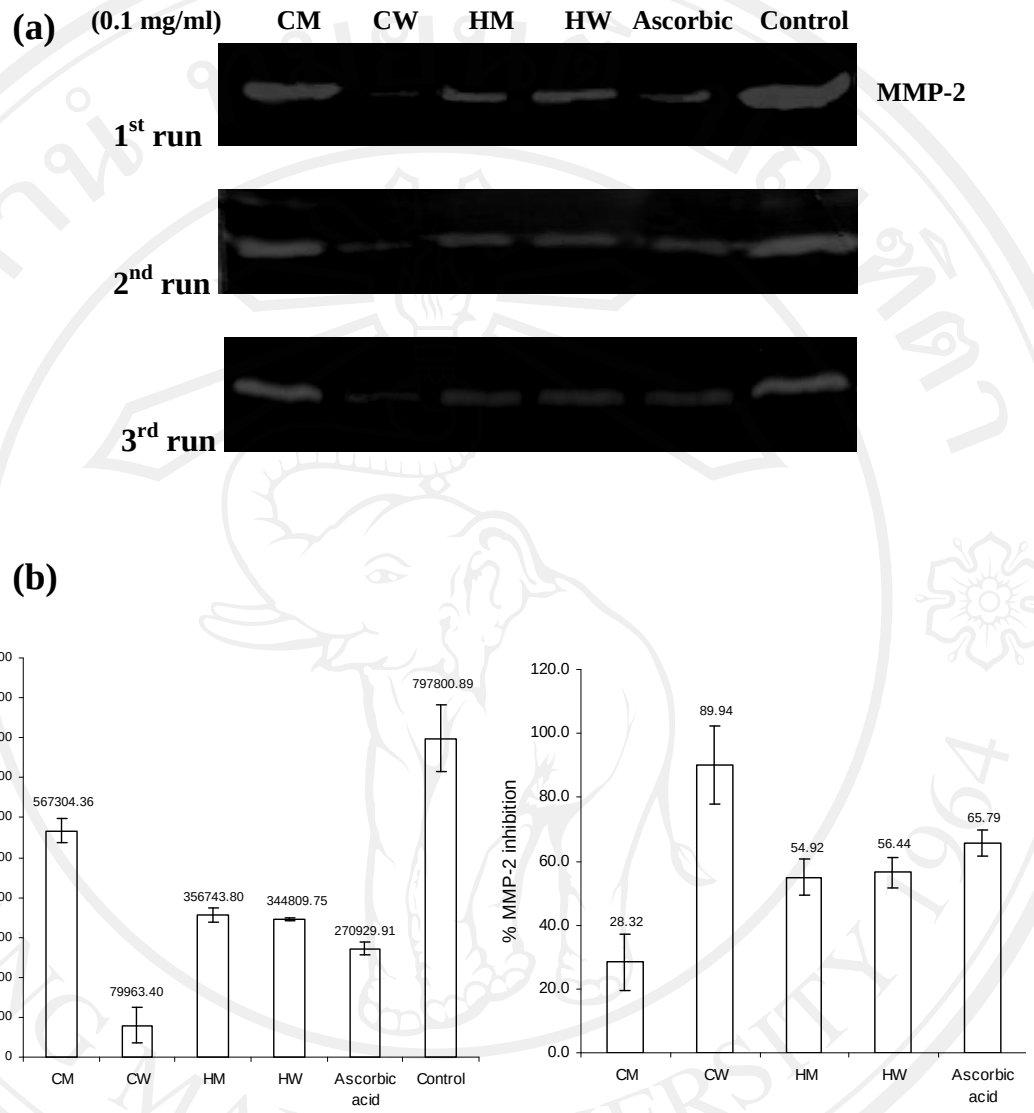


Figure 42 Comparison of the gelatinolytic activity on MMP-2 inhibition between *T. chebula* gall extracts (CW, CM, HW and HM) at 0.1 mg/ml and ascorbic acid at 0.1 mg/ml, (a): zymograms of three independent separate experiments, (b): MMP-2 contents [area × intensity; (mm^2)] (left) and the percentages of MMP-2 inhibition (right) (CM = cold methanol process; HM = hot methanol process; CW = cold aqueous process; HW = hot aqueous process)

This has suggested that the CW extract of *T. chebula* gall was a potent inhibitor of MMP-2 expresstion on early aging human skin fibroblasts (15^{th} passage). This effect may be not only from the cell proliferation stimulation, but also from the phytochemicals existing in the CW extract of this plant including alkaloids, flavonoids, tannins and xanthones. This result has also supported the traditional use for longevity of *T. chebula* gall since the indication of this plant in many Thai Lanna recipes was by cold aqueous extraction (maceration).

3.3 Biological activities of phenolic compounds isolated from galls of *Terminalia chebula* Retz. (Combretaceae)

3.3.1 Fractionation, isolation and structure elucidation of phenolic compounds from the gall extracts

The eluted solvent and yield of each fraction were as followed: fr.1: MeOH/H_2O/AcOH (20:80:1) (0.54 g), fr.2: MeOH/H_2O/AcOH (40:60:1) (0.74 g) and fr.3: MeOH/H_2O/AcOH (60:40:1) (0.50 g) Compounds **1** (43.9 mg) and **2** (27.2 mg) were isolated and purified from fr.1 at the retention times of 8.9 and 16.8 min, respectively. Compounds **3** (22.8 mg) and **4** (4.9 mg) were isolated and purified from fr.2 at the retention times of 15.2 and 23.2 min, respectively. Compounds **5** (16.7 mg) and **6** (24.6 mg) were isolated and purified from fr.3 at the retention times of 10.0 and 16.6 min, respectively. Six isolated compounds were identified as gallic acid (3,4,5-trihydroxybenzoic acid) (**1**) (Gottlieb et al., 1991), punicalagin (**2**) (Jossang et al., 1994), isoterchebulin (**3**) (Conrad et al., 2001), 1,3,6-tri-*O*-galloyl-β-D-glucopyranose (**4**) (Haddock et al., 1982), chebulagic acid (**5**) (Lee et al., 1995) and chebulinic acid

(**6**) (Kilka et al., 2004) by spectral comparison with the corresponding compounds reported in the literature.

Gallic acid (**1**): white powder, and positive to the $FeCl_3$-MeOH reagent. ^{13}C and ^{1}H NMR, see **Table 21**; HR-ESIMS (negative-ion mode) *m*/*z* 169.1214 $[M - H]^-$ (calculated for $C_7H_5O_5$, 169.0137).

Punicalagin (**2**): yellow amorphous powder. ^{13}C and ^{1}H NMR, see **Table 22**; HR-ESIMS (negative-ion mode) *m*/*z* 1083.0592 $[M - H]^-$ (calculated for $C_{48}H_{27}O_{30}$, 1083.0587). There are two groups of spectral signals of NMR due to non-acylated anomeric center of the sugar moiety, existed as a mixture of α and β forms (1:1) in solution.

Isoterchebulin (**3**): yellow amorphous powder. ^{13}C and ^{1}H NMR, see **Table 22**; HR-ESIMS (negative-ion mode) *m*/*z* 1083.0582 $[M - H]^-$ (calculated for $C_{48}H_{27}O_{30}$, 1083.0587). There are two groups of spectral signals of NMR due to the non-acylated anomeric center of the sugar moiety, existing as a mixture of α and β forms (2:1) in solution.

1,3,6-tri-*O*-Galloyl-β-D-glucopyranose (**4**): amorphous powder. ^{13}C and ^{1}H NMR, see **Table 23**; ESIMS (negative-ion mode) *m*/*z* 635 $[M - H]^-$.

Chebulagic acid (**5**): white powder. ^{13}C and ^{1}H NMR, see **Table 21**; HR-ESIMS (negative-ion mode) *m*/*z* 953.0884 $[M - H]^-$ (calculated for $C_{41}H_{31}O_{27}$, 953.0896).

Chebulinic acid (**6**): white powder. ^{13}C and ^{1}H NMR, see **Table 23**; HR-ESIMS (negative-ion mode) *m*/*z* 955.1050 $[M - H]^-$ (calculated for $C_{41}H_{31}O_{27}$, 955.1053).

Table 21 ^{13}C (100 MHz) and ^{1}H NMR (400 MHz) spectroscopic data for (**1**) and (**5**)

	1 ($CD_3COCD_3 + D_2O$)		**5** ($CD_3COCD_3 + D_2O$)		**5** (CD_3COCD_3)
Position	δ_C	δ_H [a)]	δ_C	δ_H [a)]	δ_H [a)]
1	122.2 s		91.6 d	6.51 (br s)	6.51 (br s)
2	109.7 d	7.16 (s)	70.6 d	5.45 (br s)	5.45 (br s)
3	145.7 s		61.7 d	5.86 (br s)	5.86 (br s)
4	138.3 s		66.1 d	5.19 (br d, 3.7)	5.23 (d, 3.6)
5	145.7 s		73.6 d	4.79 (d, 9.7)	4.76 (d, 9.6)
6	109.7 d	7.16 (s)	63.9 dt	4.37 (dd, 8.1, 11.0)	4.40 (dd, 4.7, 9.6)
				4.69 (dd, 9.2, 11.0)	4.82 (d, 9.6)
7	169.1 s				
Chebuloyl (Che)					
2			169.8 s		
3			66.3 d	4.84 (d, 7.4)	4.95 (d, 7.1)
4			40.9 d	5.03 (dd, 1.6, 7.4)	5.11 (dd, 1.6, 7.1)
4a			115.7 s		
5			118.1 s		
6			116.7 d	7.45 (s)	7.52 (s)
7			146.5 s		
8			140.0 s		
8a			140.8 s		
1' C=O			173.6 s		
2'			39.3 d	3.80	3.88 (td, 1.4, 7.8)
3'			29.8 d	2.17 (2H)	2.19 (2H, d, 7.8)
4' C=O			173.4 s		
1" C=O			165.6 s		
Hexahydroxydiphenyl (HHDP)					
1			117.1 s		
2			124.2 d	6.98 (s)	7.08 (s)
3			145.1 s		
4			137.6 s		
5			145.2 s		
6			110.1 d	6.98 (s)	7.08 (s)
7 C=O			166.4 s		
1'			115.2 s		
2'			125.2 d	6.61 (s)	6.66 (s)
3'			144.5 s		
4'			136.2 s		
5'			144.9 s		
6'			107.5 d	6.61 (s)	6.66 (s)
7' C=O			168.7 s		
Galloyl (Gal)					
1			119.6 s		
2			110.4 d	7.11 (s)	7.19 (s)
3			145.9 s		
4			139.9 s		
5			145.9 s		
6			110.4 d	7.11 (s)	7.19 (s)
7 C=O			165.3 s		

a) *J* values (Hz) determined are shown in parentheses.

Table 22 ^{13}C (100 MHz) and ^{1}H NMR (400 MHz) spectroscopic data for (**2**) and (**3**)

