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Original Article

CHARACTERIZATION OF THE MUCILAGES EXTRACTED FROM *HIBISCUS ROSA-SINENSIS* LINN AND *HIBISCUS MUTABILIS* LINN AND THEIR SKIN MOISTURIZING EFFECT

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ABSTRACT

Objective: The present study was aimed to investigate the physicochemical properties, chemical characterization and skin moisturizing effect of the mucilages extracted from *Hibiscus rosa-sinensis* (HR), and *H. mutabilis* (HM).

Methods: The HR and HM leaves were extracted with distilled water to obtain their mucilages. Then dried and powdered. The physicochemical properties of the mucilage powders, including pH value, swelling index, loss on drying, solubility, total ash, acid-insoluble ash and carbohydrate content were evaluated. The polysaccharides were isolated from the mucilages and purified by DEAE-650M column and identified the sugar unit constituents by acid hydrolysis, followed by TLC and HPAEC analyses. The short-term moisturizing effect of the mucilages was determined on pig skin using Corneometer®.

Results: The yield of HR and HM mucilages was 21% and 15% w/w. Each of mucilage showed the specific physicochemical properties. The main component of HR and HM mucilages was acidic polysaccharides named as AHR and AHM. The major components with the Mol% of AHR were 27% galactose, 24% rhamnose, 19% galacturonic acid, and 18% arabinose, while AHM were rich in 27% rhamnose, 25% galactose, 18% xylose, 16% arabinose and 9% galacturonic acid. The skin moisturizing effect of 0.2 % HR mucilage was significantly more effective than 0.2% HM mucilage, 0.2% hyaluronic acid, 5% propylene glycol and 5% butylene glycol at 30 min after application.

Conclusion: The results suggest that mucilage extracted from *Hibiscus rosa-sinensis* was more superior in quality than *Hibiscus mutabilis* mucilage for using as a good moisturizer in the skin care product.

Keywords: *Hibiscus rosa-sinensis*, *Hibiscus mutabilis*, Mucilage, acidic polysaccharide, Skin moisturizing effect.

INTRODUCTION

Plant mucilages are hydrocolloid and typically form the complex polysaccharides consisting of sugars and uronic acids [1]. Mucilages found in all parts of plants as in rhizomes, roots and seed endosperms may indicate primarily as energy reserves but foliar mucilages may play a role in water transport, wound responses, plant host pathogen interactions, frost tolerance and drought resistance. Because of the high concentration of hydroxyl groups in the polysaccharides, the mucilages normally have a high water holding capacity [2, 3].

In recent years, mucilages are widely used in pharmaceutical and cosmetic applications such as binder, disintegrant, emollient, emulsifier, gelling agent, granulating agent, lubricant, suspending agent, sustained release agent and skin soothing agent. Furthermore, their therapeutic values have been investigated for diabetes, immunostimulation, cancer, angiotensin converting enzyme inhibition, stomachic, anti-inflammation, wound healing and antioxidant properties. They are considered advantageous compared to synthetic materials due to their non-toxic, biodegradable, biocompatible, low cost and local availability [4-6]. The demand of natural mucilages is increasing continuously. Therefore, the new sources are being explored to meet the demands.

