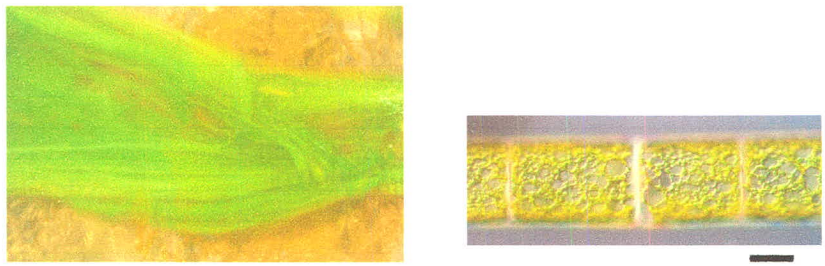
CHAPTER IV

RESULTS AND DISCUSSION

4.1 Identification of Freshwater Macroalga

The characterizations of freshwater macroalga were identified using the morphological features of its macroscopic and microscopic structures as described below.



(a) (b)

Figure 4.1 The morphology of *Rhizoclonium hieroglyphicum* (C.Agardh) Kützing

(a): in the field, (b): under light microscope

Scale bar: 50 μm

***Rhizoclonium hieroglyphicum* (C.Agardh) Kützing**

Division Chlorophyta (Green algae)

Class Ulvophyceae

Order Cladophorales

Family Cladophoraceae

Genus *Rhizoclonium*

Species *Rhizoclonium hieroglyphicum* (C.Agardh) Kützing

Poster presentations

1. **Mungmai L.**, Jiranusornkul S., Peerapornpisal Y., Sirithunyalug B. and Leelapornpisid P., 2010. Isolation, Chemical Investigation and Biological Activities of Polysaccharides from *Microspora* spp. (Microsporaceae, Chlorophyta). The Commission on Higher Education Congress III University Staff Development Consortium. October 9-11, 2010.

Oral presentations

1. Characterization and Biological Activities of Extracts from Freshwater Macroalgae (*Rhizoclonium hieroglyphicum* (C.Agardh) Kützing) on March 28-30, 2013 at The Empress Hotel, Chiang Mai, Thailand.

Rhizoclonium hieroglyphicum is classified as: Filaments slender, loose lying with basal cells, or attached by holdfast with basal lobes. Plants unbranched with cylindric cell. The filaments extended up to 35 cm. in length and vegetative cells showed 160 μm in width, 171-232 μm in length. It contained numerous nuclei and chloroplasts are reticulate, arranging in parietal, with pyrenoids, often densely packed with starch. Reproduction in freshwater species is observed by fragmentation and more rarely biflagellate zoospores [121-122, 152].

4.2 Extraction of *R. hieroglyphicum*

From the extraction of *R. hieroglyphicum* with different solvents, the aqueous extract (RW) showed a higher extraction yield (21.56±0.28%) than the ethanolic extract (RE) (12.58±0.44%). This indicated that most components of the algae dissolved in high-polarity solvents and that more polar compounds were found. Matanjun *et al.* [124] also reported that the extraction of some marine algae such as *Caulerpa racemosa*, *Sargassum polycystum* and *Padina* sp. exhibited an increasing percentage yield when the polarity of the solvents was increased. Similarly, Boonchum *et al.* [38] reported that the extraction of marine algae with water yielded a higher quantity than ethanol.

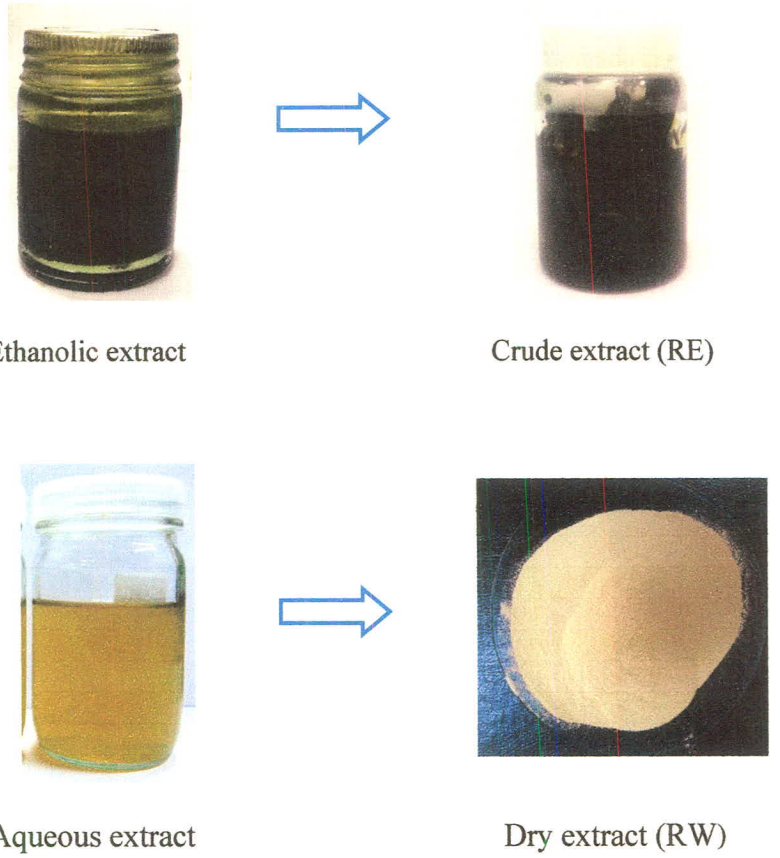


Figure 4.2 The appearance of *R. hieroglyphicum* extract

4.3 Antimicrobial activity of *R. hieroglyphicum* extracts

Regarding the application for bacterial-infected skin, the antimicrobial activity of the extracts (RW and RE) against three gram-positive bacteria, viz., *S. aureus* ATCC 29213, MRSA and *P. acne* ATCC 6919 showed that both the 2% and 5 % (w/v) extracts could not inhibit the growth of these bacteria. The same result was observed from the extract from *S. platensis*. Our results indicate that *R. hieroglyphicum* extracts did not contain active antimicrobial compounds.

Peerapornpisal *et al.* [1] also reported that the extracts of "Kai" algae (species not specified) did not react against gram-positive bacteria *Bacillus subtilis*

and *Micrococcus luteus* and also gram-negative *Escherichia coli, Klebsiella pneumonia* and *Proteus valgaris*.

Bhowmik *et al.* [152] reported that *S. platensis* at various concentrations of 1, 5 and 10 mg/ml showed antimicrobial activity against three gram negative (*Escherichia coli* MTCC443, *Pseudomonas aeruginosa* MTCC424 and *Proteus vulgaris* MTCC426) and three gram-positive bacteria (*S. aureus* MTCC96, *Bacillus subtilis* MTCC619 and *B. pumulis* MTCC1456). In contrast, Pradhan *et al.* [5] had shown that the aqueous extract of *S. platensis* had no effective antimicrobial activity against the test microorganism (*Pseudomonas putida, P. aeruginosa, P. fluorescens, Aeromonas hydrophila, Vibrio alginolyticus, V. anguillarum, V. fluvialis* and *Escherichia coli*). These results indicated that *S. platensis* possessed antimicrobial ability depended on the strain of microbial, method of extraction, different solvents of the extract, source of the extracts and conditions of the culture.

4.4 Antioxidant activities of *R. hieroglyphicum* extracts

The antioxidant activities of the extracts (RW and RE) were compared with the ethanolic extracts of *S. platensis* (SE) (Table 4.1).

The ethanolic extracts of *S. platensis* showed significantly higher levels of DPPH, ABTS scavenging activities and inhibition on lipid peroxidation by TBARS assay ($p<0.05$) than the ethanolic extract from *R. hieroglyphicum* (RE), although it contained less total phenolic compounds. It is probable that *S. platensis* contains other hydrophobic or non-polyphenolic antioxidative substances such as carotenoids, chlorophyll, phycocyanin [153] and vitamin E [154]. In addition, Hanaa *et al.* [155] reported that polyphenolic compounds in *S. platensis* also exhibited antioxidant

Table 4.1 IC_{50}, standard equivalent of antioxidant activities and total phenolic content of *R. hieroglyphicum* (C.Agardh) Kützing and *S. platensis*

Extracts	DPPH		ABTS		TBARS		Total Phenolic content
	IC_{50} (mg/ml)	GEAC mg gallic acid/g extract	IC_{50} (mg/ml)	TEAC mg trolox/ g extract	IC_{50} (mg/ml)	QEAC mg quercetin/ g extract	mg GAE/ g extract
RW extract	23.883 ± 1.123^{b}	0.921 ± 0.042^{b}	13.949 ± 0.027^{a}	2.079 ± 0.031^{a}	55.208 ± 0.269^{c}	0.09276 ± 0.002^{c}	0.1505 ± 0.003^{a}
RE extract	29.144 ± 1.330^{c}	0.755 ± 0.034^{c}	81.810 ± 3.041^{c}	0.354 ± 0.013^{c}	47.439 ± 0.843^{b}	0.11211 ± 0.000^{b}	0.7349 ± 0.004^{c}
SE extract	15.481 ± 0.621^{a}	1.349 ± 0.052^{a}	67.450 ± 0.028^{b}	0.430 ± 0.000^{b}	28.293 ± 0.697^{a}	0.16268 ± 0.001^{a}	0.5188 ± 0.001^{b}

Data shown are mean ± standard error (SD) of three replicates. $^{a, b}$ and ^{c} are statistical comparison between groups using ANOVA post hoc Tukey's b Test ($P<0.05$).

effects on lipid peroxidation and on DPPH radical scavenging activity. However, the one of antioxidants in RE extract was phenolic compounds (Table 4.1).

In ABTS scavenging assay, RW showed significantly ($p<0.05$) higher scavenging activity than RE and SE extract. The RW extract showed the highest ABTS scavenging activity with an IC_{50} of 13.949±0.027 mg/ml and TEAC value of 2.079±0.031 mg trolox/g extract, which is twice higher activity than Trolox, a vitamin E analog, and about seven and five times higher activity than RE and SE from *S. platensis*, respectively. This indicated that the higher amount of polar polyphenolic compounds contained in the RW extract showed a high reducing capability to perform electron donating. This is in line with previous studies that have shown that aqueous extracts of several algae contained higher antioxidant activity than ethanolic extracts [38, 156]. Senvirathne *et al.* [157] have also reported that DPPH radical scavenging capacities of *Ecklonia cava* increased with the increasing polarity of the solvent.

Other hydrophilic compounds found in algae, besides polyphenolic substances, may contribute to the high antioxidant activities of aqueous extracts. In addition to phenolic compounds, other hydrophilic compounds, including peptides, fucoidan and polysaccharides, have also been reported as antioxidants [156]. Polysaccharides from vascular plants and algae have been shown to possess strong antioxidant properties [10, 158-159]. The RW extract is also consisting of sulfated polysaccharide as shown in figure 4.4

In our study, the RW extract showed significantly ($p<0.05$) lower anti-lipid peroxidation activity on a TBARS assay than RE and SE extract. The result demonstrated that the polar active substances in aqueous extracts did not react well with the non-polar or low-polar free radical molecules such as the lipids.