	2 ($CD_3COCD_3 + D_2O$)[a]				**3** ($CD_3COCD_3 + D_2O$)			
	α		β		α		β	
Position	δ_C	δ_H[b]	δ_C	δ_H[b]	δ_C	δ_H[b]	δ_C	δ_H[b]
1	89.9 d	5.11 (d, 3.6)	94.1 d	4.70 (d, 7.7)	91.0 d	5.38 (d, 3.2)	94.2 d	5.07 (d, 8.3)
2	74.4 d	4.83 (t, 9.8)	76.5 d	4.66 (dd, 7.7, 9.2)	74.6 d	4.95 (dd, 3.2, 9.6)	77.1 d	4.75 (dd, 5.5, 9.6)
3	76.5 d	5.23 (t, 9.4)	78.9 d	4.92 (t, 9.2)	74.9 d	5.07 (t, 9.6)	77.3 d	5.37 (t, 9.6)
4	70.9 d	4.81 (t, 9.9)	70.7 d	4.82 (dd, 2.9, 9.9)	69.6 d	4.81 (dd, 9.2, 9.6)	69.4 d	4.77 (dd, 7.2, 9.2)
5	66.5 d	3.26 (br t, 9.0)	72.4 d	2.67 (br t, 9.6)	70.2 d	4.22 (dd, 8.7, 9.2)	74.5 d	3.91 (t, 8.7)
6	64.2 t	2.15 (br d, 10.5)	64.3 t	2.23 (br d, 10.5)	64.3 t	3.13 (d, 11.7)	64.4 t	3.13 (d, 11.7)
		4.12 (dd, 7.6, 10.5)		4.07 (dd, 9.0, 10.5)		4.54 (dd, 8.7, 11.7)		4.50 (dd, 8.7, 11.7)
	4,6-Galloyl (GAL)				Isoterchebuloyl (Ter)			
1					123.8 s		123.9 s	
2					116.0 s		116.2 s	
3					144.6 s		144.6 s	
4					137.4 s		137.4 s	
5					145.2 s		145.1 s	
6		6.69 (s)		6.76 (s)	109.1 d	6.78 (s)	109.2 d	6.75 (s)
7 C=O	168.9 s		168.8 s		167.4 s		167.3 s	
1'					107.8 s		107.9 s	
2'					113.0 s		113.0 s	
3'					136.7 s		136.7 s	
4'					139.2 s		137.5 s	
5'					147.6 s		147.7 s	
6'					124.1 s		126.2 s	
7' C=O	158.5 s		158.5 s		157.5 s		157.6 s	
1"					113.6 s		113.7 s	
2"					113.0 s		113.6 s	
3"					138.8 s		138.6 s	
4"					141.8 s		141.9 s	
5"					150.5 s		150.6 s	
6"					112.6 s		112.5 s	
7" C=O	159.0 s		158.9		159.2 s		159.2 s	
1"'					115.0 s		115.2 s	
2"'					142.4 s		142.4 s	
3"'					138.8 s		138.8 s	
4"'					137.8 s		137.6 s	
5"'					138.8 s		138.7 s	
6"'				7.02 (s)	107.3 d	6.51 (s)	107.4 d	6.53 (s)
7"' C=O	168.4 s		168.7 s		166.1 s		166.2 s	
HHDP								
1					114.5 s		114.5 s	
2					126.6 s		126.5 s	
3		6.57 (s)		6.58 (s)	107.7 d	6.61 (s)	107.6 d	6.61 (s)
4					144.8 s		144.9 s	
5					136.2 s		136.1 s	
6					144.3 s		144.3 s	
7 C=O	168.9 s		168.8 s		168.6 s		168.8 s	
1'					114.2 s		114.3 s	
2'					126.7 s		126.6 s	
3'		6.65 (s)		6.65 (s)	106.7 d	6.35 (s)	106.7 d	6.37 (s)
4'					145.0 s		145.1 s	
5'					135.9 s		136.0 s	
6'					144.3 s		144.4 s	
7' C=O	168.7 s		168.6 s		169.2 s		169.3 s	

a) Other unassigned ^{13}C NMR signals: 107.1 (d), 107.2 (d), 107.3 (2C, d), 108.7 (d), 109.3 (d), 110.5 (s), 110.6 (s), 110.9 (s), 111.5 (2C, d), 113.8 (s), 113.9 (s), 114.1 (s), 114.2 (s), 114.3 (s), 114.4 (s), 114.5 (s), 114.7 (s), 114.8 (s), 117.6 (s), 117.7 (s), 121.4 (s), 121.5 (s), 123.6 (2C, s), 124.8 (s), 124.0 (s), 124.8 (s), 124.5 (s), 126.3 (s), 126.4 (s), 126.5 (2C, s), 126.6 (s), 135.7 (s), 135.8 (s), 135.9 (s), 136.0 (s), 136.3 (s), 136.5 (s), 136.6 (s), 136.7 (s), 137.1 (s), 137.3 (s), 137.4 (s), 138.1 (s), 138.2 (s), 138.7 (s), 138.9 (s), 143.6 (s), 143.7 (s), 143.8 (s), 143.9 (2C, s), 144.0 (s), 144.3 (s), 144.5 (s), 144.7 (s), 144.8 (3C, s), 145.1 (s), 145.2 (2C, s), 145.3 (s), 147.4 (s), 147.5 (s), 147.6 (s), 148.1 (s)

b) *J* values (Hz) determined are shown in parentheses.

Table 23 ^{13}C (100 MHz) and ^{1}H NMR (400 MHz) spectroscopic data for (**4**) and (**6**)

Position	**4** ($CD_3COCD_3 + D_2O$)		**6** (CD_3COCD_3)	
	δ_C	δ_H [a)]	δ_C	δ_H [a)]
1	92.2 d	5.90 (d, 8.2)	92.2 d	6.53 (d, 2.3)
2	71.1 d	3.89	71.1 d	5.49
3	62.0 d	5.36 (t, 9.2)	62.0 d	6.35 (br s)
4	69.0 d	3.92	69.0 d	5.10 (d, 3.7)
5	75.6 d	3.98 (ddd, 1.9, 4.1, 9.6)	75.6 d	4.72
6	64.8 t	4.50 (dd, 4.1, 12.4)	64.8 t	4.77
		4.57 (dd, 1.9, 12.4)		4.88 (dd, 7.3, 10.6)
Chebuloyl (Che)				
2			169.5 s	
3			66.6 d	4.97 (d, 7.3)
4			41.2 d	5.19 (dd, 1.4, 7.3)
4a			116.1 s	
5			119.1 s	
6			117.1 d	7.56 (s)
7			146.5 s	
8			139.5 s	
8a			141.0 s	
1' C=O			173.7 s	
2'			39.4 d	3.96 (ddd, 1.4, 1.6, 10.4)
3'			30.2 d	2.27 (2H)
4' C=O			172.6 s	
1'' C=O			165.1 s	
Galloyl (Gal)				
1	120.2 s		120.2 s	
2	110.4 d	7.15 (s)	110.4 d	7.23 (s)
3	146.0 s		146.0 s	
4	139.7 s		139.7 s	
5	146.0 s		146.0 s	
6	110.4 d	7.15 (s)	110.4 d	7.23 (s)
7 C=O	164.9 s		164.9 s	
Galloyl (Gal)				
1	120.0 s		120.0 s	
2	110.4 d	7.18 (s)	110.4 d	7.29 (s)
3	146.1 s		146.1 s	
4	139.8 s		139.8 s	
5	146.1 s		146.1 s	
6	110.4 d	7.18 (s)	110.4 d	7.29 (s)
7 C=O	164.8 s		164.8 s	
Galloyl (Gal)				
1	120.0 s		120.0 s	
2	109.9 d	7.19 (s)	109.9 d	7.07 (s)
3	145.9 s		145.9 s	
4	139.0 s		139.0 s	
5	145.9 s		145.9 s	
6	109.9 d	7.19 (s)	109.9 d	7.07 (s)
7 C=O	166.4 s		166.4 s	

a) *J* values (Hz) determined are shown in parentheses.

The phenolic compounds embrace a considerable range of substances that possess a central core of polyhydric alcohol such as glucose, and hydroxyl groups, which are esterified either partially or wholly by gallic acid (gallotannins) or by hexahydroxy-diphenic acid (HHDP) and other substituents. In this study, 6 phenolic compounds have been isolated from *T. chebula* galls which have never been reported before. Their structures were identified as a simple molecule which is gallic acid (**1**) and more complicated higher molecular weight compounds including punicalagin (**2**), isoterchebulin (**3**), 1,3,6-tri-*O*-galloyl-β-D-glucopyranose (**4**), chebulagic acid (**5**) and chebulinic acid (**6**) (**Figure 43**).

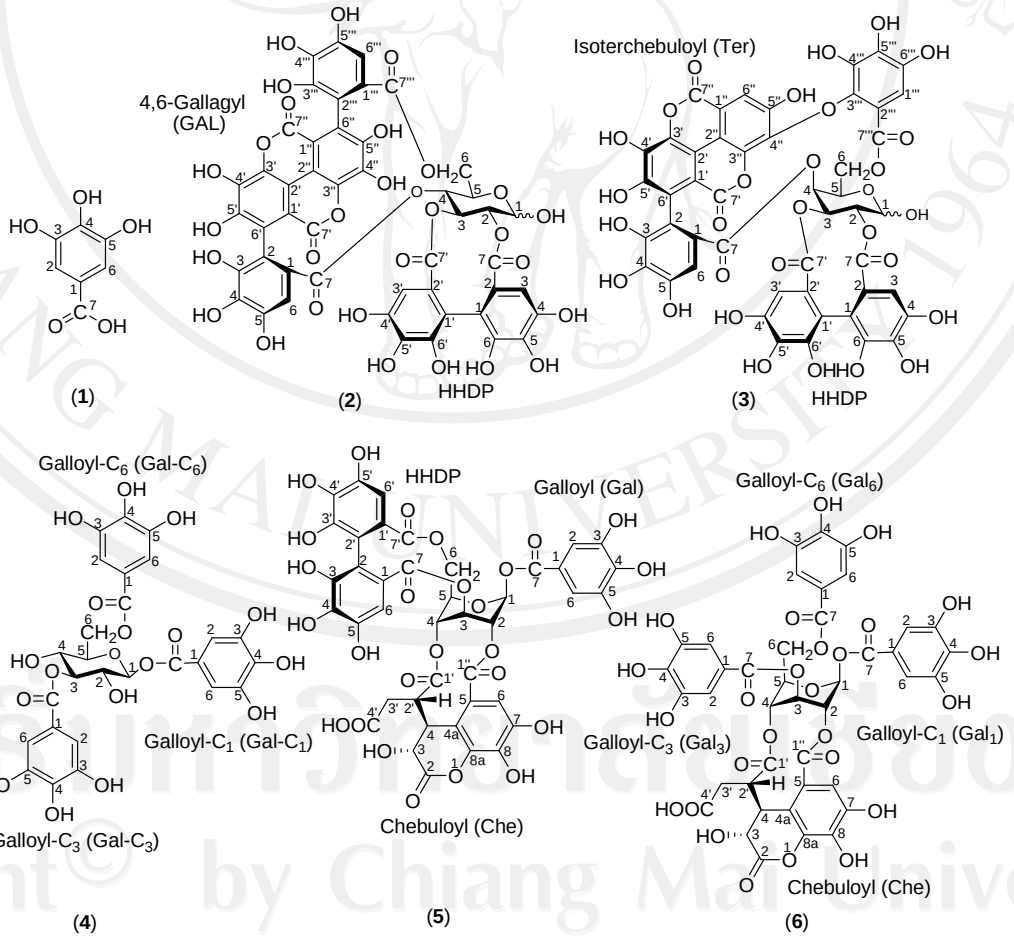


Figure 43 The chemical structures of the 6 isolated compounds from *T. chebula* galls (HHDP: hexahydroxydiphenoyl)

3.3.2 Biological assays

3.3.2.1 Free radical scavenging assay

In previous study, the cold aqueous extract of *T.chebula* galls exhibited the highest scavenging activity among the 60 extracts from the 15 medicinal plants with the SC_{50} value of 0.016 mg/ml, which were 2.37 and 3.09 times more potent than *α*-tocopherol (SC_{50} value of 0.038 mg/ml) and BHT (SC_{50} value of 0.049 mg/ml), respectively (Manosroi et al., 2010a). All fractions (Diaion fr., fr.1, 2 and 3) exhibited stronger scavenging activity on DPPH radicals, with the SC_{50} values of 0.96, 1.06, 1.08 and 1.07 µg/ml, respectively, than ascorbic acid, α-tocopherol and BHT (SC_{50} values of 4.30, 5.11 and 3.93 µg/ml, respectively). The values of the positive controls were less than our previous study of about 10 times because of the different experimental conditions (Akazawa et al., 2006 and, Tachibana et al., 2001), especially the final concentrations of DPPH in the assay systems. These two methods were different in solvent used (DMSO/methanol and 20% DMSO), DPPH concentrations (500/1200 µM), reaction mixture (10 µl of the test solution, 200 µl of EtOH, 190 µl of 0.1 M acetate buffer and 100 µl DPPH solution/50 µl of the test solution and 50 µl DPPH solution), the final concentration of DPPH solution (100/600 µM), the incubation temperature (30/25°C) and the wavelength of absorbance measurement (517/515 nm). The DPPH radical scavenging activity of the phenolic compounds isolated from *T.chebula* galls in comparison to the reference compounds were shown in **Table 24**. All isolated compounds showed higher DPPH radical scavenging activity than ascorbic acid, α-tocopherol and BHT which gave the SC_{50} value of 24.41,

11.86 and 17.83 μM, respectively. Chebulinic acid (**6**) which has 3 galloyl with 1 chebuloyl groups (1 group of *ortho*-dihydroxy) exhibited the highest DPPH radical scavenging activity (SC_{50} = 0.94 μM), whereas gallic acid (**1**) showed the lowest activity (SC_{50} = 5.17 μM). Ascorbic acid which contained no phenolic hydroxyl group gave low DPPH radical scavenging activity. From the structures of the 6 isolated phenolic compounds, galloyl and *ortho*-dihydroxyl groups may be related to the DPPH radical scavenging activity. In fact, free radical scavenging activity of phenolic compounds depends on the numbers and positions of the hydroxyl (–OH) groups, especially at the *ortho* position and with more hydroxyl groups such as dihydroxyl and galloyl groups (Bouchet et al., 1998). It has been reported that more hydroxyl groups give high radical scavenging activity because of increasing H-donating ability (Rice- Evans et al., 1996; Natella et al., 1999).