Hibiscus is the genus of shrubs, subshrubs, trees or herbs in the Malvaceae family. Its several species are widely distributed in different regions of Asian continent for horticultural and ornamental purposes because of its showy and colorful flowers [7]. *Hibiscus* plants are one of the rich sources of mucilaginous component and two species, *Hibiscus rosa-sinensis* Linn (HR) and *Hibiscus mutabilis* Linn (HM) were then selected for this study. HR is known as the shoe flower, China rose or Chinese hibiscus [8]. Leaves of this plant are traditionally used as aperients and emollients in the treatment of burning sensations and skin disease. The HR mucilage had been studied as oral disintegrant, binder and release-retarding agent [9,10]. The mucilage of leaves called Hibiscus-mucilage RL mainly consists of L-rhamnose, D-galactose, D-galacturonic acid, and D-glucuronic acid and its molecular mass was 1.0×10^7. Methylation analysis, partial hydrolysis, and nuclear magnetic resonance studies indicated its main structural features including a unique backbone chain composed of alpha-1,4-linked D-galactosyl alpha-1,2-linked L-rhamnosyl alpha-1,4-linked D-galacturonic acid units[11]. *Hibiscus mutabilis* Linn (HM) is known as cotton rose mallow or confederate rose. This plant is used in traditional medicine as emollients, stimulants and pulmonary complaints. HM extract and isolated constituents were reported to have anti-inflammatory, anti-bacterial, anti-allergic, nitric oxide scavenging, anti-proliferative, α-glucosidase inhibitory and HIV-1 reverse transcriptase inhibitory activities. HM was also reported containing flavones, flavone glycosides, anthocyanins and lectin in different parts [12, 13].

The present study deals with the isolation, physicochemical properties and chemical characterization of the mucilages extracted from the leaves of HR and HM. These *Hibiscus* mucilages have not been explored as moisturizing agent. Thus, this study intends to examine their skin moisturizing effect for further developing as topical pharmaceutical and/or cosmetic skin care products.

MATERIALS AND METHODS

Plant materials and chemicals

Fresh leaves of HR and HM were collected from the garden in Faculty of Pharmacy, Chiang Mai University, Thailand in July 2012. TLC Silica gel 60 GF_{254} and Toyopearl® DEAE 650M were purchased from Merck (Germany). The standard monosaccharides and uronic acids were obtained from Sigma-Aldrich (Germany). All other chemicals used were analytical grade.

Mucilage extraction

Each of HR and HM leaves was washed with water to remove all foreign matters, dried at 50ºC and cut into small pieces. Then, the materials were pre-extracted with 95% ethanol for 24 h at room temperature, filtered and then dried for 24 h at room temperature. Each 100 g of HR and HM dried residues were further extracted with 60°C distilled waters (1 L) for 1.5 h and kept aside for 1 h to complete the releasing of mucilage into water. The material was filtered to separate the mucilage from the residue, dried in an oven at 50°C, powdered, weighed and stored in desiccator.

Physicochemical characterization of mucilages

The obtained mucilage powders were screened for carbohydrates by preliminary standard tests; Molisch's test, Iodine test, Benedict's test, and Ruthenium Red test [1]. The physicochemical properties such as pH, swelling index, solubility, loss on drying, total ash, and acid–insoluble ash were determined according to British Pharmacopoeia procedure [14] with some modifications. The swelling index is the volume in mL taken up by 0.1 g of a sample after it has been swollen in aqueous solution for 4 h.

Isolation and purification of polysaccharide from mucilages

Each mucilage powder (1 g) was purified by treatment with 2 M sodium hydroxide (20 mL) under constant stirring for 24 h at room temperature and centrifuged at 3500 rpm for 15 min. The collected supernatants were neutralized with 8 M hydrochloric acid and precipitated with methanol (100 mL). The precipitates were then collected by centrifugation, washed with acetone, and finally dried to yield the crude polysaccharides. Fractionating to neutral and acidic polysaccharides were done by anion-exchange chromatography as follows; the crude polysaccharide (50 mg) was dissolved in 0.005 M ammonium formate buffer (pH 5), and applied to an equilibrated DEAE-650M column (1.8 cm x 25 cm). The neutral fraction was firstly eluted from column with the same buffer at a flow rate of 2.0 mL/min and the acidic fraction was further eluted by 0.35 M ammonium formate buffer. The eluting fractions were concentrated and lyophilized to obtain the main fractions. Washing with methanol and blowing with N_2 was then proceeding to give dried precipitates. The dried precipitates were further re-dissolved in distilled water and lyophilized to provide the neutral and acidic polysaccharides.