Our results strongly indicate that extracts of *R. hieroglyphicum* a potential natural antioxidant capability.

The aqueous extract of *R. hieroglyphicum* possessed higher yield and higher antioxidant activities than the ethanolic extract, therefore it was selected for further study of its chemical analysis and physiochemical properties.

4.5 Content analysis of RW extract

4.5.1 Phenol-sulfuric acid method

The total sugar content of the RW extract was determined by phenol-sulfuric acid method. This method is the first step in characterization of characterizes polysaccharides. The RW extract showed the total sugar content of 41.228±0.593 mg glucose/g dry weight. This indicated that the RW extract consisted of neutral sugars.

4.5.2 Protein and amino acid analysis

The protein content of the RW extract was determined by Bradford assay. It was found to contain very low protein in the range of 0.2756 - 0.394 ng/g crude powder. Chromatogram of amino acids was determined by gas chromatography equipped with a GC-MS as shown in Figure 4.3.

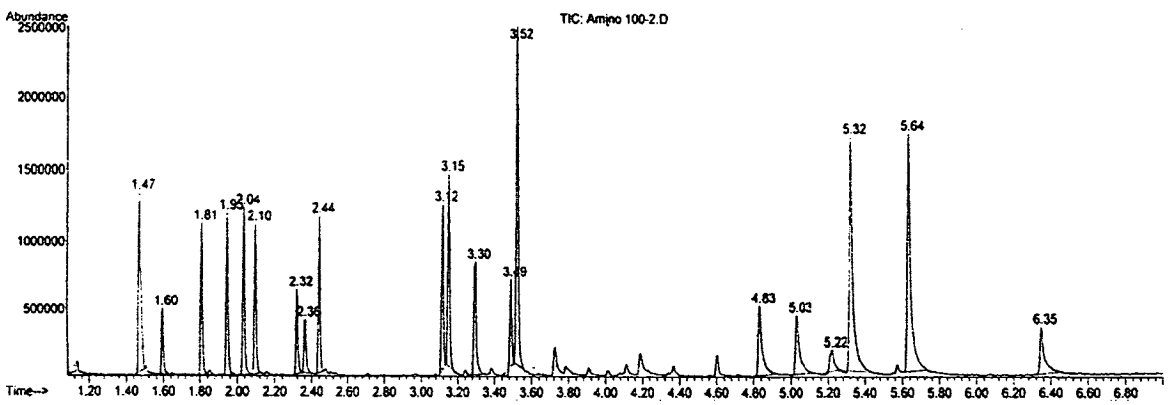


Figure 4.3 GC-MS chromatogram of the *R. hieroglyphicum* extract (RW)

According to Figure 4.2 the major abundant amino acids were tryptophan (14%), tyrosine (13%) and phenylalanine (12%). Other essential amino acids were alanine, methionine and asparagines (Table 4.2). The result demonstrated that the extract contained low levels of protein.

Table 4.2 Amino acid composition and % of total amino acid value of the RW extract

Retention time (min)	Amino acid	% of total
1.47	Alanine	7
1.60	Glycine	2
1.81	Valine	5
2.04	Leucine	5
2.10	Isoleucine	5
2.32	Threonine	3
2.36	Serine	2
2.44	Proline	5
3.12	Asparagine/Aspartic acid	6
3.15	Methionine	6
3.49	Glutamine/Glutamic acid	2
3.52	Phenylalanine	12
4.83	Lysine	5
5.03	Histidine	4
5.32	Tyrosine	13
5.64	Tryptophan	14
6.35	Cysteine	4

* The % of total amino acid value is obtained from the peak area of each amino acid

Amino acids are used as moisturizing in skin care products. Since they have a low molecular weight, so they can easily penetrate deeply into the skin. Amino acids are also humectants which keep the skin moist [160], thus, they are moisturizing ingredients of the skin. Lautenschläger reported that the amino acids also play an important role in the field of anti-aging skin care [161]. Furthermore, amino acids are substances found in the stratum corneum (SC) layer of the skin known as "natural

moisturizing factor" (NMF), which regulate skin's moisture content [56]. About half of the NMF in the skin is made up of amino acids and pyrrolidone carboxylic acid (PCA) derived from glutamate, an amino acid [162].The role of NMF is to maintain adequate skin hydration of the SC [59]. One of the causes of dry skin is due to a decreased in amino acid content [162]. Therefore, *R. hieroglyphicum* is likely to be a new source of natural moisturizer to prevent and for the treatment of dry skin. This will be tested for its moisturizing property.

4.5.3 Polysaccharide analysis

As previously reported, Peerapornpisal *et al.* [1] found that the extracts of *R. hieroglyphicum* showed high carbohydrate content determined by AOAC method [140]. Thus, polysaccharides, a type of complex carbohydrates, were also analyzed in this study using Fourier Transform Infrared (FT-IR) spectroscopy.

4.5.3.1 FT-IR spectroscopy

The FT-IR spectrum of the RW showed the absorption characteristics of polysaccharides [163-164]. As seen in Figure 4.3, the absorbance at 1249 cm^{-1} related to –S=O stretching vibration of the sulfate ester group [35, 165-169]. The region around 800-850 cm^{-1} indicated the sulfate group in agarocolloids [165-167, 169]. The absorption band at 827 cm^{-1} was a characteristic of galactose-6-sulfate [35, 165]. Therefore, the spectrum of RW was consistent with a sulfated polysaccharide, which is related to its antioxidant activity. This is similar to the findings of Qi *et al.* [170-171] and Zhao *et al.* [172], who reported that the higher sulfate content of polysaccharides from marine algae exhibited stronger antioxidant activity. Rocha de Souza *et al.* [10] also reported the beneficial effect of sulfated polysaccharide from brown and red seaweeds as antioxidants.

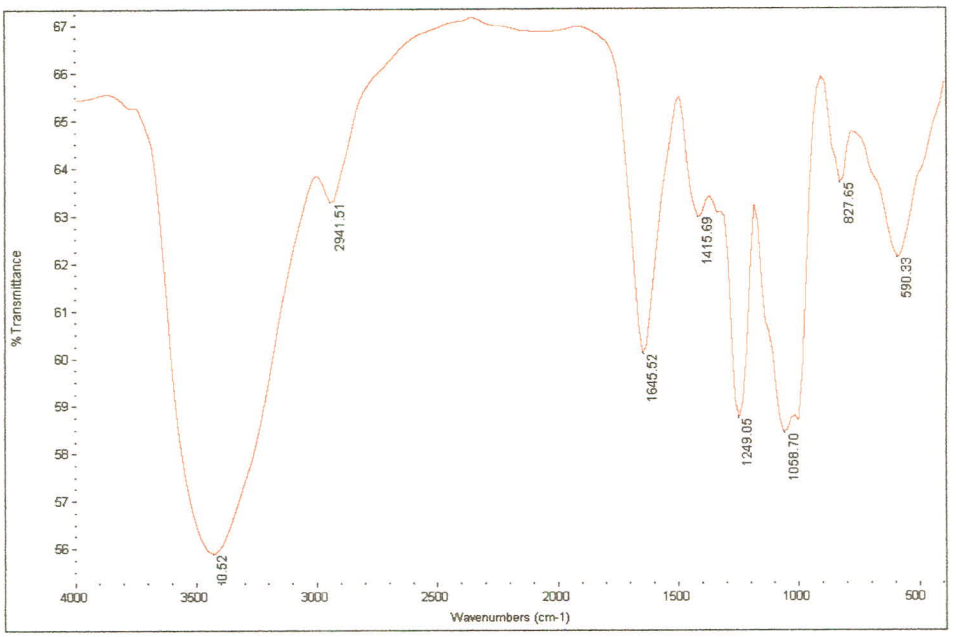


Figure 4.4 FT-IR spectrum of the *R. hieroglyphicum* (RW)

4.5.3.2 Sugar analysis and molecular weight

The RW was separated on a preparative size exclusion chromatography (SEC) system consisting of two columns filled with Sephacryl S200 and Biogel P2, into four overlapping fractions. The content of components in the 4 fractions are: Fraction1: 10 mg (3.57%), Fraction 2: 80 mg (26.35%), Fraction 3: 39 mg (12.83%) and Fraction 4: 41 mg (13.71%).

Only fractions 1 and 2 showed monosaccharide peaks while fraction 3 had traces of monosaccharides and fraction 4 was free of monosaccharide peaks. Since fraction 1 showed only a small amount of monosaccharides, so fraction 2 was selected for further investigation of its polysaccharide composition. Quantification of sugar moieties from fraction 2 was done by TLC analysis (Figure 4.5).

Fraction 2 consisted mostly of arabinose, with smaller amounts of rhamnose, xylose and galactose in the molar ratio of 12.84:1:4.72:7.36, respectively. Arabinose and galactose have also been found to be the major sugars of polysaccharides from aqueous extracts of green algae [35-36]. For different species and even within the same species, different quantities of these sugars were found.

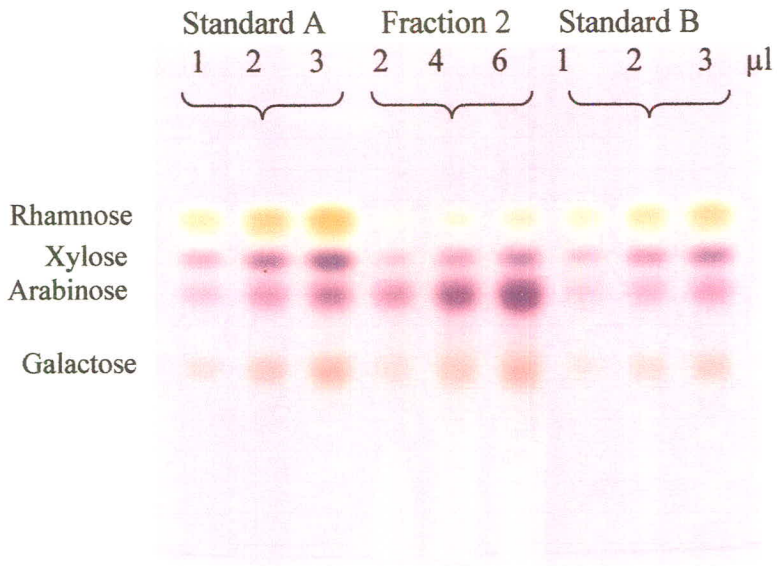


Figure 4.5 The TLC plate shows the oligosacchrides of fraction 2 compared with standards (The concentration of standard B is 50% of that standard A)

4.5.3.3 SEC profiles of RW extract

The elution profiles are based on monitoring of mass profile by refractive index detection. After calibration with dextran standards, the molecular mass distribution for this polysaccharide was between 10,000 and 350,000 [g/mol]. The high molecular component was only carbohydrate (Figure 4.6).