Table 24 DPPH radical scavenging activity of 6 phenolic compounds isolated from *T.chebula* galls

Compounds	SC_{50} (μM)
Gallic acid (**1**)	5.17
Punicalagin (**2**)	1.00
Isoterchebulin (**3**)	0.97
1,3,6-tri-*O*-Galloyl-ß-D-glucopyranose (**4**)	1.07
Chebulagic acid (**5**)	3.54
Chebulinic acid (**6**)	0.94
Ascorbic acid	24.41
α-Tocopherol	11.86
BHT	17.83

3.3.2.2 Tyrosinase and α-MSH induced melanin production on B16 murine melanoma inhibition assay

From our previous mushroom tyrosinase assay, we have found that darker solution of the mixture was obtained with longer incubation period. However, the inhibition value was calculated from the following equation: Tyrosinase inhibition (%) = 100 – (A_{sample}/ $A_{control}$) × 100. Therefore, there will be no significant difference of tyrosinase inhibition between different incubation times. Many research workers, e.g. Nerya et al (2003) have also evaluated the tyrosinase inhibition at 5 minutes the same as our study. All isolated compounds showed IC_{50} values of tyrosinase inhibition activity more than 500 μM. Chebulagic (**5**) and chebulinic acids (**6**) showed the percentages of tyrosinase inhibition activity at 500 μM of 28.8 ± 0.41 and 46.8 ± 2.86%, respectively, which were less than arbutin (IC_{50} values = 170.0 μM) and kojic acid (IC_{50} values = 16.2 μM) of 54.7 ± 1.08 and 66.4 ± 0.22%, respectively. To confirm the inhibition activity of mushroom tyrosinase reaction, the inhibition of α-MSH induced melanin production on B16 murine melanoma cell line has also performed. All isolated compounds showed less melanin content on B16 murine melanoma cells with slightly less cell viability than kojic acid and arbutin (**Table 25**). Punicalagin **(2)** showed the highest inhibition acitivity of α-MSH induced melanin production on B16 murine melanoma among all test samples. All isolated compounds from *T. chebula* galls may not directly inhibit tyrosinase enzyme, but inhibit other pathways during the melanin synthesis in the cells. Ellagic acid, which was the substitute group in isoterchebulin (**3**) and punicalagin (**2**), has been shown to give

depigmentation by interacting with the copper ions at the tyrosinase active site (Briganti et al., 2003). A correlation between melanogenesis and reactive oxygen species (ROS) has also been reported by Yasui and Sakurai, 2003. Oxidative stress which may be induced by increasing the generation of ROS and other free radicals in the skin, can promote melanin biosynthesis and DNA damage by inducing the proliferation of melanocytes. Compounds which can scavenge free radicals have also been reported to have the depigmentation effects by reducing hyperpigmentation via scavenging the ROS generated in the cells from UV or α-MSH exposure (Karg et al., 1993; Ma et al., 2001).

Table 25 Melanogenesis inhibition on B16 murine melanoma cells of 6 phenolic compounds isolated from *T.chebula* galls

Compounds[a)]	**Melanogenesis inhibition and cytotoxicity on B16 cells**		**Correlation ratio**[b)]
	Mean ± SD (%)		
	Melanin contents	**Cell viability**	
Gallic acid (**1**)	68.8 ± 3.83	85.7 ± 9.22	0.80
Punicalagin (**2**)	58.9 ± 4.65	81.6 ± 5.82	0.72
Isoterchebulin (**3**)	75.9 ± 4.15	89.8 ± 3.88	0.85
1,3,6-tri-*O*-Galloyl-ß-D-glucopyranose (**4**)	79.0 ± 3.87	92.8 ± 5.24	0.85
Chebulagic acid (**5**)	60.1 ± 1.99	80.5 ± 5.19	0.74
Chebulinic acid **(6**)	69.0 ± 4.45	80.6 ± 3.07	0.86
Arbutin	90.5 ± 3.61	98.3 ± 3.11	0.92
Kojic acid	91.5 ± 1.99	103.4 ± 6.53	0.88

a) All compounds were at the concentration of 10 μM

b) The correlation ratio was obtained from the melanin contents divided by the number of cell viability

3.3.2.3 Cytotoxic activity on human tumor cell lines by MTT assay

The cytotoxic activities (EC_{50} values) of all isolated compounds on human tumor cell lines were in the range of 19.3 to >100 μM (**Table 26**). All compounds exhibited cytotoxic effects, but less than cisplatin in all human tumor cell lines (HL60, A549, AZ521 and SK-BR-3). Gallic acid (**1**) gave the highest cytotoxic effect in HL60, AZ521 and SK-BR-3 cell lines, while isoterchebulin (**3**) and 1,3,6-tri-*O*-galloyl-β-D-glucopyranose (**4**) showed the lowest activity in all tumor cells. Gallic acid, a naturally occurring polyphenol, is obtained by the hydrolysis of tannins in plants. It is a well known

Table 26 Cytotoxic activity (EC_{50}) on four human tumor cell lines of the 6 phenolic compounds isolated from *T.chebula* galls

Compounds	Cytotoxicity EC_{50} (μM)			
	HL60	**A549**	**AZ521**	**SK-BR-3**
Gallic acid (**1**)	19.3	-	71.3	43.5
Punicalagin (**2**)	30.5	-	-	-
Isoterchebulin (**3**)	-	-	-	-
1,3,6-tri-*O*-Galloyl-β-D-glucopyranose (**4**)	-	-	-	-
Chebulagic acid (**5**)	26.8	-	93.2	-
Chebulinic acid **(6)**	36.3	-	-	-
Cisplatin[a)]	1.9	24.9	5.1	13.7
5-Fluorouracil[a)]	9.5	-	11.3	-

Note: a) = Reference compound, - = EC_{50} more than 100 μM

antioxidant, antimutagenic, cytotoxic and anticarcinogenic agent in a variety of *in vivo* and *in vitro* studies. Its three adjacent hydroxyl groups are believed to be responsible for its cytotoxic potential (Saleem et al., 2002). Lee et al. (1995) have demonstrated the moderate cytotoxicity against lung carcinoma

(A549) of the methanolic extract of *T. chebula* fruit and the four isolated compounds including gallic acid (**1**), 1,2,3,4,6-penta-*O*-galloyl-β-D-gulcopyranose, chebulagic acid (**5**), and chebulinic acid (**6**). Chebulinic acid (**6**) has been reported to exhibit higher cytotoxic activity than gallic acid (**1**) while this study indicated almost the same cytotoxicity effect as gallic acid (>100 μM) on A549.

3.3.2.4 Nitric oxide (NO) production from macrophages stimulated by LPS

The inorganic free nitric oxide (NO) has been implicated in physiological and pathological processes, such as vasodilation, nonspecific host defense, ischemia reperfusion injury, and chronic or acute inflammation. However, all isolated compounds except gallic acid (**1**) which were glycosides core (**Figure 43**) showed weak or no activity (>100 μM) in NO production inhibition assay with no toxicity on RAW264.7 macrophage cells. Gallic acid (**1**) can inhibit NO production with the IC_{50} value of 14.0 μM which was higher than the reference compound, L-NMMA (IC_{50} value 32.1 μM). This NO production inhibition activity result has agreed with the report of Tao et al. (2002). Glycosides such as stilbene glycosides showed less activity than their corresponding aglycons. This is due to the less permeability of the glycosides which have the hydrophilic nature to the cell membranes thereby being difficult to reach to the active site (Tao et al., 2002). Thus, the inhibitory activities of gallic acid against NO production in LPS-activated macrophages appeared to support the traditional use of *T. chebula* galls for the treatment of inflammation in many Thai medicinal plant recipes.

3.4 Entrapment of the semi-purified fraction and gallic acid in the selected niosomal formulation

3.4.1 Total phenolic contents and identification of the phenolic compounds in the selected semi-purified fraction

The eluted solvent and the yield of each fraction were as followed: fr.1: MeOH/H_2O/AcOH (2:8:0.1) (0.54 g), fr.2: MeOH/H_2O/AcOH (4:6:0.1) (0.74 g) and fr.3: MeOH/H_2O/AcOH (6:4:0.1) (0.50 g). The sample was dissolved by 10% solvent-A. The compounds which were isolated from the cold water crude extract of *T. chebula* galls including gallic acid, 1,3,6-tri-*O*-galloyl-β-D-glucopyranose, punicalagin, isoterchebulin, chebulagic acid and chebulinic acid were eluted at 9.2, 13.7, 16.5, 30.1, 34.5, 41.9 min, respectively (Manosroi et al., 2010b). After fractionation of the cold aqueous crude extract of *T.chebula* galls by Diaion column and ODS column chromatography, the semi purifed MeOH/H_2O/AcOH (2:8:0.1) fraction which gave the highest total phenolic contents of 21.43 ± 1.31 mg GAE/g (**Table 27**) was selected to load in niosomes. A gradient HPLC chromatogram of this selected semi-purified fraction was shown in **Figure 44**. The peaks were identified by comparing the retention times with the isolated phenolic compounds (Manosroi et al., 2010b). The contents of gallic acid, 1,3,6-tri-*O*-galloyl-β-D-glucopyranose and punicalagin in the semi-purified fraction calculated using area under the peak were found at 15.3, 17.8 and 38.4%, respectively.

3.4.2 Characteristics of niosomes

The mean size of niosomes, entrapment efficiency, zeta potential and deformability index were demonstrated in **Table 28**. A decrease in vesicular size was observed when 25% ethanol was incorporated. It has been reported that the higher ethanol

concentration in the elastic vesicles, the lesser membrane thickness was observed owing to the formation of a phase with interpenetrating hydrocarbon chains (Dubey et al., 2007; Barry and Cullis 1995). Ethanol may also modify the net negative charge of the system leading to a decrease in the mean particle size of elastic niosomes (Lasic et al., 1998). Both elastic and non-elastic niosomes loaded with gallic acid or semi-purified fraction gave larger mean sizes than the unloaded (blank) niosomes. All loaded niosomal formulations exhibited negative zeta potential value, larger size and higher negative zeta potential value than the unloaded (blank) vesicles. In the buffer system, the vesicles such as niosomes are surrounded by the counter ions which are opposite charges to the surface charges of niosomes (McLaughlin et al., 1971). The partial positive charge distribution of cholesterol molecules in the niosomal composition may be neutralized and dominated by the phosphate ions of the buffer system resulting in the negative zeta potential values. Visualized by negative staining

Table 27 Percentage yields and total phenolic contents of fractions by column chromatography from the cold aqueous *T. chebula* gall crude extract

Column	Fraction No.	Eluting solvent	Eluting solvent ratio	% yield of the fraction	Total phenolic contents (mg GAE/g)
Dianion	-	MeOH/H_2O	1:1	34.76	36.90 ± 0.37
ODS	F1	MeOH/H_2O/AcOH	2:8:0.1	6.75	21.43 ± 1.31
	F2	MeOH/H_2O/AcOH	4:6:0.1	9.25	7.67 ± 2.98
	F3	MeOH/H_2O/AcOH	6:4:0.1	6.25	2.81 ± 0.06

Note: The total phenolic content was expressed in the term of mg of gallic acid equivalents (GAE) per 100 g of the crude extract. The calibration equation for gallic acid was $y = 7.8829x + 0.0477$ ($r^2 = 0.9994$)