Determination of monosaccharide composition

Thin-layer chromatography (TLC)

Two mg of each polysaccharide as crude, neutral and acidic parts were hydrolyzed with 0.2 mL of 2 M trifluoroacetic acid (TFA) under constant stirring at 100ºC for 2 h and then TFA was removed under nitrogen gas. Each hydrolysate was dissolved in 0.4 mL methanol and applied onto TLC using mobile phase system of CH_3CN: H_2O (17:3), and detected by spraying with thymol-sulfuric acid reagent and heated at 100ºC [15]. The R_f values of the hydrolysates were compared with standard monosaccharides. Standard monosaccharide 1 (S1) composed of 1 mg/mL of D-mannose (Man), L-rhamnose (Rha), D-galactose (Gal), D-xylose (Xyl) and L-arabinose (Ara). Standard monosaccharide 2 (S2) composed of 0.25 mg/ml of Rha, Xyl, Ara, D-glucose (Glc) and 0.5 mg/mL of D-galacturonic acid (GalA) and D-glucuronic acid (GlcA).

High performance anion exchange chromatography (HPAEC)

The evaluations of the monosaccharide composition of each 2 mg/mL of hydrolysed HR and HM acidic polysaccharide fraction were performed on a Dionex system (Dionex, USA) using a CarboPac™PA10 analytical column (4 mm×250 mm) and a CarboPac™PA10 guard column (4 mm×50 mm). Detection was carried out by PDA-100 photodiode array detector. The column was eluted at a flow rate of 1 mL/min with a gradient elution from 200 mM sodium hydroxide (eluent A), H_2

Short-term skin moisturizing test

The moisturizing efficacy of 0.2% HR mucilage and 0.2% HM mucilage were examined and compared with the commercial moisturizers; 5% glycerin, 5% propylene glycol (PG), 5% butylene glycol (BG) and 0.2% hyaluronic acid (HA). The tested skins were prepared from the back of 6 months age pigs, removed the fat layer off and cut into 3 x 3 cm. The prepared skins were incubated at room temperature (25 ± 1°C) with 50-60% RH at least 30 min before use. Then, 60 µL of each test sample were applied on the skin surface. The moisture content was measured before applying and at time 10, 20 and 30 min after applying the sample using Corneometer®. All of the measurements were done in triplicate. Skin moisturizing efficacy (%) was calculated as: Skin moisturizing efficacy (%) = [(At - A0)/A0] x 100 where At = skin capacitance at a specified time and A0 = skin capacitance at the base line. This method had been modified from O'Goshi *et al* [17] and Leelapornpisid *et al* [18].

Statistical analysis

The experiments were done in triplicate. Statistical analysis was performed by one-way ANOVA post hoc LSD test; $p<0.05$ were considered statistically significant as compared between groups in each time. Results were processed by SPSS software version 17.0**.**

RESULTS AND DISCUSSION

Mucilage extraction

The mucilage powders from leaves of HR and HM were light brown color with characteristic odor and yielded 21%, and 15% w/w, respectively. The yield of HR mucilage in this study was higher than that reported by Shaha *et al* [9], who found in 17%.

Chemical and physicochemical characterizations

Preliminary confirmative tests for dried mucilages as shown in table 1, both mucilages showed the presence of carbohydrate and mucilage by the positive results with Molisch's test and ruthenium red test, respectively. On treatment of both mucilages with iodine, they showed yellow solution confirming the absent of starch.

Table 1: Preliminary confirmative tests for dried mucilages

Tests	Observations	Inferences of mucilage	
		HR	HM
Molisch's test	Violet green ring observed at the junction of the two layers.	Carbohydrate present	Carbohydrate present
Ruthenium red test	Pink color develops on powdered particles.	Mucilage present	Mucilage present
Iodine test	The color of solution does not change.	Starch is absent	Starch is absent