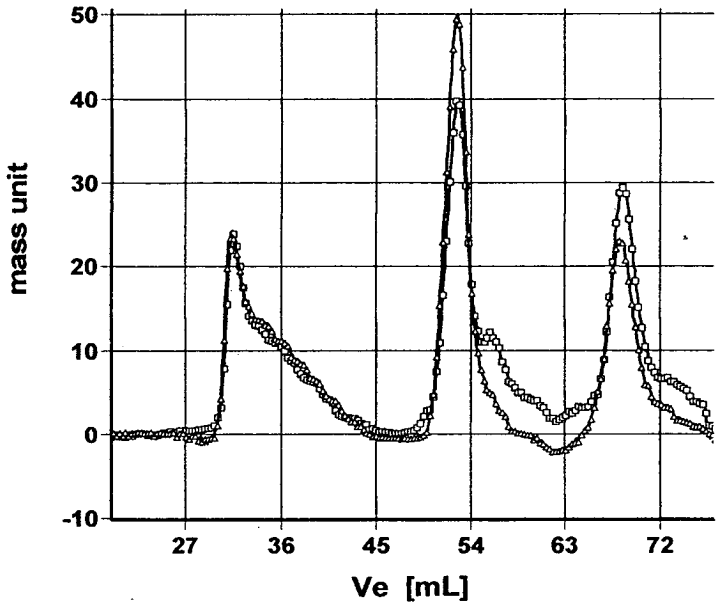


Figure 4.6 SEC profiles of RW extract before (□) and after (Δ) MeOH treatment

The elution profiles were determined by SEC-mass/molar after calibration with Dextran standards. The weight average molecular weight (Mw) was 125,000 g/mol, number average molecular weight (Mn) was 57,000 g/mol and polydispersity Mw/Mn was 2.2 (Figure 4.7).

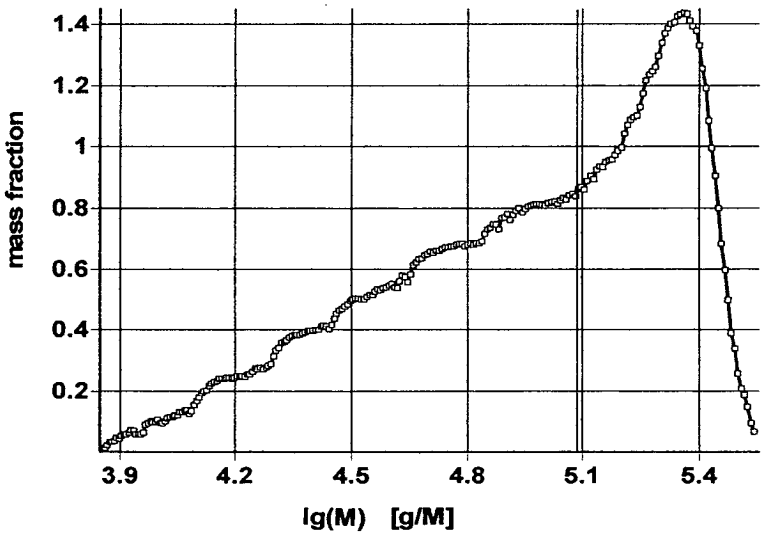
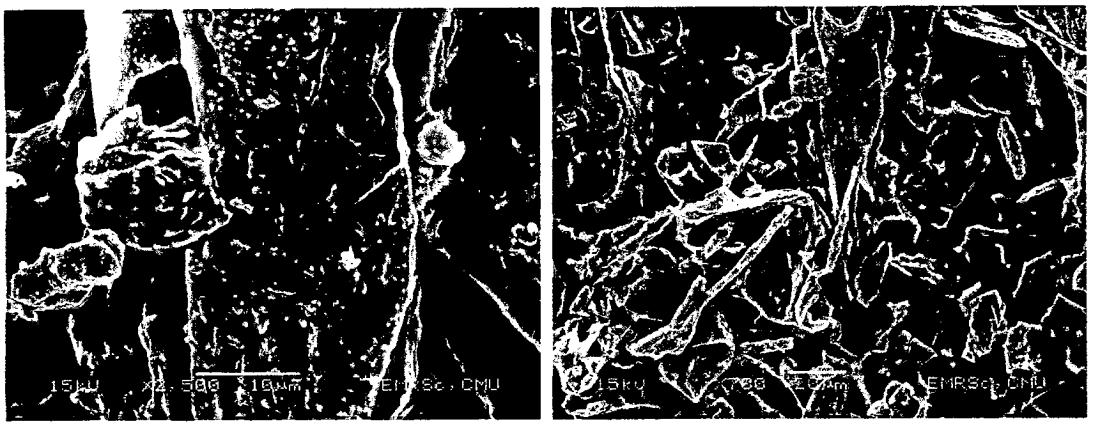


Figure 4.7 Molecular weight distributions from SEC-mass/molar: distribution of mass fractions and distribution of molar fractions

4.6 Physicochemical properties of the RW extract

4.6.1 Morphology and observation of the RW using Scanning Electron Microscope (SEM)



(a) (b)

Figure 4.8 SEM images of RW extract powder (a): Magnification: 2,500x. Scale bar: 10 μm. (b): Magnification: 700x. Scale bar: 20 μm.

SEM images of RW extract powder were shown in Figure 4.8. The particle sizes of RW extract powder were approximately 20-50 μm. In generally, the average particle sizes of commercial polysaccharide powders are in the range of 50-150 μm [173]. The solubility and dissolution rate of polysaccharide increase with reduction in particle size. Therefore, the particle size of polysaccharide is the factor affecting to solubility and dissolution rate. Particles with smaller size will have larger surface area which would increase the absorption rate of the particles and their hygroscopicity.

4.6.2 Textural properties

The texture profile of RW and kappa-carrageenan gels showed peaks of compression and adhesivness. The negative area for the first compression cycle represented the work needed to overcome the attractive forces between the surfaces of

the probe and the sample (Figure 4.9). The RW gel showed similarly of the compression and adhesiveness peaks after the second determination indicating the reformation of gel structure. While the profile of kappa-carrageenan, no adhesivness was observed, and the second compression was very low due to its gel structure broke down from the first compression as shown in Figure 4.10. The hardness is determined as the height of the peak force. Both gel exhibited similar force of gel break down at approximately 2.7 N.

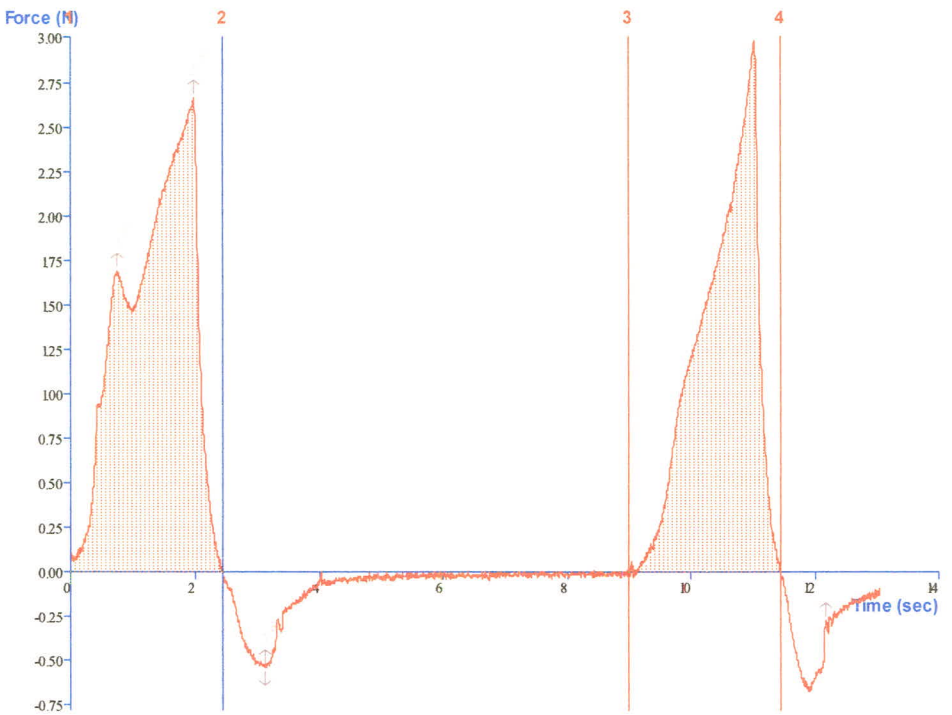


Figure 4.9 Texture profile analysis of the *R. hieroglyphicum* extract (RW)

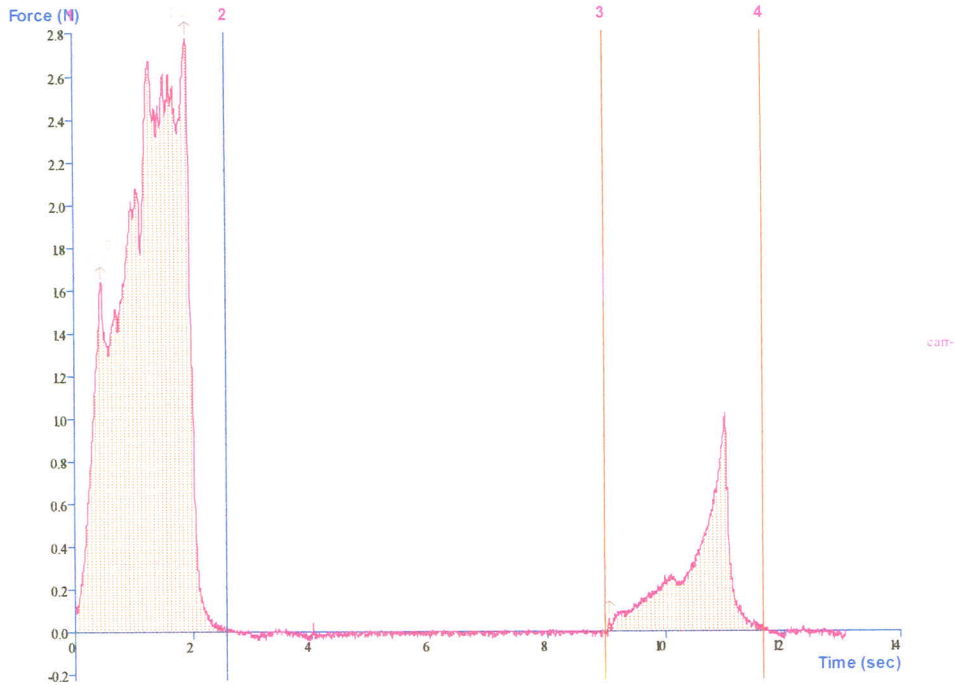


Figure 4.10 Texture profile analysis of the kappa-carrageenan

The gelling properties of the RW in terms of gel strength, rupture force, deformation, cohesiveness and flexibility were compared to kappa-carrageenan as shown in Table 4.3. The textural properties were directly proportional to the concentration of the samples. The gel strength of 10% RW was found to be 52.68±2.56 g/cm^2, which was slightly lower than that of 1% carrageenan (62.39±4.42 g/cm^2). This result was related to the rupture force where the 10% RW (160.17±14.45 g) was also slightly lower than the 1% carrageenan (168.98±1.85 g). This indicated that the gel strength and rupture force of the 10% RW was similar to 1% carrageenan. However, no significant difference was observed in the above results. The cohesiveness of 10% RW (89.26±3.82 g) exhibited significantly higher than that of 1% carrageenan (20.06±3.27 g) indicating that the gel of 10% RW possesses the strong internal bonding of the gel matrix.