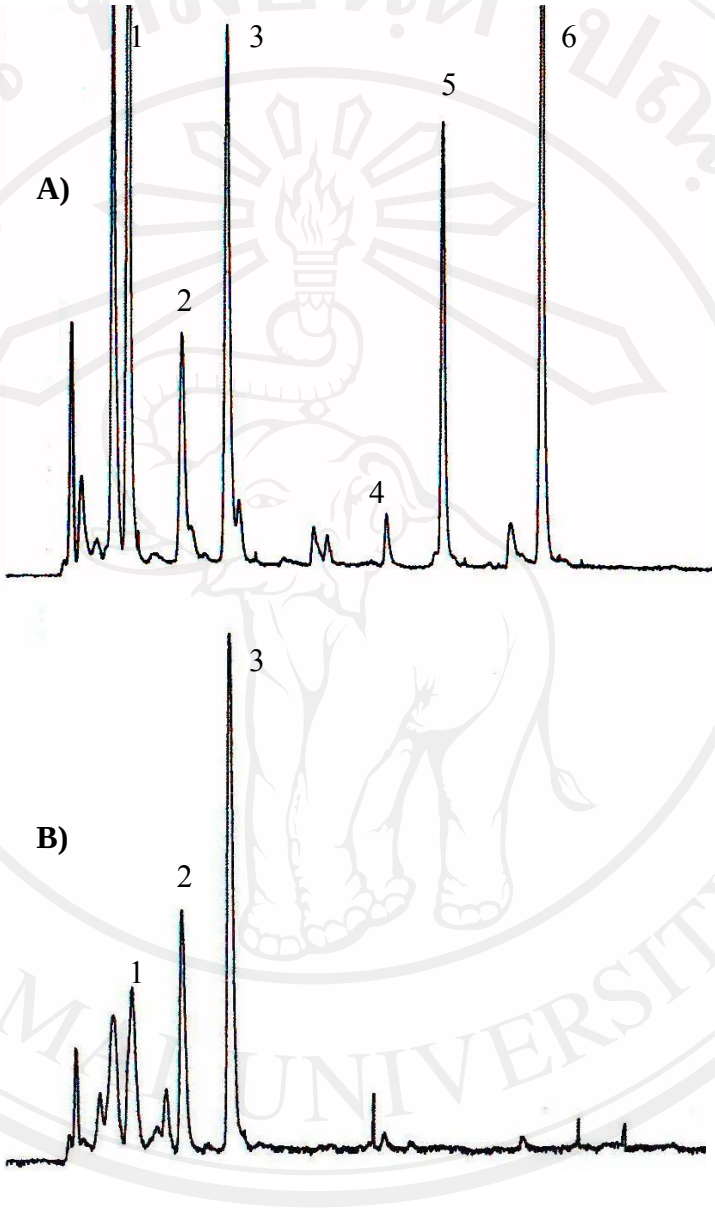


Figure 44 The gradient HPLC chromatogram of A) cold water crude extract of *T. chebula* galls containing phenolic compounds including gallic acid (**1**), 1,3,6-tri-*O*-galloyl-β-D-glucopyranose (**2**), punicalagin (**3**), isoterchebulin (**4**), chebulagic acid (**5**) and chebulinic acid (**6**) and B) the semi-purified fraction

of TEM, both non-elastic and elastic niosomes loaded with gallic acid or the semi-purified fraction was in the mixture of unilamellar and multilamellar structures (**Figure 45**). The entrapment efficiencies of pure gallic acid and gallic acid in the semi-purified fraction in elastic niosomes (55.18 ± 3.87 and 24.34 ± 1.53 %) were higher than in non-elastic niosomes (29.72 ± 1.68 and 20.10 ± 3.91%) of about 1.86 and 1.21 times, respectively. This may be due to an increasing solubility in the niosomal membrane of gallic acid and semi-purified fraction by ethanol containing in the elastic niosomes thereby facilitating their entrapment in the vesicles.

For vesicular deformability, the elastic niosomes loaded with pure gallic acid (10.98 ± 2.75) or semi-purified fraction (10.75 ± 3.47) showed similar DI to the unloaded (blank) elastic niosomes (11.36 ± 1.55), while higher than the non-elastic niosomes loaded with gallic acid (2.50 ± 0.78) and the semi-purified fraction (2.04 ± 1.13) of about 4.39 and 5.27 times, respectively (**Table 28**). Ethanol in the elastic niosomes may interact with the polar head group region of the surfactant molecules (Tween 61) resulting in the reduction of the melting point, thereby increasing the fluidity of the vesicles (Touitou et al., 2000; Dayan and Touitou 2000). However, non-elastic niosomes also showed some deformability. In fact, a proper amount of surfactant molecule within the lipid bilayer can provoke a disruption and fluidization of the bilayers (Touitou et al., 2000). Both elastic and non-elastic niosomes, indicated slight different of DI when loaded with gallic acid or the semi-purified fraction (**Table 28**), indicating that the loaded compounds did not have any effects on the vesicular membrane elasticity, since the polar hydroxyl groups of gallic acid and other phenolic compounds in the semi-purified fraction were located in the aqueous inner core, but not in the niosomal membranes.

Table 28 Size, entrapment efficiency, zeta potential and deformability index (DI) of non-elastic and elastic niosomes loaded with gallic acid or the semi-purified fraction containing gallic acid

Formulations	Initial size (nm)	Size after filtration (nm)	% Entrapment efficiency of gallic acid	Zeta potential	Deformability index (DI)
Non-elastic niosomes					
blank	214.40 ± 10.41	85.71 ± 11.91	-	-38.50 ± 2.58	2.79 ± 0.34
loaded with gallic acid	235.07 ± 7.43	47.45 ± 6.72	29.72 ± 1.68	-46.64 ± 0.78	2.50 ± 0.78
loaded with the semi-purified fraction	395.95 ± 31.69	77.00 ± 13.18	20.10 ± 3.91	-47.11 ± 0.73	2.04 ± 1.13
Elastic niosomes					
blank	146.57 ± 11.19	139.22 ± 21.18	-	-32.76 ± 1.13	11.36 ± 1.55
loaded with gallic acid	171.15 ± 4.03	115.79 ± 3.38	55.18 ± 3.87	-35.55 ± 0.31	10.98 ± 2.75
loaded with the semi-purified fraction	230.13 ± 56.69	96.08 ± 32.88	24.34 ± 1.53	-44.56 ± 1.89	10.75 ± 3.47

Values represent mean ± SD. (n = 3)

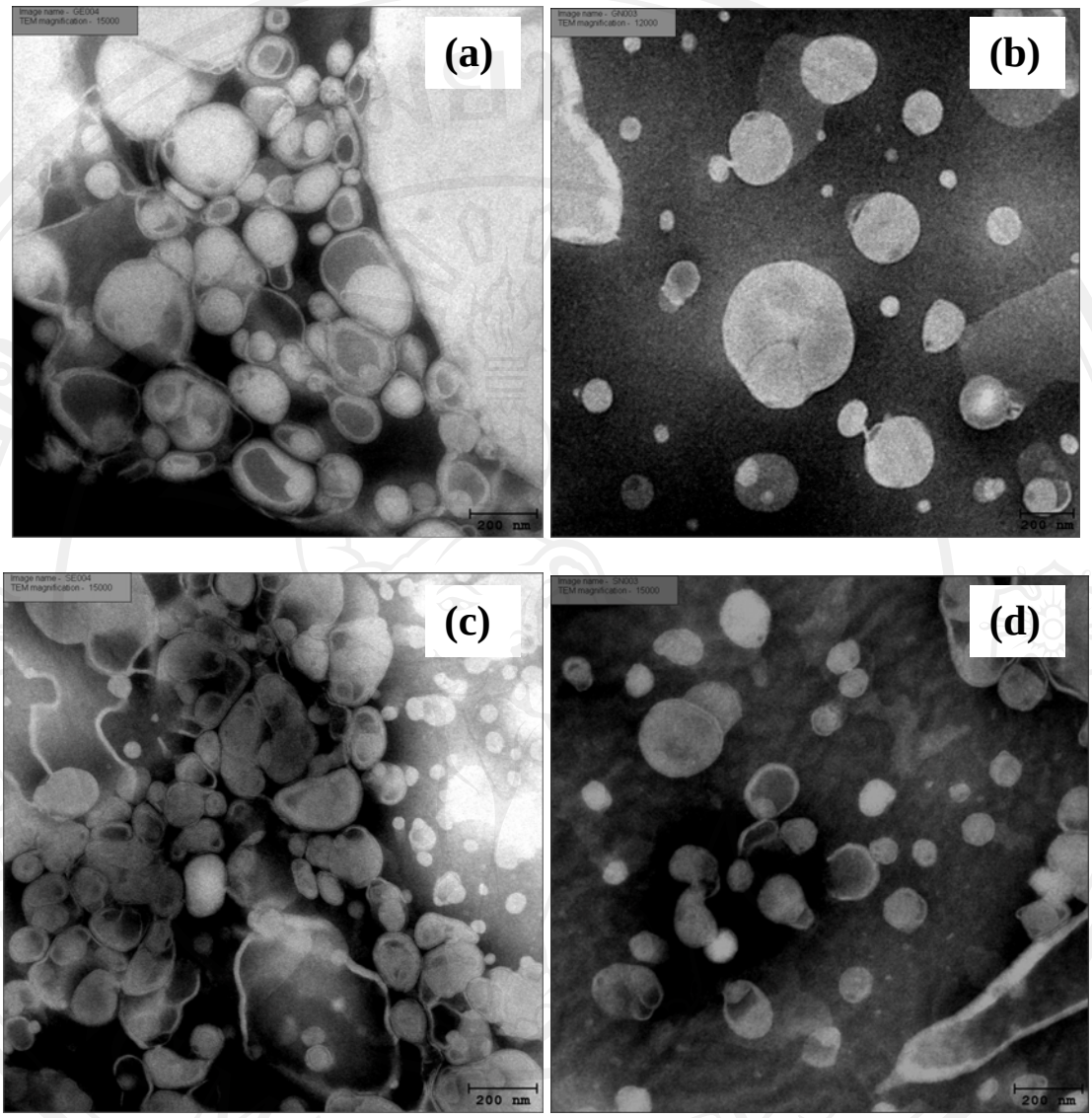


Figure 45 Negative-staining TEM images of elastic and non-elastic niosomes (Tween 61 mixed with cholesterol at 1:1 molar ratio, 20 mM) loaded with gallic acid and the semi-purified fraction containing gallic acid: (a) elastic niosomes loaded with gallic acid (GE) (15,000×); (b) non-elastic niosomes loaded with gallic acid (GN) (12,000×); (c) elastic niosomes loaded with the semi-purified fraction (SE) (15,000×) and (d) non-elastic niosomes loaded with the semi-purified fraction (SN) (15,000×)

Figure 46 showed the percentage remaining of gallic acid in various formulations at different storage temperatures for 3 months. Stored at room temperature (27 ± 2°C) and 45 ± 2°C for 3 months, both pure gallic acid (GS) and gallic acid in the semi-purified fraction (SS) in buffer solution were not only physical unstable with color change to brownish, but also the percentages of gallic acid remaining were less than 45 and 30% at room temperature (27 ± 2°C) and 45 ± 2°C, respectively. However, at 4 ± 2°C, the percentages of gallic acid remaining after 3 months of GS and SS were more than 60%. The increased temperature showed the less amounts of the remained gallic acid. These may be due to the decarboxylation of gallic acid to pyrogallol at high temperature (Jennifer et al., 1998). When gallic acid or the semi-purified fraction containing gallic acid was loaded in elastic (GE and SE) and non-elastic niosomes (GN and SN), they showed no sedimentation, no layer separation and no color change with higher percentages of gallic acid remaining at all temperatures for 3 months. The percentages of gallic acid remaining after 3 months of GE, SE, GN and SN were 62.87, 64.55, 72.09 and 75.99; 74.32, 75.99, 83.00 and 85.29; 40.14, 43.54, 48.89 and 49.98% at room temperature (27 ± 2°C), 4 ± 2°C and 45 ± 2°C, respectively. The percentages of gallic acid remaining in non-elastic niosomes were higher than elastic niosomes of about 1.12–1.21 times. This may be due to the evaporation of ethanol at elevated temperature during storage which may fluidize the vesicular membrane and facilitate the leakage of gallic acid from the elastic vesicles. In all systems, the percentages remaining of gallic acid in the semi-purified fraction which contained the mixture of phenolic compounds including gallic acid were slightly higher than the pure gallic acid. The polyphenol pro-oxidant activity of other phytochemicals in the

semi-purified fraction, such as punicalagin may have some degradation protection for gallic acid (Rice-Evans et al., 1996; Halliwell, 2008).