The 0.2% w/v solution of HR and HM mucilages in water gave pH of 6.56±0.03, and 6.32±0.03, respectively. Both mucilages were almost neutral pH, which may be less irritation for their application as skin care product [19]. The swelling index of HR mucilage (200±0.00 mL/g) was higher than HM mucilage (150±0.00 mL/g). The difference in swelling index was associated with the abilities of mucilage to absorb moisture and water holding capacity [20]. Due to the polysaccharide matrix can hold water causing important swelling and viscous solution. Both mucilages were soluble in water with light brown solution and they were practically insoluble in most of organic solvents. The loss on drying of HR mucilage (3.45±0.21%) was slightly more than HM mucilage (3.27±0.13%). Total ash and acid-insoluble ash contents are considerable indices to demonstrate the quality as well as purity of mucilage. The total ash

content of HM mucilage (22.18±0.05%) was more than that of HR mucilage (19.63±0.08%), while the acid-insoluble ash content of HR mucilage (0.43±0.13%) was slightly higher than that of HR mucilage (0.26±0.11%), respectively.

The acid-insoluble ash contents were found very low in both mucilages, which indicated the small content of some contaminants. The physicochemical properties of HR and HM mucilages are summarized in table 2.

Table 2: Physicochemical characterizations of dried mucilages

Parameters	HR mucilage	HM mucilage
pH	6.56±0.03	6.32±0.03
Swelling index (ml/g)	200±0.00	150±0.00
Solubility	Soluble in water and insoluble in methanol, ethanol, and acetone	Soluble in water and insoluble in methanol, ethanol, and acetone
Loss on drying (%)	3.45±0.21	3.27±0.13
Total ash (%)	19.63±0.08	22.18±0.05
Acid–insoluble ash (%)	0.43±0.13	0.26±0.11

*Values are expressed in mean ± SD, n=3.

Isolation and purification of polysaccharide from mucilage

Polysaccharides of malvaceous mucilage were isolated by precipitation in 2M sodium hydroxide and methanol. The light brown crude polysaccharides were obtained with the yields of 34% and 20% w/w from HR and HM mucilages. Each of the crude polysaccharides was further separated into two main fractions on DEAE-650M column. The neutral polysaccharide fraction was firstly eluted with 0.005 M ammonium formate buffer while the acidic fraction was later obtained from 0.35 M ammonium formate buffer elution. HR polysaccharides consisted 70% of acidic polysaccharide (AHR) and 26% of neutral polysaccharide (NHR), whereas HM polysaccharide consisted 43% of acidic polysaccharide (AHM) and 33% of neutral polysaccharide (NHM). The acidic part was a major component of both mucilages, which were according to other malvaceous mucilages reported in the leaves of Malva sylvestris [21], Adansonia digitata [22] and *Althea officinalis* [23].

Analysis of monosaccharide compositions

The composition analysis of polysaccharide is typically based on hydrolysis procedure using TFA due to its efficacy at the hydrolyzing glycoside bonds without resulting in extensive destruction of the monosaccharide components and due to its volatility, which reduces its interference with subsequent procedures [16]. According to TLC chromatogram as shown in **Fig. 1**, the acid hydrolysis of AHR and AHM consisted of Rha, Gal, GalA, Xyl, Ara and GlcA.

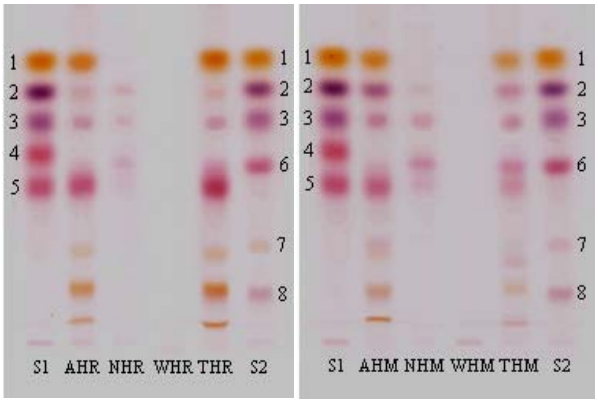

Fig. 1: TLC chromatograms of malvaceous polysaccharide; 1= rhamnose, 2=xylose, 3=arabinose, 4=mannose, 5=galactose, 6=glucose, 7=glucuronic acid, 8=galacturonic acid, S1=standard 1, S2=standard 2, AHR=acidic polysaccharide of HR, NHR=neutral polysaccharide of HR, WHR=HR polysaccharide without acid hydrolysis, THR=total polysaccharide of HR after hydrolysis, AHM=acidic polysaccharide of HM, NHM=neutral polysaccharide of HM, WHM=HM polysaccharide without acid hydrolysis, THM=total polysaccharide of HM after hydrolysis