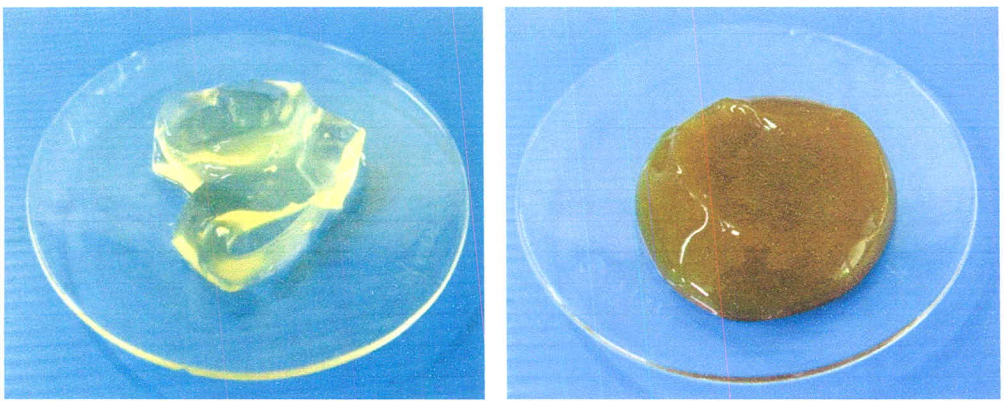
Table 4.3 Gelling properties of the RW extract compared to kappa-carrageenan

	Gel strength (g/cm^2)	Rupture force (g)	Deformation (mm)	Cohesiveness (g)	Flexibility ($mm/gx10^2$)
RW-10%	52.68±2.56	160.17±14.45	5.87±3.54	**89.26±3.82	3.58±1.89
Carrageenan-1%	62.39±4.42	168.98±1.85	2.29±0.03	**20.06±3.27	1.36±0.02

Data shown are mean ± standard error (SD) of three replicates.

** is statistical comparison at 99% confidence level

Note: RW extract formed gel at 10% w/w



Kappa-Carrageenan-1% RW-10%

Figure 4.11 Visual appearance of the kappa-carrageenan and RW gels

4.6.3 Gelling and melting temperatures

The gelling and melting temperatures of the RW were ranged from 50-55 °C and 70-85 °C, respectively. The RW showed the ability to form thermo-reversible gel like carrageenan in that it could be re-melted on heating and form gel again on cooling. This gel-forming property has led to a number of practical applications where it is used as a food additive and other applications in microbiology, biochemistry, biology and many industries [174]. The conventional gel melts on heating and resets

on cooling. This cycle can be repeated for an indefinite number of times. The RW formed gels at high concentrations, with a concentration for gelation of 10%, while carrageenan did at a concentration of 1%. Interestingly, gelation of RW does not require the presence of other products or ions for gelation.

4.6.4 Solubility tests

The solubility of RW was investigated in various solvents at room temperature and 60^{0}C (Figure 4.12-4.13). The RW extract was slightly soluble in water (1:30 to 1:50) and 1%Tween 80 solution (1:20 to 1:50) but swelling and formed gel in 1:10 to 1:15 water. The extract in 1:20 water and 1%Tween 80 (1:10 to 1:15) were slightly soluble with some swelling as shown in Table 4.4. It was sparingly soluble in H_2O:ethanol (1:10 to 1:50) with separation after 10 mins, but was insoluble in glycerin, propylene glycol, mineral oil, jojoba oil and PEG-7 glyceryl cocoate. This result resembled in both temperatures but at 60 °C, the RW indicated higher solubility. The water solubility of RW extract is due to the consisting sugar units and amino acids which are incompatible with alcohol and non polar solvents.

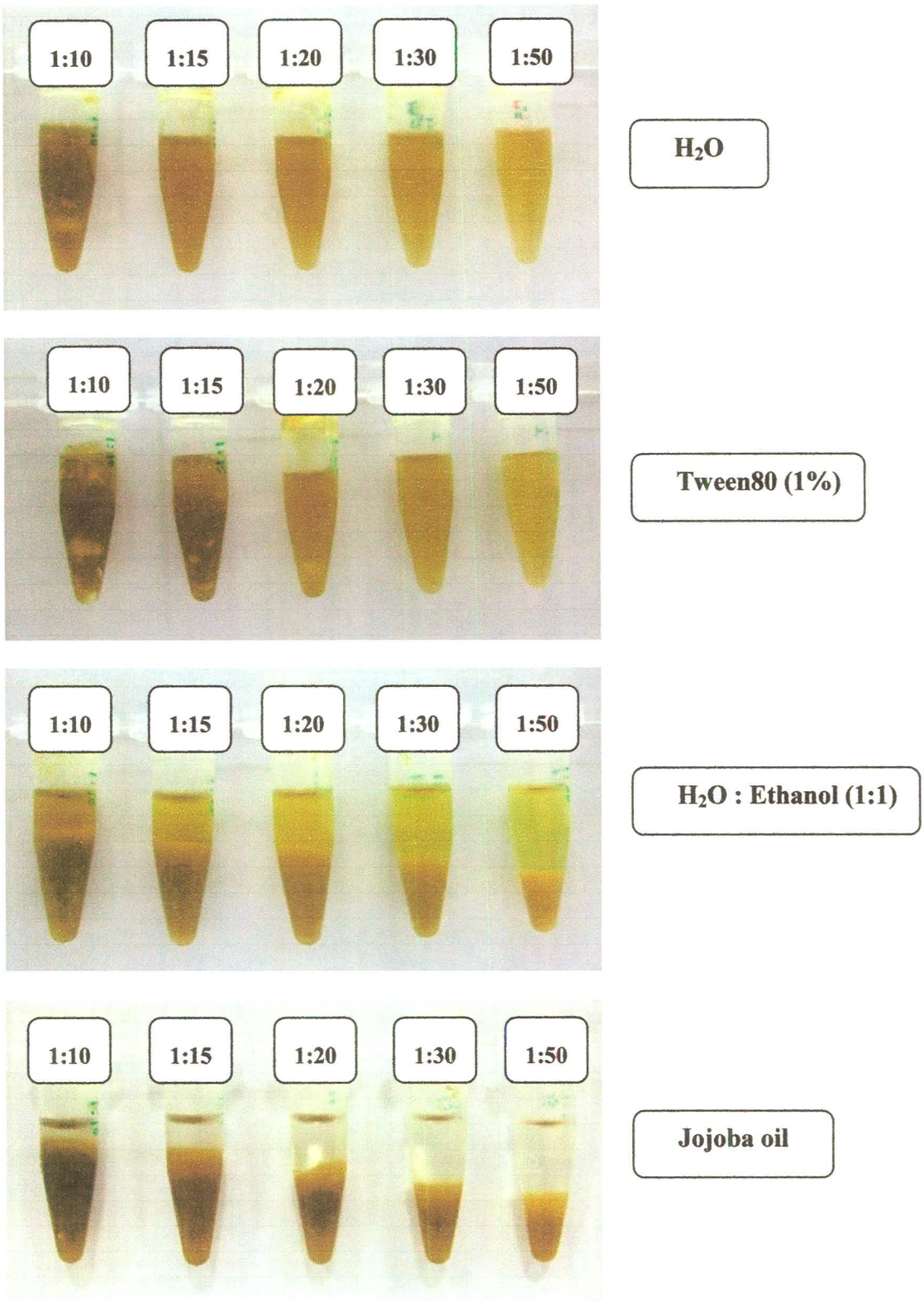


Figure 4.12 Characteristic solubility of the *R. hieroglyphicum* extract (RW) at room temperature

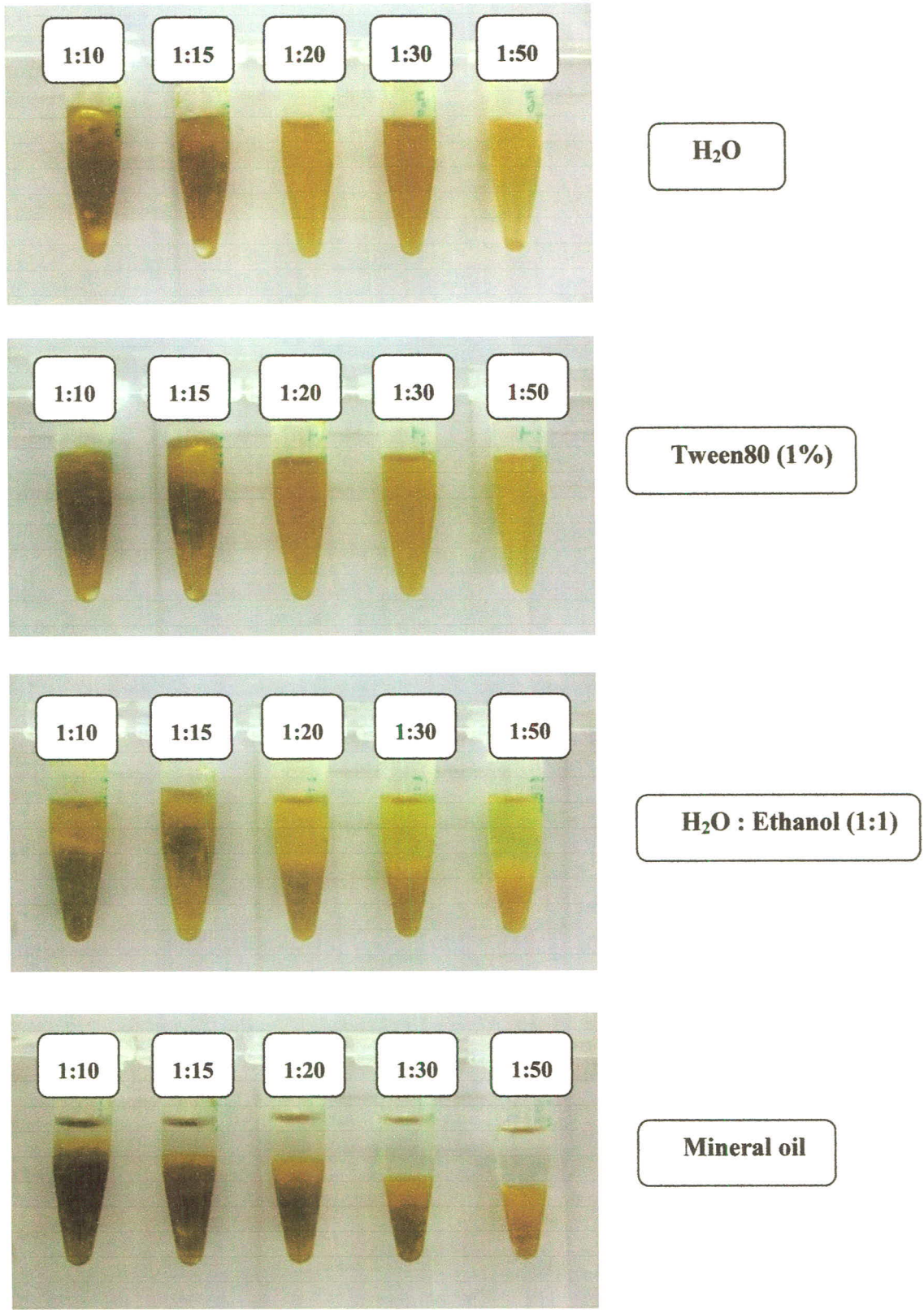


Figure 4.13 Characteristic solubility of the *R. hieroglyphicum* extract (RW) at 60 °C