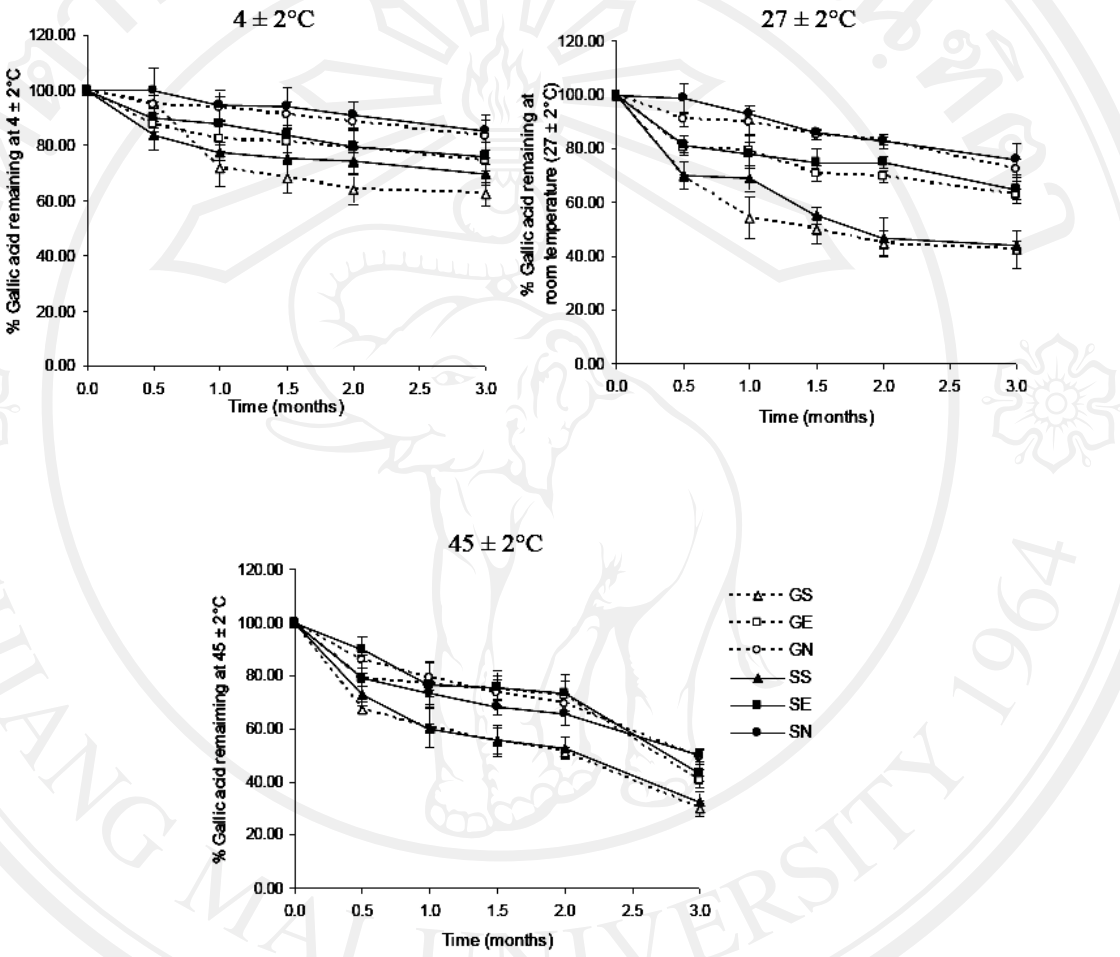


Figure 46 The percentages of gallic acid remaining in various formulations at different storage temperatures (27 ± 2°C, 4 ± 2°C and 45 ± 2°C) for 3 months (GS = gallic acid in phosphate buffer solution, GE = elastic niosomes loaded with gallic acid, GN = non-elastic niosomes loaded with gallic acid, SS = the semi-purified fraction in phosphate buffer solution, SE = elastic niosomes loaded with the semi-purified fraction, SN = non-elastic niosomes loaded with the semi-purified fraction)

3.5 Preparation of gel formulations containing bioactive compounds

3.5.1 Preparation of gel base formulations

3.5.1.1 Physical stability determination and selection of the gel base formulations

The compositions of gel base formulations and physical appearances (color, odor, texture and pH) of gel base formulations kept at 4 ± 2, 27 ± 2 and 45 ± 2°C for 3 months were shown in **Tables 29** and **30**, respectively. After 3 months, Carbopol® 980 gel showed the best physical appearances with only the tiny changes of pH values were observed. Thus, Carbopol® 980 gel base formulation was selected in incorporate with the niosomes.

Table 29 The compositions of gel base formulations

Formulation Nos.	Composition	%
1	Methyl cellulose	1.00
	Propylene glycol	10.00
	Conc. paraben	1.00
	Water	88.00
2	Sodium carboxy methyl cellulose	1.50
	Propylene glycol	10.00
	Conc. paraben	1.00
	Water	87.50
3	Carbopol® 934	0.60
	Propylene glycol	10.00
	99% triethanolamine	0.40
	Conc. paraben	1.00
	Water	89.00
4	Carbopol® 980	0.60
	Propylene glycol	10.00
	99% triethanolamine	0.40
	Conc. paraben	1.00
	Water	89.00

Table 30 Physical appearances and characteristics (color, odor, texture and pH) of the four gel base formulations kept at 4 ± 2, 27 ± 2 and 45 ± 2°C for 3 months

Formulation Nos	At initial	1 month	2 months	3 months
1. (Methyl cellulose gel base)				
4 ± 2°C	transparence, odorless, rough texture, pH = 5.20	transparence, odorless, rough texture, pH = 5.20	transparence, odorless, rough texture, pH = 5.11	transparence, slight smell of oil, rough texture, pH = 5.00
27 ± 2°C	transparence, odorless, rough texture, pH = 5.20	transparence, odorless, rough texture, pH = 5.20	transparence, odorless, rough texture, pH = 5.17	transparence, slight smell of oil, rough texture, pH = 4.95
45 ± 2°C	transparence, odorless, rough texture, pH = 5.20	transparence, slight smell of oil, rough texture, pH = 5.10	transparence, slight smell of oil, rough texture, pH = 5.00	transparence, slight smell of oil, rough texture, pH = 4.88
2. (Sodium carboxy methyl cellulose gel base)				
4 ± 2°C	transparence, odorless, smooth texture, pH = 5.00	transparence, odorless, smooth texture, pH = 5.00	transparence, odorless, smooth texture, pH = 5.00	transparence, odorless, smooth texture, pH = 5.00
27 ± 2°C	transparence, odorless, smooth texture, pH = 5.00	transparence, odorless, smooth texture, pH = 5.00	transparence, odorless, smooth texture, pH = 5.05	transparence, odorless, smooth texture, pH = 5.11
45 ± 2°C	transparence, odorless, smooth texture, pH = 5.00	transparence, odorless, smooth texture, pH = 5.00	transparence, odorless, rough texture, pH = 5.35	transparence, odorless, rough texture, pH = 5.50

Table 30 Physical appearances and characteristics (color, odor, texture and pH) of the four gel base formulations kept at 4 ± 2, 27 ± 2 and 45 ± 2°C for 3 months (continued)

Formulations	At initial	1 month	2 months	3 months
3. (Carbopol® 934 gel base)				
4 ± 2°C	transparence, odorless, rough texture, pH = 6.12	transparence, odorless, rough texture, pH = 6.00	transparence, odorless, rough texture, pH = 6.00	transparence, slight smell of oil, rough texture, pH = 5.88
27 ± 2°C	transparence, odorless, rough texture, pH = 6.12	transparence, odorless, rough texture, pH = 5.88	transparence, slight smell of oil, rough texture, pH = 5.85	transparence, slight smell, rough texture, pH = 5.83
45 ± 2°C	transparence, odorless, rough texture, pH = 6.12	transparence, slight smell of oil, rough texture, pH = 5.50	transparence, slight smell of oil, rough texture, pH = 5.50	transparence, slight smell of oil, rough texture, pH = 5.37
4. (Carbopol® 980 gel base)				
4 ± 2°C	transparence, odorless, smooth texture, pH = 5.00	transparence, odorless, smooth texture, pH = 5.00	transparence, odorless, smooth texture, pH = 5.00	transparence, odorless, smooth texture, pH = 5.00
27 ± 2°C	transparence, odorless, smooth texture, pH = 5.00	transparence, odorless, smooth texture, pH = 5.00	transparence, odorless, smooth texture, pH = 5.00	transparence, odorless, smooth texture, pH = 5.07
45 ± 2°C	transparence, odorless, smooth texture, pH = 5.00	transparence, odorless, smooth texture, pH = 5.00	transparence, odorless, smooth texture, pH = 5.10	transparence, odorless, smooth texture, pH = 5.10

3.5.2 Preparation of gel containing the semi-purified fraction loaded in elastic niosomes

3.5.2.1 Physical stability

The gel formulations incorporated with the elastic and non-elastic niosomes loaded with gallic acid or semi-purified fraction from *T. chebula* galls extract gave good physical stability with no sedimentation, no layer separation and no color change at all temperatures (4, 27 and 45°C) for 3 months. **Figure 47** showed negative-staining TEM images of various gel formulations stored at 3 different temperatures (27 ± 2, 4 ± 2 and 45 ± 2°C) at 3 months.

3.5.2.2 *Chemical stability*

Figure 48 showed the percentages remaining of gallic acid in various gel formulations at different storage temperatures for 3 months. At all temperatures, gallic acid or the semi-purified fraction containing gallic acid loaded in elastic and non-elastic niosomes and incorporated in gel formulations (GE and SE; GN and SN, respectively) showed higher percentages of gallic acid than those unloaded in niosomes and incorporated in gel (GS and SS). The percentages of gallic acid remaining in GE, SE, GN and SN were 83.00, 85.29, 84.32 and 85.99; 76.80, 75.99, 77.09 and 79.54; 67.74, 68.54, 68.89 and 69.98% when stored for 3 months at 4 ± 2, 27 ± 2 and 45 ± 2°C, respectively. The percentages of gallic acid remaining after 3 months in all gel formulations stored at higher temperatures were less than at lower temperature owing to the decarboxylation of gallic acid to pyrogallol at high temperature (Jennifer et al., 1998). However, the percentages of gallic acid remaining in elastic niosomes were slightly lower than those in the non-elastic niosomes but not significant. This may be due to the evaporation of ethanol at elevated temperature

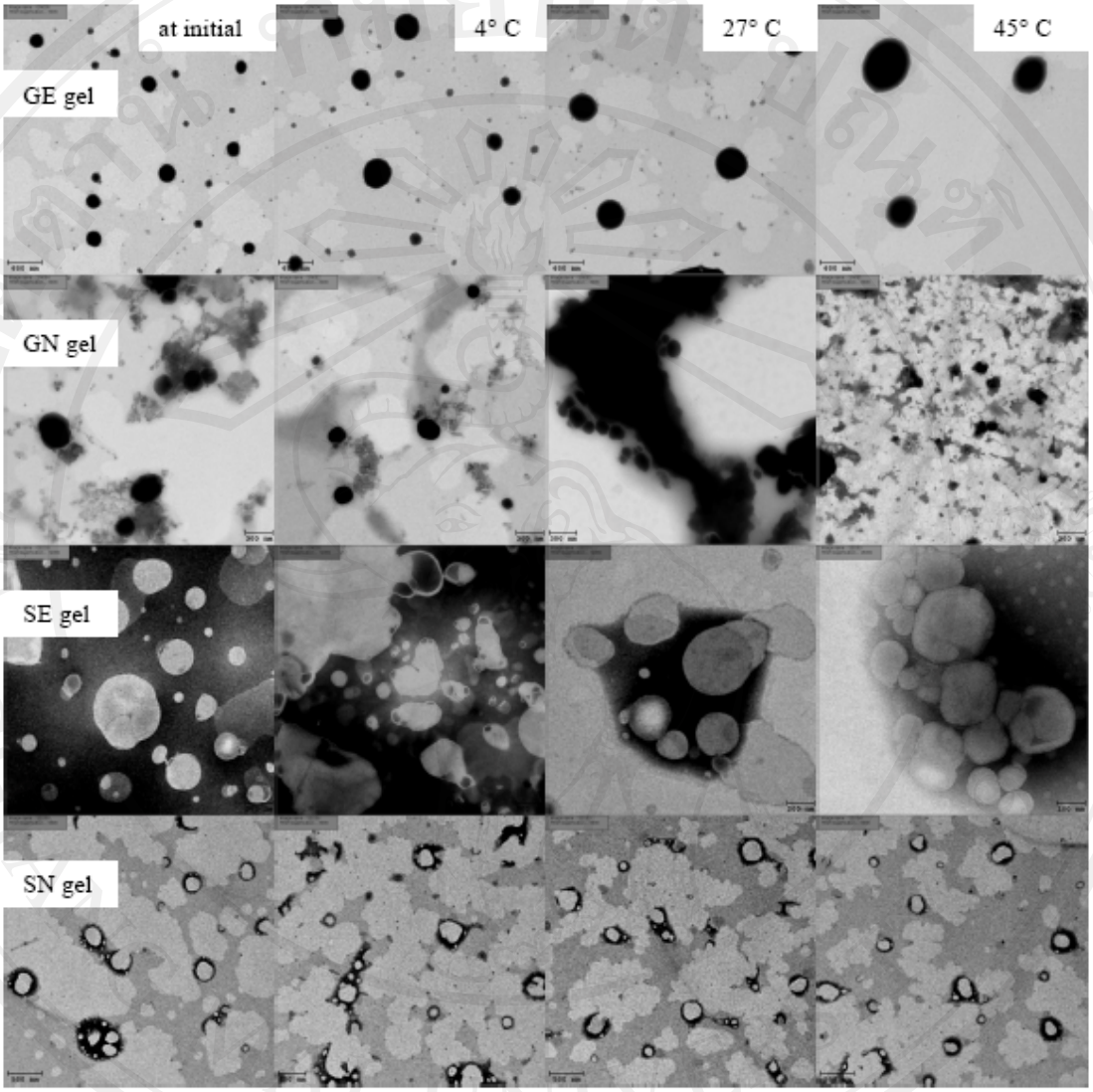


Figure 47 Negative-staining TEM images of gels containing elastic and non-elastic niosomes (Tween 61 mixed with cholesterol at 1:1 molar ratio, 20 mM) loaded with gallic acid (0.5% w/w) and the semi-purified fraction containing gallic acid (0.08% w/w): (a) elastic niosomes loaded with gallic acid (GE) (15,000×); (b) non-elastic niosomes loaded with gallic acid (GN) (12,000×); (c) elastic niosomes loaded with the semi-purified fraction (SE) (15,000×) and (d) non-elastic niosomes loaded with the semi-purified fraction (SN) (15,000×)