0 (eluent B), and a mixture of 200 mM sodium hydroxide and 200 mM sodium acetate (eluent C) using the following program: 0-18 min 6% A and 94% B, 18-35 min 50 to 90% C in A. Each monosaccharide concentration was determined by Chromeleon management system and compared with standard curves of Rha, Xyl, Ara, Gal, Glc, GalA, and GlcA [16].

The hydrolyzed NHR and NHM mainly contained Xyl, Ara and Glc, whereas mannose was absent in both polysaccharides. HR and HM polysaccharides without acid hydrolysis designated as WHR and WHM respectively were not detected any monosaccharide and uronic acid. The qualitative analysis revealed both HR and HM mucilages were the heterogeneous mixture of polysaccharides consisted of neutral monosaccharides and uronic acids.

Quantitative analysis of monosaccharide composition of AHR and AHM after acid hydrolysis was further investigated by HPAEC, and results are shown in table 3 and **Fig. 3-4**. From the HPAEC chromatogram of standard monosaccharides (**Fig. 2**), the retention times of Rha, Ara, Gal, Glc, Xyl, GalA and GlcA were at 9, 10, 13, 14, 16, 26 and 27 minutes, respectively. The main components with the Mol% of AHR were 27% Gal, 24% Rha, 19% GalA, and 18% Ara, while of AHM were rich in 27% Gal, 25% Rha, 18% Xyl, 16% Ara and 9% GalA. AHR was composed of Rha, Ara, Gal, Xyl, GalA and GlcA in the mole ratio of 5:4:5:1:4:1 while the mole ratio of AHM was 5:3:5:4:2:1. The uronic acids ratio of AHR is considerably greater than AHM. The mole ratio of AHM is firstly reported here. In case of AHR, their monosaccharide compositions are similar to the previous report by Shimizu *et al* [11] who found that major constituent was the acidic polysaccharide composed of Rha, Gal, GalA, and GlcA with the mole ratio of 5:8:3:2 that is partially different from our study.

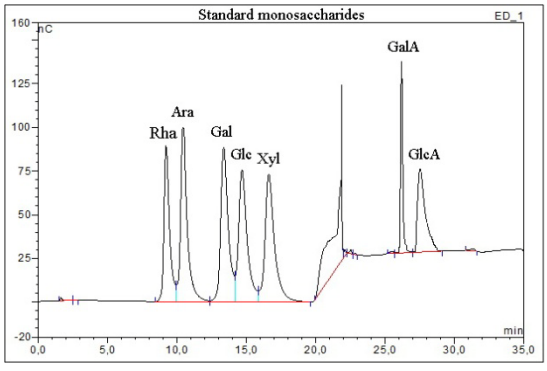

Fig. 2: HPAEC chromatogram of standard monosaccharides

Short-term skin moisturizing test

The moisturizing efficacy of the tested substances was evaluated on pig skin model. The selected pig skin model relies on a number of factors, including cost, availability, ease of handling, functional and structural similarity to humans [24]. This study was accomplished using Corneometer® by the measurement of skin electrical capacitance (which is related to water content in the stratum corneum). From **Fig. 5**, the short-term moisturizing effect of all tested substances showed significantly difference from control

(untreated area) at $p<0.05$ and showed the decrease of moisturizing efficacy with times. 5% Glycerin exhibited the highest skin moisturizing effect, followed by 0.2% HR mucilage, 0.2% HA, 0.2% HM mucilage, 5% BG, 5% PG and water. The moisturizing effect of HR mucilage was significantly better than that of HM mucilage that may be related to the uronic acid content.