Table 4.4 Dissolution and visual appearance of the *R. hieroglyphicum* extract (RW)

Solvents	Ratio (RW extract:solvent)	Room temperature		60 °C	
		Dissolution	Appearances	Dissolution	Appearances
H_2O	1:10	+	G, SW	++	G, SW
	1:15	+	G, SW	++	G, SW
	1:20	+	SW	++	SW
	1:30	++	BS	+++	BS
	1:50	++	BS	+++	BS
Propylene glycol	1:10	-	SP	-	SP
	1:15	-	SP	-	SP
	1:20	-	SP	-	SP
	1:30	-	SP	-	SP
	1:50	-	SP	-	SP
Tween80 (1%)	1:10	+	SW	+	SW
	1:15	+	SW	+	SW
	1:20	+	BS	++	BS
	1:30	+	BS	++	BS
	1:50	++	BS	+++	BS
Glycerin	1:10	-	SP	-	SP
	1:15	-	SP	-	SP
	1:20	-	SP	-	SP
	1:30	-	SP	-	SP
	1:50	-	SP	-	SP
H_2O : Ethanol (1:1)	1:10	+	SP	+	SP
	1:15	+	SP	+	SP
	1:20	+	SP	+	SP
	1:30	+	SP	+	SP
	1:50	+	SP	+	SP

Dissolution: +++ Very good, ++ Good, + Little, - Insoluble

G :Gelling, **SW:** Swelling, **SP:** Separation, **BS:** Brown solution

Table 4.4 (Cont.) Dissolution and visual appearance of the *R. hieroglyphicum* extract (RW)

Solvents	Ratio (RW extract:solvent)	Room temperature		60 °C	
		Dissolution	Appearances	Dissolution	Appearances
H_2O : Ethanol (1:2)	1:10	+	SP	+	SP
	1:15	+	SP	+	SP
	1:20	+	SP	+	SP
	1:30	+	SP	+	SP
	1:50	+	SP	+	SP
H_2O : Ethanol (2:1)	1:10	+	SP	+	SP
	1:15	+	SP	+	SP
	1:20	+	SP	+	SP
	1:30	+	SP	+	SP
	1:50	+	SP	+	SP
Mineral oil	1:10	-	SP	-	SP
	1:15	-	SP	-	SP
	1:20	-	SP	-	SP
	1:30	-	SP	-	SP
	1:50	-	SP	-	SP
Jojoba oil	1:10	-	SP	-	SP
	1:15	-	SP	-	SP
	1:20	-	SP	-	SP
	1:30	-	SP	-	SP
	1:50	-	SP	-	SP
PEG-7 glyceryl cocoate	1:10	-	SP	-	SP
	1:15	-	SP	-	SP
	1:20	-	SP	-	SP
	1:30	-	SP	-	SP
	1:50	-	SP	-	SP

Dissolution: +++ Very good, ++ Good, + Little, - Insoluble

G :Gelling, **SW:** Swelling, **SP:** Separation, **BS:** Brown solution

4.6.5 Acid-base tolerance and stability test

The RW extract in water at concentration ratio of 1:10 (10% w/v), 1:20 (5% w/v) and 1:50 (2% w/v) were tested for acid-base tolerance (Figure 4.13 and 4.14). Initially, their pH was about 6.3. It was found that, the 10% w/v RW formed gel at pH 4 and above in the same manner as agar and carrageenan [174-175], but was a low viscosity solution at pH 2 and 3 (Table 4.5). The RW extract at 5% w/v, the solution at pH 2 and 3 showed lower viscosity, while the viscosity increased when the pH of solution was higher than 3. At pH 2-9, it was stable at all conditions (room temperature, 4°C, 45°C and heating/cooling) for except pH 4, it showed unstable at 45°C and heating/cooling condition (Table 4.6). The 2% w/v RW, the solution at pH 2 and 3 also showed lower viscosity whereas its viscosity increased when the pH of solution was 7 and above. At pH 4-6, the 2% w/v RW was stable at room temperature and 4°C except at 45°C and heating/cooling condition which indicated that the viscosity was slightly increased as shown in Table 4.7. From these results, the RW extract at high concentration (10% w/v) presented the properties as other acidic polysaccharides such as agar and carrageenan, so it may be used as thickening and gelling agent in pharmaceutical as well as cosmetic products. Otherwise, at low concentration (2% and 5% w/v) it was soluble in water and stable under tested conditions depending on pH and temperature. The RW was then further investigated for other property such as skin moisturizer for cosmetic purpose.

Table 4.5 Acid-base stability test of the RW extract (10% w/v in H_2O) before and after 1 month storage at various conditions

pH	Conditions									
	Observed-immediately		RT		4°C		45°C		H/C (8 cycles)	
	Appearances	Color	Appearances	Color	Appearances	Color	Appearances	Color	Appearances	Color
C	G V++++	B	G V++++	B	G V++++	B	G V++++	DB	G V+++	DB
2	S V+++	B	S V+++	B	S V+++	B	S V+++	DB	S V+++	DB
3	S V+++	B	S V+++	B	S V+++	B	S V+++	DB	S V+++	DB
4	G V++++	B	G V++++	B	G V++++	B	G V++++	DB	G V+++	DB
5	G V++++	B	G V++++	B	G V++++	B	G V++++	DB	G V+++	DB
6	G V++++	B	G V++++	B	G V++++	B	G V++++	DB	G V+++	DB
7	G V++++	DB	G V++++	DB	G V++++	DB	G V++++	DB	G V+++	DB
8	G V++++	DB	G V++++	DB	G V++++	DB	G V++++	DB	G V+++	DB
9	G V++++	DB	G V++++	DB	G V++++	DB	G V++++	DB	G V+++	DB

G : Gelling , V : Viscosity, S : Solution, B : Brown, DB : Dark brown

++++ = Extremely, +++ = Very much, ++ = Medium, + = slightly

C = control (pH 6.3)

Table 4.6 Acid-base stability test of the RW extract (5% w/v in H_2O) before and after 1 month storage at various conditions

pH	Conditions									
	Observed-immediately		RT		4°C		45°C		H/C (8 cycles)	
	Appearances	Color	Appearances	Color	Appearances	Color	Appearances	Color	Appearances	Color
C	S V++	B	S V+	B	S V+	B	S V++	B	S V++	B
2	S	DB	S	DB	S	DB	S	DB	S	DB
3	S	DB	S	DB	S	DB	S	DB	S	DB
4	S V++	B	S V+	B	S V+	B	S	DB	S	DB
5	S V++	B	S V++	B	S V++	B	S V+	B	S V+	B
6	S V++	B	S V++	B	S V++	B	S V++	B	S V++	B
7	S V+++	B	S V++	B	S V++	B	S V++	DB	S V++	DB
8	S V++++	DB	S V+++	DB	S V+++	DB	S V++	DB	S V++	DB
9	S V++++	DB	S V+++	DB	S V+++	DB	S V+++	DB	S V++	DB

G : Gelling , V : Viscosity, S : Solution, B : Brown, DB : Dark brown

++++ = Extremely, +++ = Very much, ++ = Medium, + = slightly

C = control (pH 6.3)

Table 4.7 Acid-base stability test of the RW extract (2% w/v in H_2O) before and after 1 month storage at various conditions

pH	Conditions									
	Observed-immediately		RT		4°C		45°C		H/C (8 cycles)	
	Appearances	Color	Appearances	Color	Appearances	Color	Appearances	Color	Appearances	Color
C	S	B	S	B	S	B	S V++	B	S	B
2	S	DB	S	DB	S	DB	S	DB	S	DB
3	S	DB	S	DB	S	DB	S	DB	S	DB
4	S	DB	S	B	S	B	S	DB	S	DB
5	S	B	S	B	S	B	S V+	B	S	B
6	S	B	S	B	S	B	S V++	B	S V+	B
7	S V++	B	S V+	B	S V+	B	S V++	B	S V+	DB
8	S V++	DB	S V+	DB	S V+	DB	S V++	DB	S V++	DB
9	S V++	DB	S V+	DB	S V+	DB	S V++	DB	S V+	DB

G : Gelling , V : Viscosity, S : Solution, B : Brown, DB : Dark brown

++++ = Extremely, +++ = Very much, ++ = Medium, + = slightly

C = control (pH 6.3)