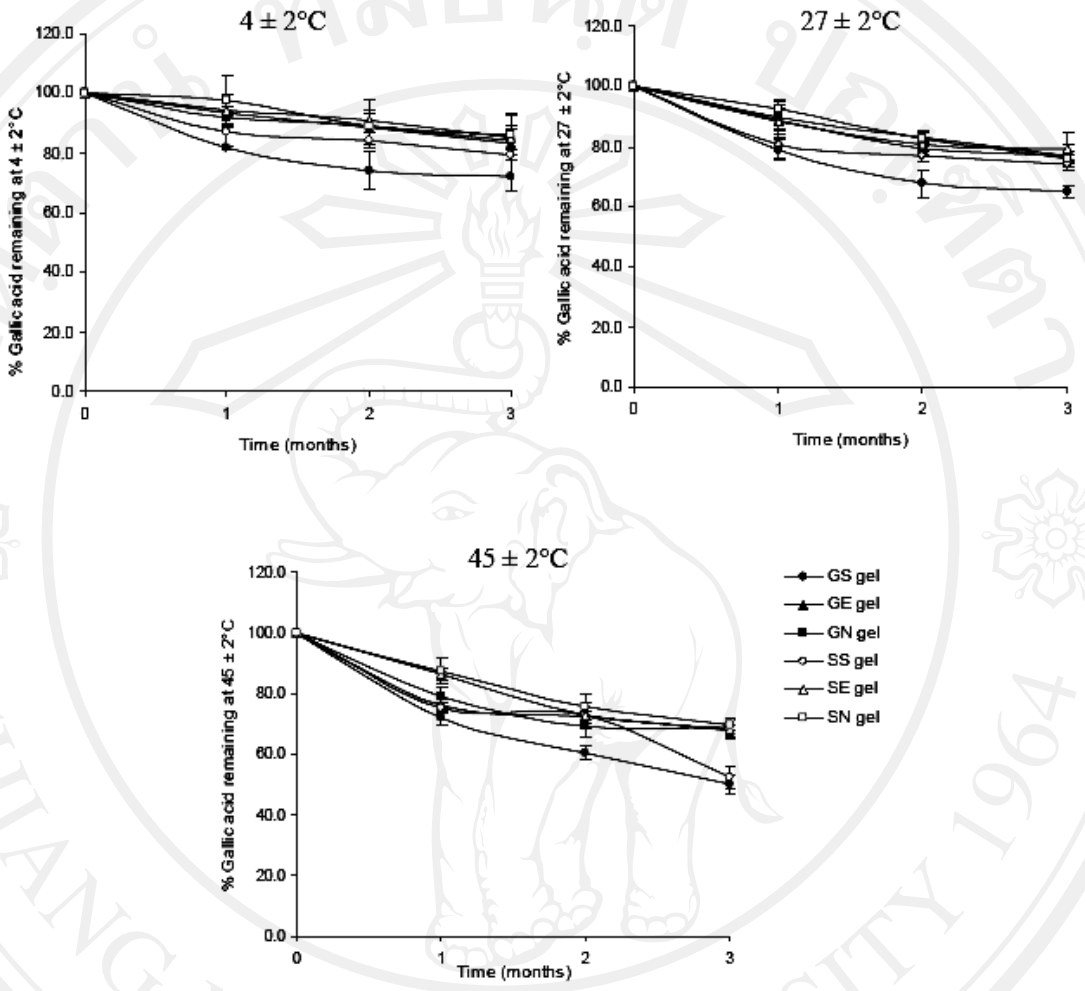


Figure 48 The percentages of gallic acid remaining in various gel formulations at different storage temperatures (27 ± 2, 4 ± 2 and 45 ± 2°C) for 3 months (GS gel: gel containing the unloaded gallic acid; GN gel: gel containing non-elastic niosomes loaded with gallic acid; GE gel: gel containing elastic niosomes loaded with gallic acid; SS gel: gel containing the unloaded semi-purified fraction; SN gel: gel containing non-elastic niosomes loaded with the semi-purified fraction and SE gel: gel containing elastic niosomes loaded with the semi-purified fraction).

during storage which may fluidize the vesicular membrane and facilitate the leakage of gallic acid from the elastic vesicles. When incorporated in gel, gallic acid and the semi-purified fraction loaded in niosomes was protected from thermal degradation more than those loaded in niosomes but not incorporated in gel of about 1.31 times (Manosroi et al., 2010c). In addition, the gel structure may retard the leakage of the gallic acid from the vesicles, thereby preventing its degradation from the thermal effects (Bochot et al, 1998; Ruel-Gariepy et al, 1994; Glavas-Dodov et al, 2002).

3.6 Transdermal absorption of gel containing elastic niosomes loaded with gallic acid from *Terminalia chebula* Retz. (Combretaceae) galls

The cumulative amounts and percentages of gallic acid per area in different skin strata from various gel formulations after 12 h investigated by Franz diffusion cells were presented (**Figure 49** and **Table 31**). Each 0.5 g of GS, GE and GN gel containing 0.5% w/w of gallic acid, and of SS, SE and SN gel containing the semi-purified fraction with 0.08% w/w of gallic acid was placed onto the donor compartment. The cumulative amounts ($\mu g/cm^2$, $n = 3$) through skin of gallic acid in all gel formulations increased with times. The gel containing pure gallic acid (GS) exhibited the highest cumulative amount after 12 h at 898.98 ± 18.96, 21.87 ± 1.00 and 15.89 ± 0.75 $\mu g/cm^2$ which were 35.96, 0.87 and 0.64% in SC, VED and the receiving solution, respectively (**Table 31**). The gel containing the semi-purified fraction (SS) showed the lowest cumulative amount after 12 h of gallic acid at 1.12 ± 0.86 and 0.55 ± 0.17 $\mu g/cm^2$ which were 0.19 and 0.09% in VED and the receiving solution, respectively. For SC, the semi-purified fraction in gel (SS) showed the percentages of gallic acid at 41.01% higher than the fraction loaded in elastic (SE)

(32.92%) and non-elastic niosomes (SN) (33.99%) gel. Gallic acid in the pure form appeared to penetrate into the skin more efficiently, while gallic acid in the semi-purified fraction exhibited lower penetration with some were remained on the upper layer of the skin (SC). This can be explained with two reasons. First, the pure gallic acid which was a small molecule (MW = 170.12) and had higher amount (2,500 μg of gallic acid) at initial in the donor chamber of about 6 times of gallic acid in the semi-purified fraction (400 μg of gallic acid) may result in higher penetration. Second, gallic acid in the semi-purified fraction may be highly polymerized or associated with other polyphenolic compounds by hydrogen bonding (Haslam, 1996) thereby retarding the skin permeation from its large complex molecular structure.

Gel containing pure gallic acid loaded in elastic (GE) and non-elastic niosomes (GN) showed lower cumulative amounts at 12 h of gallic acid in SC and VED of 821.44 ± 42.70 and 837 ± 31.35; 8.84 ± 0.49 and 6.67 ± 1.63 μg/cm^2, respectively, than the unloaded gallic acid in gel (GS) (898.98 ± 18.96 and 21.87 ± 1.00 μg/cm^2 in SC and VED, respectively). However, gel containing the semi-purified fraction loaded in elastic and non-elastic loaded niosomes (SE, SN) showed higher cumulative amounts at 12 h of gallic acid in VED and the receiving solution of 4.21 ± 1.11 and 1.99 ± 1.42; 2.22 ± 0.31 and 1.32 ± 0.52 μg/cm^2, respectively, than the unloaded semi-purified fraction in gel (SS) (1.12 ± 0.86 and 0.55 ± 0.07 μg/cm^2 in VED and the receiving solution, respectively) (**Figure 49**). Elastic niosomes loaded with pure gallic acid (GE) and the semi-purified fraction (SE) showed higher percentages of gallic acid amount at 12 h of 0.35 and 0.70; 0.21 and 0.22% in VED and receiving solution, respectively, than their non-elastic niosomes loaded with pure gallic acid (GN) and the semi-purified fraction (SN) (0.27 and 0.33; 0.14 and 0.22%

in VED and receiving solution, respectively) (**Table 28**). Therefore, both elastic and non-elastic niosomes demonstrated the skin permeation retardation of the loaded gallic acid, whereas exhibited the skin permeation enhancement of gallic acid in the semi-purified fraction. Hence, elastic and non-elastic niosomes appeared to reduce the systemic effect of the loaded pure gallic acid since about less than 3.0 and 4.5 times of the loaded in elastic and non-elastic niosomes than the unloaded pure gallic acid was found in the receiving solution. For gallic acid in the semi-purified fraction loaded in elastic niosomes, the niosomes did not only enhance transdermal permeation of gallic acid to VED, but also reduced the remaining gallic acid on the upper layer of the skin (SC). This may be not only due to the small vesicular size and high deformability of elastic niosomes (**Table 31**), but ethanol may also interact with the polar head group region of the Tween 61 molecules and provide the reduction of vesicular melting point. This resulted in the soft and flexible characteristics of the elastic vesicles that enhanced the penetration of gallic acid in the semi-purified fraction into the deeper layers of the skin. Ethanol may also fluidize the bilayer structure of SC (Kirjavainen et al., 1999) leading to the enhancement of gallic acid penetration.

3.7 *In vivo* anti-aging evaluation of gel containing niosomes loaded with phenolic compounds from *Terminalia chebula* galls

3.7.1 Skin irritation evaluation

3.7.1.1 Rabbit skin irritation by the closed patch test

The calculated PIIs of all gel formulations in rabbit skin irritation by the closed patch test at 72 h were in the range of 0.00–0.33 except the gallic acid gel (PII = 0.44–0.56, slight irritation), the gel base (PII = 0.11, negligible) and the positive control (5%

SLS, PII = 0.78–1.22, slight irritant) (**Table 32**). Thus, all developed gel formulations gave no irritation, except the gallic acid gel. The irritation was from gallic acid which is a small molecule (MW = 170.12), since the gel base gave no skin irritation. In the material safety data sheet, gallic acid may cause irritation to the skin with redness or minor inflammation (Mallinckrodt Chemical, J.T. Baker, NJ, U.S.A.). This may be also due to its concentrations in the gel which was about 6 times (2,500 µg of gallic acid) more than that in the semi-purified fraction (400 µg of gallic acid). In addition, gallic acid containing in the semi-purified fraction may be highly polymerized or

Table 31 The percentages of gallic acid amounts (%) in SC (stratum cormeum), VED (viable epidermis and dermis) and receiving solution following transdermal absorption across excised rat skin from various gel formulations by Franz diffusion cells after 12 h

Gel formulations containing	Percentages of gallic acid amount (%)		
	SC	VED	Receiving solution
Gallic acid in phosphate buffer solution (GS)	35.96 ± 7.59	0.87 ± 0.14	0.64 ± 0.19
Elastic niosomes loaded with gallic acid (GE)	32.86 ± 9.94	0.35 ± 0.10	0.21 ± 0.08
Non-elastic niosomes loaded with gallic acid (GN)	33.51 ± 8.60	0.27 ± 0.02	0.14 ± 0.04
Semi-purified fraction in phosphate buffer solution (SS)	41.01 ± 8.90	0.19 ± 0.06	0.09 ± 0.03
Elastic niosomes loaded with the semi-purified fraction (SE)	32.92 ± 3.42	0.70 ± 0.15	0.37 ± 0.14
Non-elastic niosomes loaded with the semi-purified fraction (SN)	33.99 ± 2.70	0.33 ± 0.07	0.22 ± 0.01

Note: Values represent mean ± SD. (n = 3). GS, GE and GN gel containing 0.5% w/w gallic acid, and SS, SE and SN gel containing the semi-purified fraction with 0.08% w/w of gallic acid.