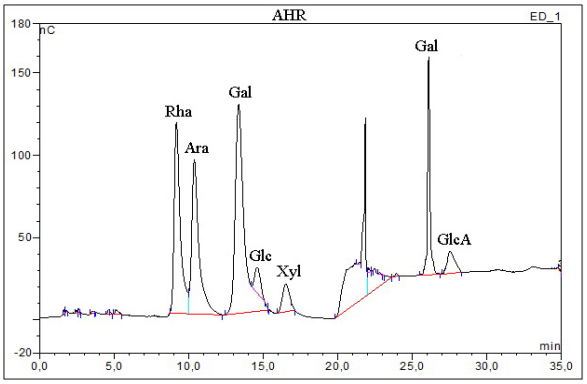

Fig. 3: HPAEC chromatogram of hydrolysed AHR.

Due to the high negative charge resulting from the carboxyl and hydroxyl group of GalA and GlcA, HR mucilage may have higher water holding capacity and maintain skin water content [25, 26]. Moreover, polysaccharide may moisten the skin by enhancing the skin barrier and/or increasing dermal mucopolysaccharides [27]. Interestingly, it was found that HR mucilage could keep moisture on pig skin longer than HA, BG and PG, especially at 30 min after application. The results indicated that HR mucilage could be an effective moisturizer for cosmetic products.

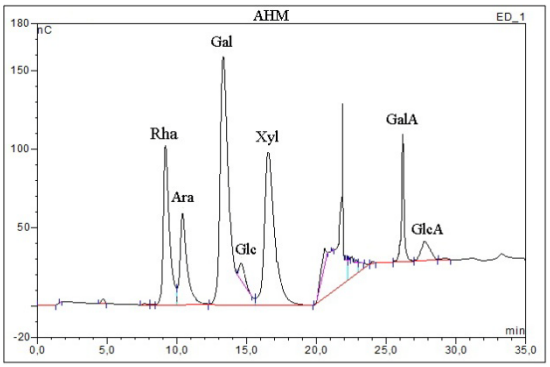

Fig. 4: HPAEC chromatogram of hydrolysed AHM

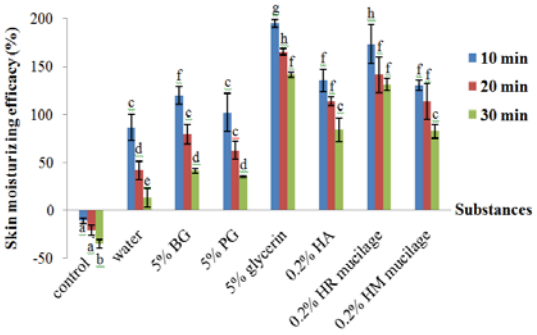

Fig. 5: Skin moisturizing efficacy on pig skin after application of the tested substances at 10, 20 and 30 min measured by Corneometer®. (Data shown are mean ± SD. Mean with different letters (a-h) differ significantly at $p < 0.05$)

Table 3: Monosaccharide composition of the HR and HM acidic polysaccharides

Sample	Mol%						
	Rha	Ara	Gal	Glc	Xyl	Gal A	Glc A
AHR	24	18	27	3	6	19	3
AHM	25	16	27	2	18	9	3

*Individual compositions were identified and quantified based on elution of known standards.

CONCLUSION

The mucilage extracted from two *Hibiscus* leaves showed their specific physicochemical characteristics. The major monosaccharides of these mucilages are Rha, Gal, GalA, Ara and Xyl in various quantitative ratios, which affect to the different physical, chemical and biological properties. The results revealed that HR mucilage was superior in skin moisturizing effect to HM mucilage. Thus, HR mucilage is a promising skin moisturizer. The moisturizing effect in volunteers and development of cosmetic product containing mucilage will be further investigated.

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CONFLICT OF INTERESTS

Declared None.

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