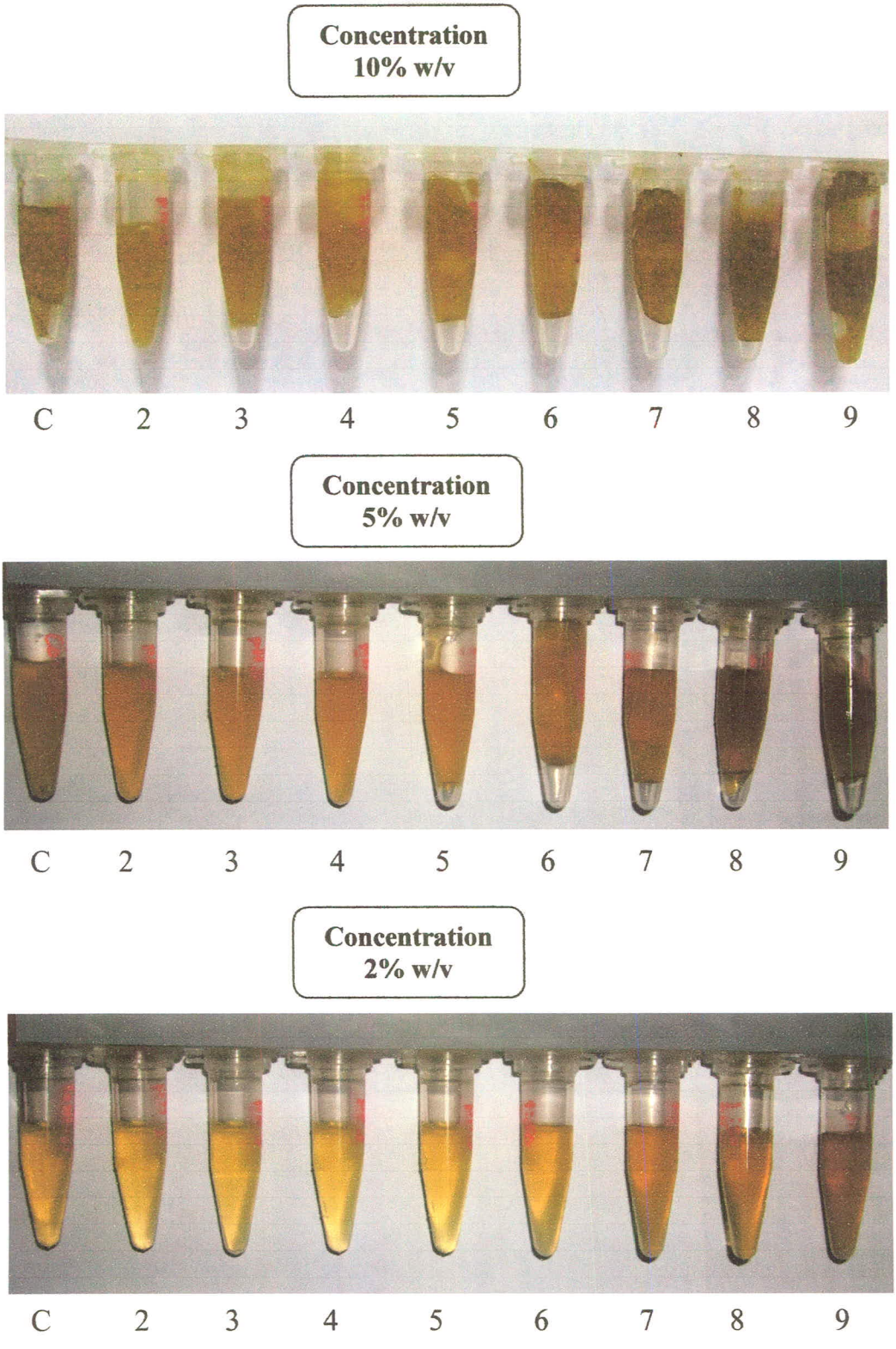


Figure 4.14 Visual appearance of the RW extract at pH 2-9

4.7 Primary skin irritation testing on animal [142-143]

Skin primary irritation test was examined in three albino rabbits (NZW rabbits) for product safety. The results are shown in Figure 4.15. Erythema and edema reactions were scored based on Draize scoring system and calculated in terms of PII values.

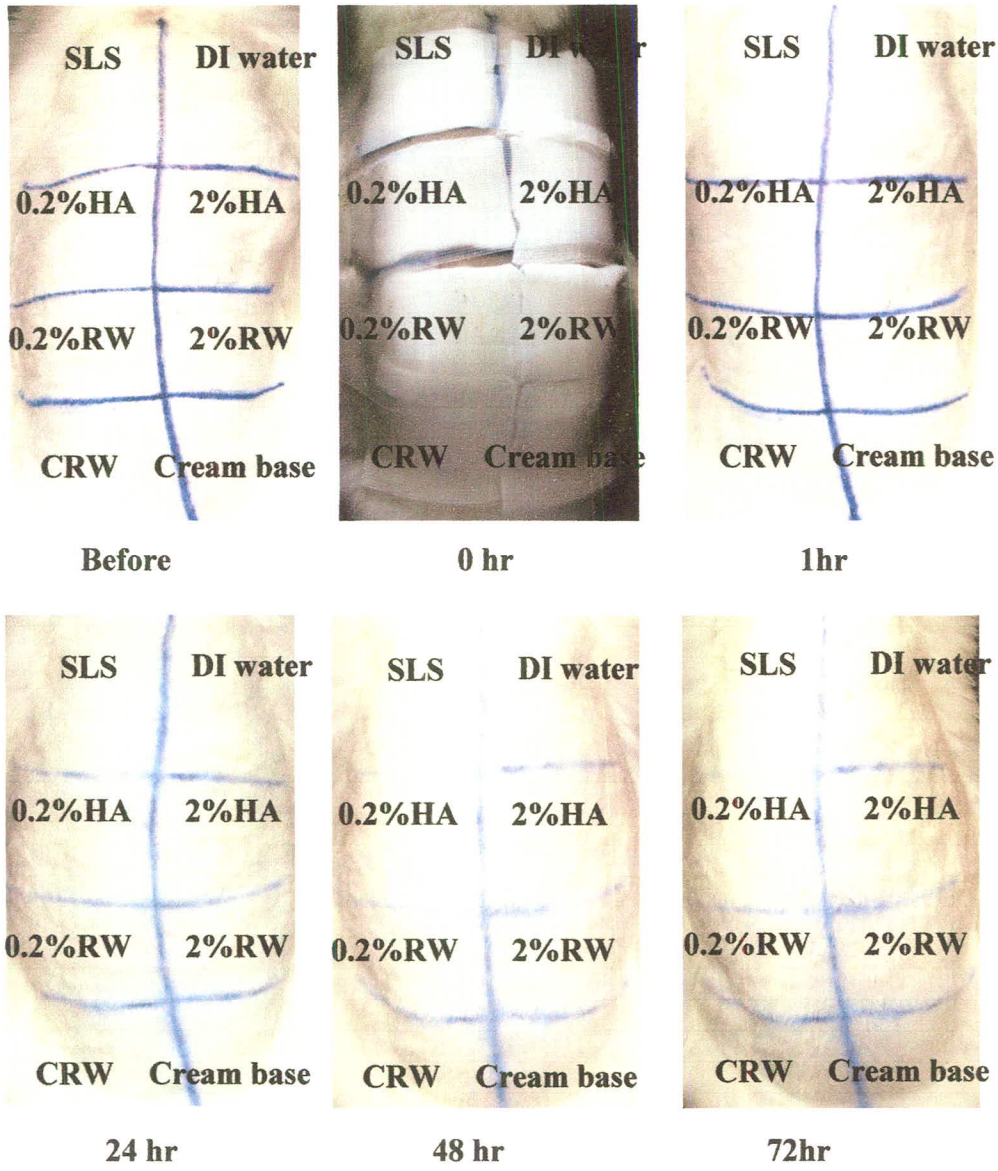


Figure 4.15 Rabbit skin primary irritation test of test substances

SLS = Salicylic acid, HA = Hyaluronic acid, RW = *R. heiroglyphicum* extract

CRW = *R. heiroglyphicum* extract cream

Erythema and edema index, which is often used to assess skin irritation in safety tests [176], according to the PII values (Table 4.8), all of test substances 0.2%HA, 2%HA, 0.2%RE, 2%RE, CRE cream, cream base and deionized water (negative control) gave no irritation whereas 1 % w/v SLS (positive control) revealed slight irritation.

Table 4.8 Primary dermal irritation index (PDII) and skin irritation reaction in rabbits

Test substances	PDII value	Classification of skin reaction
0.2%HA	0.00	No irritation
2%HA	0.00	No irritation
0.2%RW	0.00	No irritation
2%RW	0.04	No irritation
CRW cream	0.12	No irritation
Cream base	0.04	No irritation
Positive (1 % w/v SLS)	1.09	Slight irritation
Negative (DI water)	0.00	No irritation

4.8 Moisturizing test

4.8.1 Moisturizing test on pig skin

From pig skin model, the moisturizing effect of all tested substances showed significantly difference from untreated area ($p<0.05$) whereas 5PG exhibited the highest increasing of moisture content, followed by 0.1 HA, 0.1 RW and 5G (Figure 4.16). Interestingly, it was found that RW extract could keep moisture on pig skin longer than HA when compared at 30 min, this may be due to the higher humectancy effect of the extract.

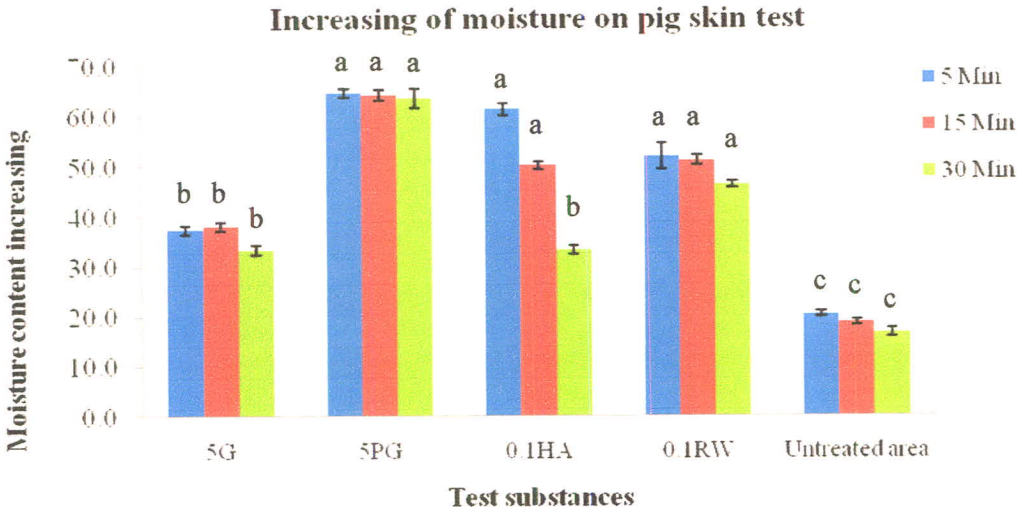


Figure 4.16 Moisture increasing on pig skin after application of the tested substances for 5, 15 and 30 min. (Data shown are mean ± standard error (SD) of three replicates. Letters a, b and c are statistical comparison between groups in each time.)

G : Glycerol PG : Propylene glycol,

HA : Hyaluronic acid RW : *R. hieroglyphicum* extract

4.8.2 Moisturizing test on the human skin

From the test on human skin, the results revealed that the moisture content increased in all test substances which was significantly difference against untreated areas ($p<0.05$) and showed the increase of moisture content with times. Glycerin is more effective than propylene glycol due to the more swelling and hydrating to the stratum corneum [177] and higher water absorption capacity (Figure 4.17). For glycerin, the result was contrast to the test on pig skin showing the lowest moisture increasing capacity. This may be due to the differences in pig skin structure from human skin that caused glycerin which is more viscous than others to lower absorbed

into pig skin leading to lower hydrating effect. In addition, at 30 min, the moisturizing effect on human skin of RW extract was not significantly different from carrageenan but less than glycerine and hyaluronic acid and also more than propylene glycol and sodium alginate. This indicated that RW extract could be an effective moisturizer which can be used in cosmetic products.