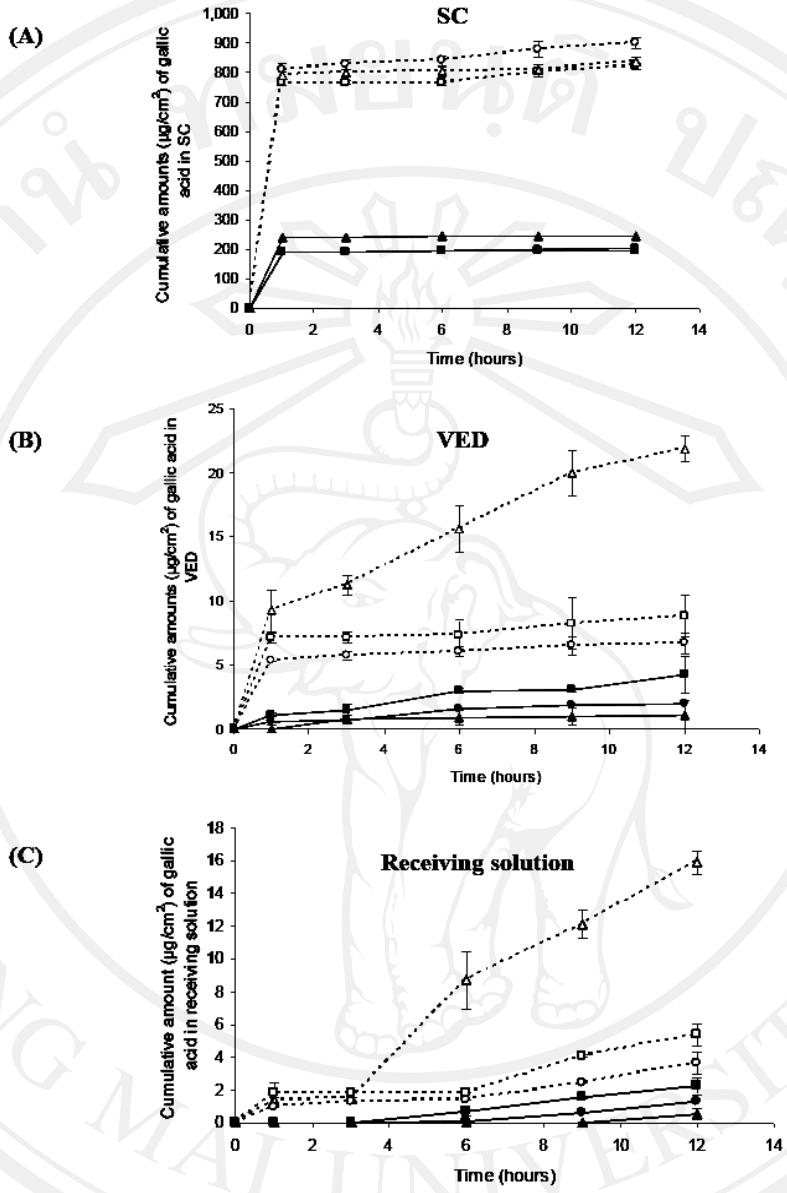


Figure 49 Cumulative amounts (µg/cm^2) of gallic acid from various gel formulations versus time (hours) in SC (stratum corneum) (A), VED (viable epidermis and dermis) (B) and receiving solution (C) following transdermal absorption across excised rat skin by vertical Franz diffusion cells (- - -△- - - = GS; gallic acid in phosphate buffer solution, - - -□- - - = GE; elastic niosomes loaded with gallic acid, - - -○- - - = GN; non-elastic niosomes loaded with gallic acid, ——▲—— = SS; the semi-purified fraction in phosphate buffer solution, ——■—— = SE; elastic niosomes loaded with the semi-purified fraction, ——●—— = SN; non-elastic niosomes loaded with the semi-purified fraction)

associated with other polyphenolic compounds by hydrogen bonding (Haslam, 1996) thereby retarding its skin permeation from this large complex molecular structure. Moreover, the higher *in vitro* rat skin permeation investigated by Franz diffusion cells of pure gallic acid than gallic acid containing in the semi-purified fraction has also supported this skin irritation results (Jennifer et al., 1998; Manosroi et al, 2010a). However, gallic acid loaded in non-elastic or elastic niosomes showed no irritation in rabbit skin, because of the reduction of the direct contact between gallic acid and the skin. Thus, all gel formulations except the gel containing the pure gallic acid were used for *in vivo* anti-aging evaluation in human volunteers.

Table 32 Primary irritation index (PII) and category of irritation based on PII of various gel formulations.

Samples	Primary irritation index (PII)			Category of irritation based on PII
	24 h	48 h	72 h	
Gel base	0.11	0.11	0.00	Negligible
BN gel	0.11	0.11	0.00	Negligible
BE gel	0.22	0.11	0.00	Negligible
GS gel	0.44	0.56	0.56	Slight irritation
GN gel	0.33	0.22	0.11	Negligible
GE gel	0.22	0.22	0.22	Negligible
SS gel	0.33	0.33	0.22	Negligible
SN gel	0.11	0.11	0.11	Negligible
SE gel	0.22	0.11	0.11	Negligible
5% Sodium lauryl sulfate (positive control)	1.22	1.00	0.78	Slight irritation
Untreated area (negative control)	0.00	0.00	0.00	Negligible

Note: Grading scale for skin irritation effect following OECD Test Guideline 404.
Primary Irritation Index (PII) = [(∑ *erythema grade at 24/48/72 h* + ∑ *edema grade at 24/48/72 h)/ 3* × *number of animals*].
BN gel: gel containing blamk non-elastic niosomes; BE gel: gel containing blank elastic niosomes; GS gel: gel containing the unloaded gallic acid; GN gel: gel containing non-elastic niosomes loaded with gallic acid; GE gel: gel containing elastic niosomes loaded with gallic; SS gel: gel containing the unloaded semi-purified fraction; SN gel: gel containing non-elastic niosomes loaded with the semi-purified fraction and SE gel: gel containing elastic niosomes loaded with the semi-purified fraction

3.7.1.2 Human skin erythema measured by Mexameter®

The erythema measurements of all gel samples except the gel containing pure gallic acid performed after 2, 4, 6 and 8 weeks of application revealed no statistically significant differences ($p > 0.05$, Student's paired *t*-test) in comparing to the initial values on day 0 and the untreated area. Therefore, all gels including the commercial product did not induce any human skin irritation and were well tolerated.

3.7.2 Human skin anti-aging evaluation

3.7.2.1 Skin elasticity

Parameters obtained from Cutometer® explain various skin mechanical properties, such as skin elastic extension, elastic recovery and deformation. During aging, changes of the skin elastic recovery (Ur/Uf) has been reported to decrease, whereas changes of the skin elastic extension (Uv/Ue) has been shown to increase progressively (Escoffier et al., 1989; Takema et al., 1994; Fujimura et al., 2007). These skin mechanical properties are related to the decrease in interstitial fluid viscosity as a result from low amount of glycosaminoglycans and soluble collagen, as well as the decrease in elastic properties of collagen and elastin fibers due to the damage, disintegration, fragmentation, or changes of the protein fiber structure (Dobrev 1998; Dobrev 2002). Thus, an increase in Ur/Uf value and a decrease in Uv/Ue value will indicate the improvement of skin elastic properties. In this study, the values of Ur/Uf and Uv/Ue elastic parameters of various topical gel formulations after 8 weeks of application in 31 volunteers measured by Cutometer® were shown in **Table 33** and **Figure 50**. All Ur/Uf values increased while all Uv/Ue values decreased with times in all treated areas. However, the gel containing gallic

acid loaded in elastic niosomes, the unloaded semi-purified fraction, and the gels containing the semi-purified fraction loaded in niosomes and elastic niosomes (GE, SS, SN and SE) showed the significant increase of Ur/Uf at 8 weeks of application. Moreover, SN and SE gel also gave the significant decrease of Uv/Ue at 6 and 8 weeks of application. The parameter changes (%), which were determined from the values after and before application time, the application of GE, SS, SN and SE gels for 8 weeks gave the parameter changes of the Ur/Uf value at +53.92, +30.32, +28.73 and +32.57%, respectively. The parameter changes of the Uv/Ue values were −21.25 and −22.63% when applied with SN and SE gel, respectively (**Table 33**). The phenolic compounds, especially gallic acid, which were the constituents in the semi-purified fraction may improve skin elasticity in the dermis by scavenging the free radical and inhibiting the MMP-2 activity. This has been evidenced that some phenolic compounds, such as tannins have been reported as a direct human neutrophil elastase (HNE) inhibitor. The decrease in skin elasticity, also accompanied by neutrophils, which supposed to participate in the aging process of human skin and the release of enzymatically active HNE (Tsuji et al., 2001). Also, some hydrolysable tannins from root of *Sanguisorba officinalis* L. applied on the rat hind limb skin following UVB exposure have been reported to inhibit wrinkle formation, maintain skin elasticity and prevent the decrease of dermal elastic fiber linearity in a dose dependent manner (Mimaki et al., 2001; Tsukahara et al., 2001)

3.7.2.2 Skin surface microstructure

Skin roughness measurement was based on the principle of measuring the depth of furrows according to the shadow and brightness size due to the inflection under illumination. The maximum roughness (Rm) and average roughness (Ra) values can be used to indicate an increase of progressive aging. The comparison of skin surface rougness before and after 8 weeks of application of the gels containing non-elastic or elastic niosomes loaded with gallic acid or semi-purified fraction measured by skin replica and Visiometer®, was shown in **Figures 51** and **52**. Rm and Ra values decreased with times in all treated groups. All samples including gel base, GN, GE, SS, SN, SE and the commercial gel, showed the significant difference of the decrease of the Ra values between before and after 8 weeks of application with the parameter changes of −19.05, −28.28, −30.95, −31.55, −39.47, −35.28 and −22.43%, respectively. Also, for the Rm values, gel base, GN, GE, SS, SN and SE gels demonstrated a significant decrease with the parameter changes of −24.62, −26.08, −28.66, −30.87, −29.43 and −32.38%, respectively (**Table 33**). For the untreated after

Table 33 Percentage changes of skin parameter (%) after 8 weeks of applications of various gel formulations and the negative control (the untreated area).

	Parameter changes (%)							
	Gel base	GN	GE	SS	SN	SE	Commercial product	Untreated area
Ra	–19.05	–28.28	–30.95	–31.55	–39.47	–35.28	–22.43	+6.30
Rm	–24.62	–26.08	–28.66	–30.87	–29.43	–32.38	–3.38	–3.14
Ur/Uf	+22.95	+47.51	+53.92	+30.32	+28.73	+32.57	+19.89	–14.32
Uv/Ue	–14.62	–8.40	–17.69	–12.85	–21.25	–22.63	–14.93	+2.90

Note: Ra: the average roughness; Rm: the maximum roughness; Ur/Uf: skin elastic recovery; Uv/Ue: skin elastic extension; GN gel: gel containing non-elastic niosomes loaded with gallic acid; GE gel: gel containing elastic niosomes loaded with gallic; SS gel: gel containing the unloaded semi-purified fraction; SN gel: gel containing non-elastic niosomes loaded with the semi-purified fraction and SE

gel: gel containing elastic niosomes loaded with the semi-purified fraction; □: decreased value; + : increased value.