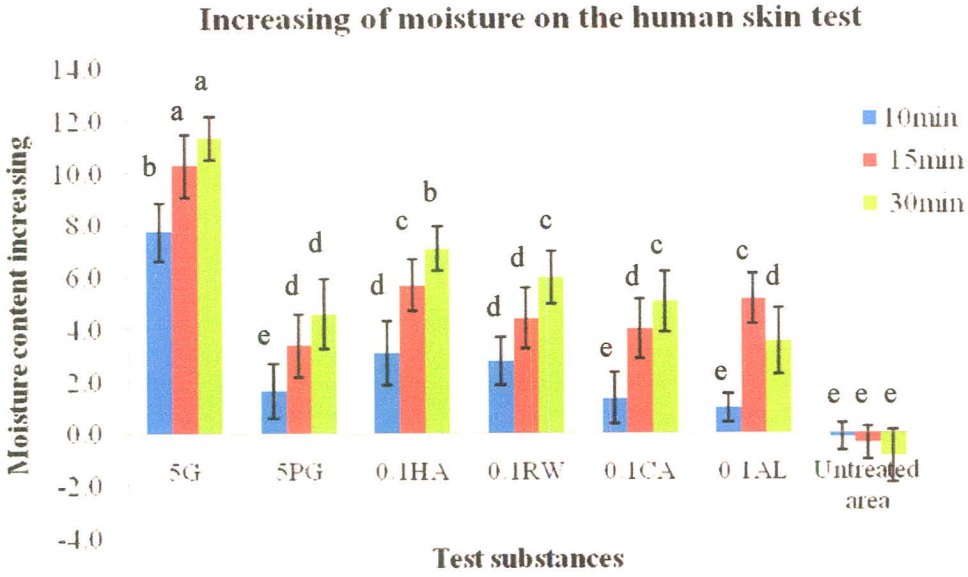


Figure 4.17 Moisture increasing on human skin after application of the test substances for 10, 15 and 30 min. (Data shown are mean ± standard error (SD) of three replicates. Letters a, b, c, d and e are statistical comparison between groups in each time.)

G : Glycerol PG : Propylene glycol,

HA : Hyaluronic acid RW : *R. hieroglyphicum* extract

CA : Carrageenan AL : Sodium alginate

The results in both pig and human skin tests indicated that RW extract was an effective skin moisturizer comparable to HA, propylene glycol and glycerin which was further formulated into skin cream.

4.9 Formulation and stability test of the cream base

4.9.1 Formulation of the cream base

Five cream formulas (A, B, C, D and E) were investigated for their physical properties: pH, texture, color, consistency, separation and precipitation, spread ability, feel on skin and short-term stability in a week as shown in Table 4.9. Formula E presented the unstable condition–cracking, while the others showed good appearances with a white color, smooth and tender textures. The pH values of formulas A and B showed nearly no difference from freshly-prepared cream. Furthermore, formula A had the best homogeneous texture and very good spread ability on skin. Therefore, formula A was chosen for stability test under the conditions at room temperature, at room temperature in the dark, 4°C and 45°C for 1 month and the heating/cooling condition of 8 cycles before being developed into active cream.

Table 4.9 Physical appearances of five cream base formulations

Formula	pH	Texture	Color	Consistency	Separation and Precipitation	Spread ability	Feel on skin	Short-term stability (in 1 week)
A	5.5	Soft & smooth	White	Tender	X	Very Good	Soft	stable
B	5.5	Soft & smooth	White	Tender	X	Very Good	Soft	stable
C	6.0	Soft & smooth	White	Tender	X	Good	Soft	stable
D	6.0	Soft & smooth	White	Tender	X	Good	Soft	stable
E	6.0	Soft & smooth	White	Tender	X	Good	Soft	Unstable (cracking)

X: No Separation and Precipitation

4.9.2 Stability test of selected cream base

Formula A was stable after the stability test and appearance, spread ability on skin of cream was very good, therefore, formula A was chosen for developing into the further alga extract cream (Table 4.10).

Table 4.10 Physical appearances of A cream base after stability test for 1 month

Storage condition	pH	Texture	Color	Viscosity (Pascal)	Consistency	Separation and Precipitation	Spread ability	Feel on skin
RT	5.5	Soft & smooth	White	1.904	Tender	X	Very Good	Soft
RT-D	5.5	Soft & smooth	White	1.593	Tender	X	Very Good	Soft
4 °C	5.5	Soft & smooth	White	1.838	Tender	X	Very Good	Soft
45 °C	5.5	Soft & smooth	White	1.434	Tender	X	Very Good	Soft
H/C (8 cycles)	5.5	Soft & smooth	White	1.671	Tender	X	Good	Soft

RT : Room temperature, RT-D : Room temperature in the dark

H/C :Heating/Cooling cycle X: Not Separation and Precipitation

4.10 Formulation and stability test of moisturizing creams

4.10.1 Formulation of moisturizing creams

The creams were determined for their physical properties, pH, spreadability, viscosity (Pas) and feel on skin. In addition, the stability was tested in various conditions as mentioned above in 3.15. In this result, the physical properties (color, smoothness and unstable conditions) of all tests creams did not change after test conditions (Table 4.11).

Table 4.11 Physical appearances of active formulations and their cream base

Formula	Texture	Color	pH	Spreadability	Feel on skin
CB	Soft & smooth	White	5.5	Very Good	Soft
CPG	Soft & smooth	White	5.5	Very Good	Soft
CG	Soft & smooth	White	5.5	Very Good	Soft
CHA 0.5	Soft & smooth	White	5.5	Very Good	Soft
CHA 0.3	Soft & smooth	White	5.5	Very Good	Soft
CRW 0.5	Soft & smooth	Yellowish	5.5	Very Good	Soft
CRW 0.3	Soft & smooth	Yellowish	5.5	Very Good	Soft

CB : Cream base CPG : Cream base + Propylene glycol,

CG : Cream base + Glycerol CHA : Cream base +Hyaluronic acid

CRW : Cream base +*R. hieroglyphicum* extract

The pH of CHA increased whereas CRW decreased, which may be due to the effect of heat to the substances. The viscosity of all creams was almost unchanged except at 45°C and heating/cooling condition (Table 4.12), which may be due to heat affected. The heat affected to pH and viscosity but not the physical appearances, therefore, the CHA and CRW cream should not be stored at high temperature for long peroid. Regarding the pH and viscosity of the sample after heating/cooling condition, the CHA0.3 and CRW0.3 were likely to be more stable than the CHA0.5 and CRW0.5 creams (Table 4.12). Therefore, CHA0.3 and CRW0.3 were selected for further study.

Table 4.12 pH and viscosity of test creams after various storage conditions for 6 months and the heating/cooling condition of 8 cycles

Test creams	Start		RT		4°C		45°C		H/C	
	pH	Viscosity (Pas)	pH	Viscosity (Pas)	pH	Viscosity (Pas)	pH	Viscosity (Pas)	pH	Viscosity (Pas)
CB	5.5	3.15 ± 0.21^{a}	5.5	2.99 ± 0.07^{a}	5.5	2.77 ± 0.09^{ab}	5.5	2.16 ± 0.01^{b}	5.5	2.88 ± 0.10^{ab}
CPG	5.5	3.33 ± 0.14^{a}	5.5	3.29 ± 0.01^{a}	5.5	3.11 ± 0.01^{a}	5.5	1.81 ± 0.03^{b}	5.5	3.15 ± 0.04^{b}
CG	5.5	3.18 ± 0.28^{a}	5.5	3.45 ± 0.16^{a}	5.5	3.03 ± 0.03^{a}	5.5	1.81 ± 0.04^{b}	5.5	3.01 ± 0.06^{b}
CHA0.5	5.5	2.87 ± 0.02^{a}	5.5	1.51 ± 0.01^{b}	5.5	1.23 ± 0.01^{d}	6.5	1.44 ± 0.01^{bc}	6.5	1.33 ± 0.07^{cd}
CHA0.3	5.5	2.04 ± 0.07^{a}	5.5	2.04 ± 0.02^{a}	5.5	1.73 ± 0.04^{b}	6.5	1.71 ± 0.12^{b}	5.5	1.89 ± 0.14^{ab}
CRW0.5	5.5	2.26 ± 0.28^{a}	5.5	2.26 ± 0.05^{a}	5.5	2.22 ± 0.04^{a}	4.5	1.63 ± 0.12^{b}	4.0	2.21 ± 0.09^{a}
CRW0.3	5.5	3.27 ± 0.11^{a}	5.5	3.12 ± 0.09^{a}	5.5	2.42 ± 0.11^{b}	4.5	1.39 ± 0.01^{c}	5.5	2.57 ± 0.06^{b}

CB : Cream base

CPG : Cream base + Propylene glycol

CG : Cream base + Glycerol

CHA : Cream base +Hyaluronic acid

CRW : Cream base +*R. hieroglyphicum* extract

4.11 Clinical evaluation

4.11.1 Skin irritation test

The skin irritation test was to determine the safety of the test creams. The dermal irritancy potential of the test substances is shown in Table 4.13. All test substances were found to be non-irritating with low primary dermal irritation index value (PDII < 0.5).

Table 4.13 Primary dermal irritation index (PDII) and skin irritation reaction in 30 volunteers

Test substances	PDII value	Classification of skin reaction
CB cream	0.00	No irritation
CPG cream	0.00	No irritation
CG cream	0.00	No irritation
CHA cream	0.06	No irritation
CRW cream	0.16	No irritation
Positive (1 % w/v SLS)	1.18	Slight irritation
Negative (DI water)	0.00	No irritation

4.11.2 Skin moisturizing test

The moisturizing effect of the test creams was evaluated on 30 healthy volunteers. Form Figure 4.18, the data showed an increased trend with the highest moisture content measured 15 min after the application. The moisture content in all tested areas was decreased after 30 min and 1 hour, which may be due to some moisture evaporated from skin in an air-conditioned room. However, the cream-treated areas still showed higher moisture content than the untreated area ($p<0.05$). This indicated that the test creams had the desired moisturizing effect on skin with different capability.

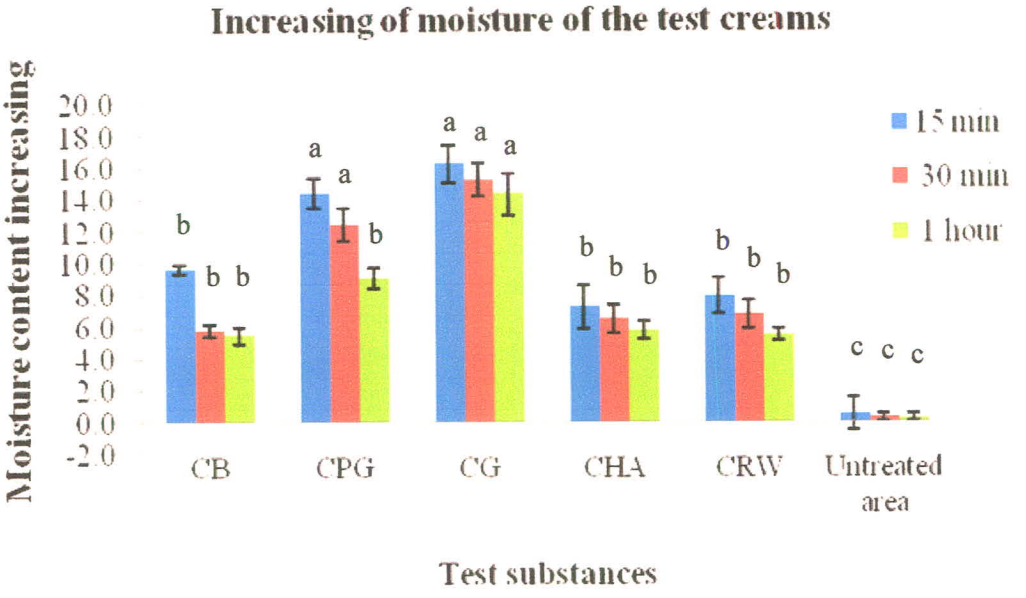


Figure 4.18 Moisture increasing after application of the test cream for 15, 30 min and 1 hour (Data shown are mean ± standard error (SD) of three replicates. Letters a, b and c are statistical comparison between groups in each time.)

CB : Cream base CPG : Cream base + Propylene glycol

CG : Cream base + Glycerol CHA : Cream base +Hyaluronic acid

CRW : Cream base +*R. hieroglyphicum* extract

Regarding a short-term moisturizing effect (15, 30 min and 1 hour) as shown in Figure 4.18, the CG showed the highest moisturizer capacity, and significantly different from CHA, CRW and CB. In addition, there was no statistically significant difference between CHA and CRW implying that RW was comparable to HA. The moisturizing effect of cream base is due to occlusive effect of the containing oily material while the PG, G, HA and RW creams showed the additional moisturizing effect on the skin due to their humectancy capability. There were no significant changes on the untreated areas of the volunteers during the period of the experiment.

For a long-term moisturizing effect after one week application of the test creams by the volunteers as shown in Figure 4.19, CG showed the highest moisture content, but not significantly different from other test creams *($p<0.05$)*. These results demonstrated that *R.hieroglyphicum* extract possessed a good moisturizing effect on human skin for long-term use similar to the effect of glycerin and hyaluronic acid. The application of HA and RW creams could maintain good skin physiological function due to the humectancy effect.

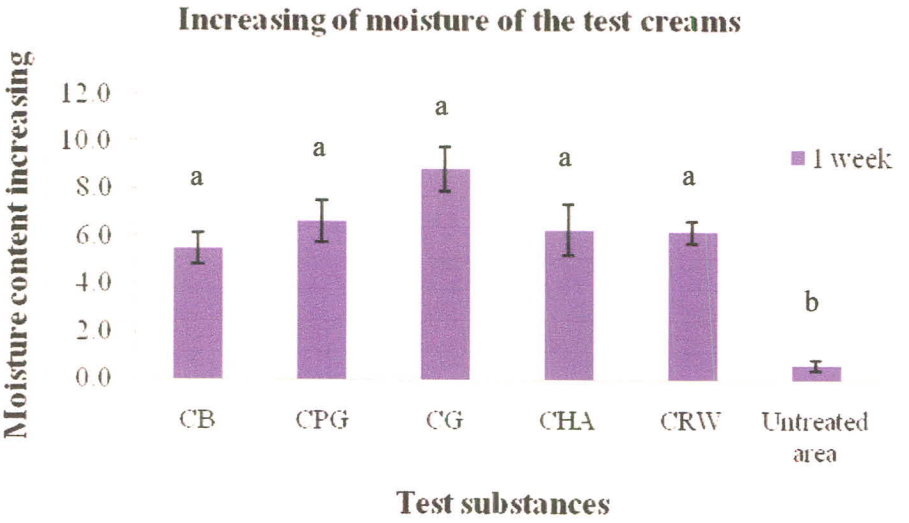


Figure 4.19 Moisture increasing after application of the test cream for 1 week (Data shown are mean ± standard error (SD) of three replicates. Letters a and b are statistical comparison between groups in each time.)

Traditionally, humectants, occlusive agents and emollients have been and continue to be the mainstay of the medical and cosmetic treatments for xerotic skin and skin moisturizing products. The most widely-used and effective humectant is glycerin because of its excellent hygroscopicity. Application of glycerin leading to swelling and hydrating the stratum corneum will thus smooth down the scales.

Benefits of the long-term application of glycerin can be seen and felt. The effect is the smoothening of the skin surface [175]. Therefore, application of glycerin results in a higher moisturizer effect, probably from its humectant effect while the effect of propylene glycol is different. In this study, propylene glycol provides a lower moisturizer content than glycerin after 1 week of application.

Hyaluronic acid is a biopolymer naturally occurring in the skin and other tissues. It is an important component of the skin matrix and also a popular skin care ingredient. Hyaluronic acid is a highly effective humectants, since it can hold thousands of times its weight in water. It is mostly used in moisturizing formulas and provides effective skin surface hydration [177]. The present findings indicate that the extract of *R.hieroglyphicum*, which is also biopolymer, provides the moisturizing effect that resemble hyaluronic acid and glycerin. This result demonstrated that the extract of *R. hieroglyphicum* is a hygroscopic substance and probably a class of sugar units containing as in hyaluronic acid that provides the same function as a humectant on the skin's surface. These substances are supposed to penetrate into the skin and increase the degree of hydration of the SC [55].

Moreover, the human primary skin irritation test exhibited that the RW caused no irritation to human skin, either immediately or during the entire course of the experiment. Therefore, it has a high potential for use in cosmetics and personal care products as well as topical pharmaceutical products, and is safe for human consumption by the topical route.

4.11.3 Satisfaction of RW cream and test creams by volunteers

The volunteers' feelings reflected in a questionnaire, the range of satisfaction with moisturizing creams from "like extremely to like moderately" exhibited more

than 80% in all topics. The result here indicated that the subjects thought the product application could add and retain moisture to the skin of the volunteers. Also, cream can control skin moisture for the whole day and even for one week under long-term application. Moreover, the range of satisfaction showed that the subjects satisfaction with CRW was similar to CHA (Figure 4.20). Additionally, there was no report of skin irritation or allergic reaction during the period of application.

Figure 4.20 Satisfaction of volunteers on moisturizing effect of the test creams

The satisfaction of the volunteers was completed by the questionnaire regarding product appearance and skin feeling.

Table 4.14 The percentage of satisfaction on CRW and CHA cream

Topic	Satisfaction on product (%)									
	CRW cream					CHA cream				
	Very good	**Good**	**Average**	**Poor**	**Very poor**	**Very good**	**Good**	**Average**	**Poor**	**Very poor**
1. Color	41.9	41.9	13.2	3.0	0	51.6	41.9	3.2	3.3	0
2. Odor	48.3	35.4	9.6	6.7	0	38.7	48.4	6.4	6.5	0
3. Texture	32.2	35.4	25.7	6.7	0	12.9	58.1	25.8	3.2	0
Topic	**Satisfaction on skin feeling (%)**					**Satisfaction on skin feeling (%)**				
	Very good	**Good**	**Average**	**Poor**	**Very poor**	**Very good**	**Good**	**Average**	**Poor**	**Very poor**
1. Skin moisture	41.9	41.9	16.2	0	0	16.1	64.5	19.4	0	0
2. Softness of cream	22.5	58.0	19.5	0	0	9.7	61.3	29	0	0
3. Spreadability	29.1	61.3	9.6	0	0	16.1	48.4	35.5	0	0
4. Skin absorption	32.3	32.3	29.0	7.0	0	16.1	48.4	32.3	3.2	0
5. Cream glossy	18.9	51.6	22.5	7.0	0	12.9	51.6	32.2	3.3	0
6. Film forming	25.8	58.1	16.1	0	0	9.7	45.2	32.3	12.8	0
7.Overall satisfaction	33.1	46.9	15.7	4.3	0	38.1	44.5	14.3	3.1	0

Table 4.15 The percentage of satisfaction on CG and CPG cream

Topic	Satisfaction on product (%)									
	CG cream					CPG cream				
	Very good	**Good**	**Average**	**Poor**	**Very poor**	**Very good**	**Good**	**Average**	**Poor**	**Very poor**
1. Color	35.5	58.1	6.4	0	0	29.0	67.7	3.3	0	0
2. Odor	41.9	41.9	9.7	6.5	0	38.7	45.2	16.1	0	0
3. Texture	19.4	48.4	32.2	0	0	25.8	51.6	22.6	0	0
Topic	**Satisfaction on skin feeling (%)**					**Satisfaction on skin feeling (%)**				
	Very good	**Good**	**Average**	**Poor**	**Very poor**	**Very good**	**Good**	**Average**	**Poor**	**Very poor**
1. Skin moisture	22.5	58.1	19.4	0	0	48.4	35.5	16.1	0	0
2. Softness of cream	19.4	51.6	29.0	0	0	25.8	41.9	31.3	0	0
3. Spreadability	16.1	58.1	25.8	0	0	22.5	41.9	29.0	6.6	0
4. Skin absorption	19.4	54.8	25.6	0	0	19.4	51.6	22.5	6.5	0
5. Cream glossy	3.2	67.7	25.6	3.5	0	25.8	29.0	35.5	9.7	0
6. Film forming	12.9	51.6	35.5	0	0	16.1	45.2	29.0	9.7	0
7.Overall satisfaction	20.0	41.2	36.2	2.6	0	24.0	39.4	34.6	2.0	0

Table 4.16 The percentage of satisfaction on CB cream

Topic	Satisfaction on product (%)				
	CB cream				
	Very good	**Good**	**Average**	**Poor**	**Very poor**
1. Color	45.1	41.9	13	0	0
2. Odor	41.9	41.9	6.5	9.7	0
3. Texture	16.2	54.8	29.0	0	0
Topic	**Satisfaction on skin feeling (%)**				
	Very good	**Good**	**Average**	**Poor**	**Very poor**
1. Skin moisture	32.3	38.7	25.8	3.2	0
2. Softness of cream	22.6	48.4	25.8	3.2	0
3. Spreadability	19.4	40.3	40.3	0	0
4. Skin absorption	19.4	45.2	32.3	3.1	0
5. Cream glossy	12.9	41.9	35.5	9.7	0
6. Film forming	22.5	41.9	29.0	6.6	0
7.Overall satisfaction	23.3	42.7	34.0	0	0