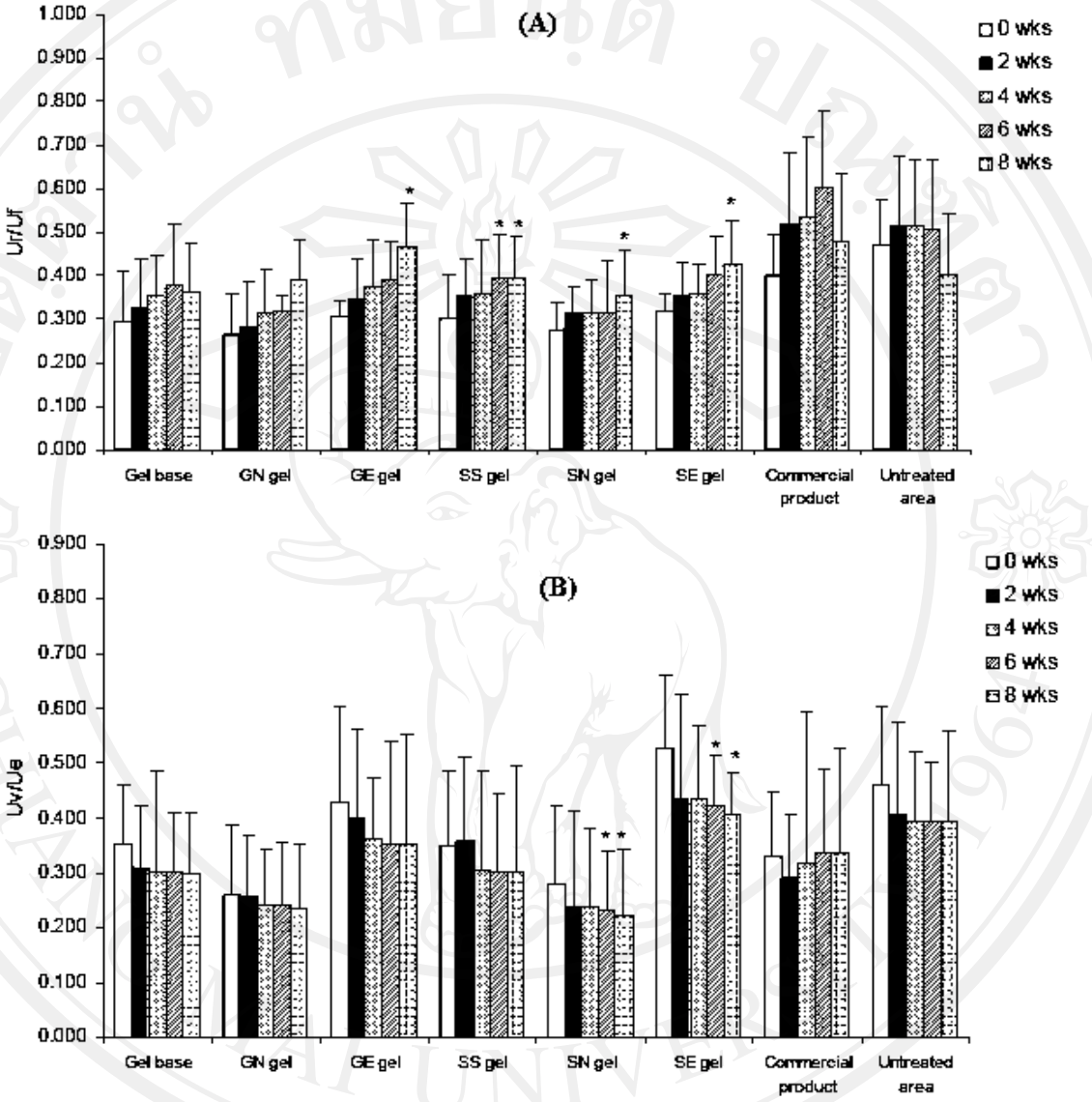


Figure 50 Changes of the skin elastic recovery or Ur/Uf (A) and changes of the skin elastic extension or Uv/Ue (B) of various topical gel formulations in 31 human volunteers after application for 8 weeks. Student paired *t*-test was used to calculate the significant differences. *$p < 0.05$ compared to before application (0 wks) (GN gel: gel containing non-elastic niosomes loaded with gallic acid; GE gel: gel containing elastic niosomes loaded with gallic acid; SS gel: gel containing the unloaded semi-purified fraction; SN gel: gel containing non-elastic niosomes loaded with the semi-purified fraction and SE gel: gel containing elastic niosomes loaded with the semi-purified fraction).

8 weeks of application, the Ra and Rm values showed only a slight increase. There was no significant difference of the roughness parameter values between the gels containing non-elastic and elastic niosomes loaded with gallic acid or semi-purified fraction (GE, GN, SE and SN). As known, the degeneration of the elastic fibers in the skin leads to a decrease of skin elasticity and wrinkle formation. The phenolic compounds which were the constituents in the semi-purified fraction may improve skin elastic resulting in the reduction of skin roughness.

3.7.2.3 Skin hydration and pigmentation

The skin hydration and pigmentation values measured by Corneometer® and Mexameter® before and after 8 weeks of topical application of all samples were not significantly different ($p > 0.05$, Student paired *t*-test)(data not shown). This indicated that all developed gel formulations and the commercial product did not improve any epidermal moisture content and pigmentation in human skin after 8 weeks of application. In order to improve skin hydration, it is necessary to maintain the normal conditions of the skin and to prevent the dryness of the skin by reducing the transepidermal water loss via the occlusive effect. In fact, the moisturizing effects of several cosmetic formulations can be influenced by many factors, including the base compositions and the concentration of the active ingredients (Li et al., 2001; Savic et al., 2004). Generally, gel formulations have only slight occlusive properties in comparing to other cosmetic formulation such as cream. Thus, all developed gels were expected not to improve skin hydration.

For skin pigmentation, UV is a major environmental factor that can cause aged-skin hyperpigmentation. The formulations which can reduce skin

pigmentation should have the active compounds that can limit the extent of UV penetration through the epidermal layers by reflecting, scattering or absorbing the UV rays and scavenging the reactive oxygen radicals that may lead to oxidative DNA damage (Stanojevic et al., 2004). In addition, the active compounds should inhibit tyrosinase enzyme. All developed gel formulations showed no significant difference in skin pigmentation between before and after 8 weeks of application. This agreed with our previous study that *T. chebula* gall extracts gave only slight *in vitro* mushroom tyrosinase inhibitory activity (Manosroi et al., 2010a).

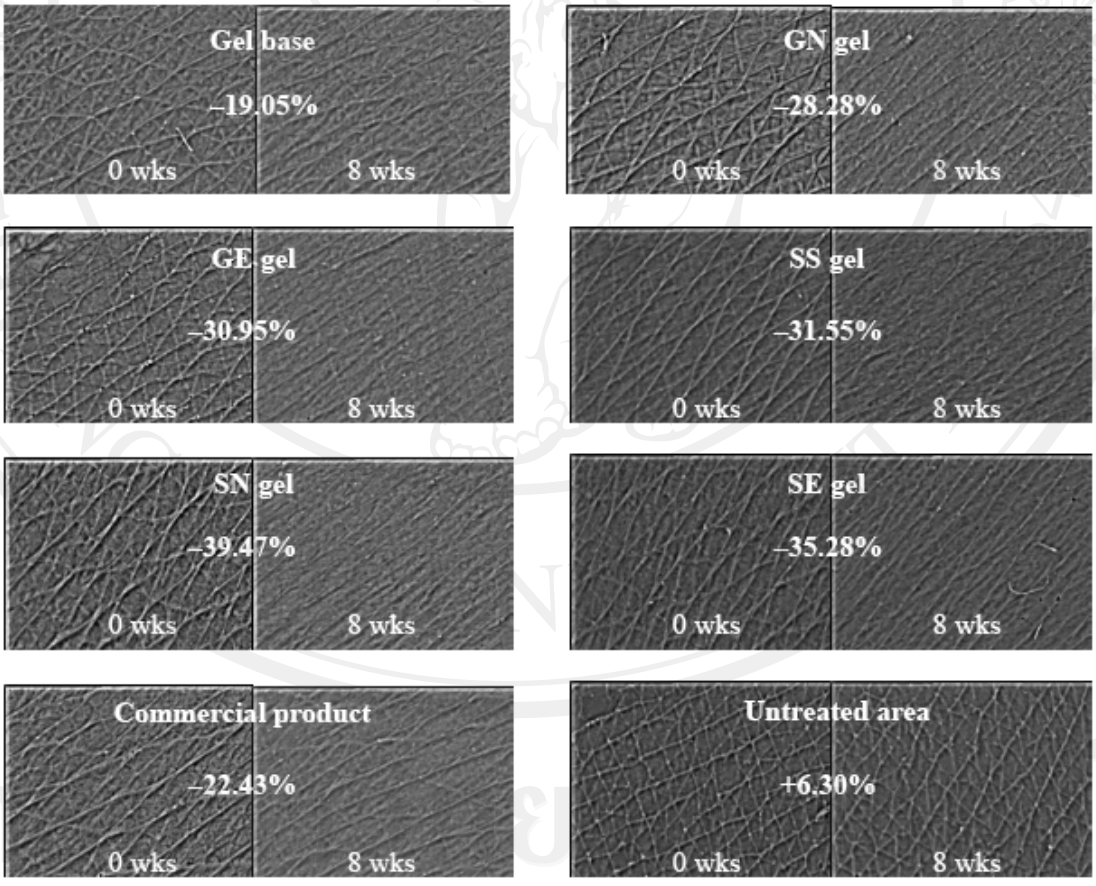


Figure 51 Comparison of the skin roughness before (left) and after application for 8 weeks (right) and % changes of the arithmetic average roughness (Ra) values of various topical formulations

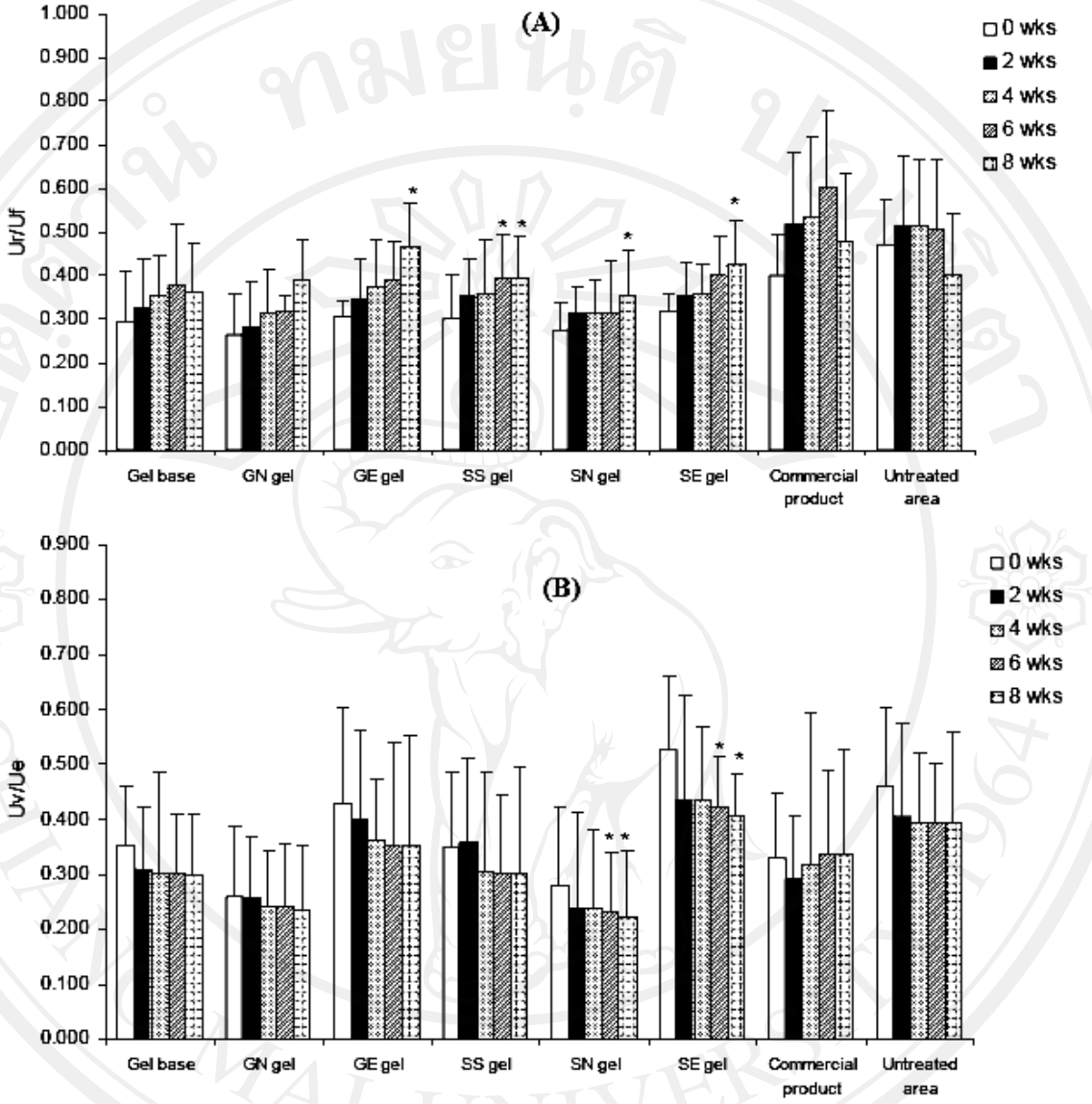


Figure 52 Changes of the skin elastic recovery or Ur/Uf (A) and changes of the skin elastic extension or Uv/Ue (B) of various topical gel formulations in 31 human volunteers after application for 8 weeks. Student paired *t*-test was used to calculate the significant differences. *$p < 0.05$ compared to before application (0 wk) (GN gel: gel containing non-elastic niosomes loaded with gallic acid; GE gel: gel containing elastic niosomes loaded with gallic acid; SS gel: gel containing the unloaded semi-purified fraction; SN gel: gel containing non-elastic niosomes loaded with the semi-purified fraction and SE gel: gel containing elastic niosomes loaded with the semi-purified fraction).