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Research Article

Development of roselle ointment with antibacterial effects

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ABSTRACT

Hibiscus sabdariffa (roselle or krachiap daeng in Thai) is a plant commonly used as a beverage and herbal medicine for diuretic purposes. There is no previous development of topical formulation from quality-controlled roselle extract. To develop an ointment containing the roselle extract and investigate for the physical, chemical, and biological stabilities. The ointment was developed from roselle extract. Physical properties including organoleptic characteristics, viscosity, pH and microbial contamination were investigated. Phytochemical characteristics were investigated by thin layer chromatographic technique. Total anthocyanin contents were analyzed by pH differential methods. In vitro antibacterial activities against clinical isolated bacteria were evaluated by agar well diffusion method. The stability test of the ointment was conducted according to ASEAN guideline on stability study and shelf-life of traditional medicine (2013). Roselle ointment appeared as dark red semi-solid with unique odor, smooth texture, pH 3.81 ± 0.02 and no microbial contamination. Thin layer chromatography showed the major bands corresponded to delphinidin-3-sambubioside, cyanidin-3-sambubioside, delphinidin-3-glucoside, and cyanidin-3-glucoside. The ointment kept in the refrigerator (2 – 8 °C) showed the highest remaining total anthocyanin content (1.50 ± 0.00 mg% cyanidin-3-glucoside equivalent (mg% C3GE) of the ointment) after 4 weeks. Roselle ointment in all storage conditions still promoted inhibitory effects against *Staphylococcus aureus* and *Staphylococcus intermedius* with the inhibition zone ranging from 9.61 ± 0.10 to 11.97 ± 0.05 mm at the end of stability test. Roselle ointment was developed with good physical, chemical, and biological activities. The refrigerator was recommended as storage condition to prolong chemical stability of the ointment.

Keywords:
Hibiscus sabdariffa; Roselle; Ointment; Anthocyanins; Antibacterial activity; Stability

1. INTRODUCTION

Hibiscus sabdariffa or roselle is a plant belongs to the Malvaceae family. The calyces of roselle have been used in traditional medicines and commonly consumed as food and drinks. Infusions of calyces of roselle have been widely used in traditional medicines of many countries[1]. For Thai traditional medicine, roselle has been used as antibacterial, hypocholesterolemia, diuretic, mild laxative, and antihypertensive agents[2]. According to Thai Herbal Pharmacopoeia 2023, the calyces of roselle have been indicated as diuretic agent[3]. There are several bioactive phytochemicals found in roselle including phenolic acids (hydroxybenzoic and hydroxycinnamic acids), flavonoids (flavonols, flavanols, flavones, flavanones, anthocyanins), and others[4] Anthocyanins are compounds responsible for the red color of roselle calyx. The major anthocyanins in roselle calyces are delphinidin-3-sambubioside and cyanidin-3-sambubioside while cyanidin-3-glucoside and delphinidin-3-glucoside are also found[5].

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Various infectious diseases result in health problems due to increasing strains of microorganisms and developing resistance to antibiotics. It has created serious global public health threats that exert a significant burden in terms of patient morbidity and financial crises[6]. Moreover, the prescription of antimicrobial drugs for bacterial infections is the standard treatments not only for human uses but also for animal therapies. Therefore, the new antimicrobial substances from plant sources for veterinary medicine have received a lot of attention in recent years.

Previous studies reported the antibacterial effects of roselle calyx aqueous and ethanol extracts against the tested bacteria (*Salmonella typhimurium* DT104, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Bacillus cereus*) with the minimum inhibitory concentration the (MIC) values ranged from 112 - 144 and 72 - 96 µg/mL, respectively[7].

Nowadays, there is no previous development of topical formulation from quality-controlled roselle extract. Therefore, the objectives of this study were to develop an ointment containing the roselle extract and investigate for the physical, chemical, and biological stabilities.

2. MATERIALS AND METHODS

2.1. Preparation of plant material

The dried roselle calyces were purchased from local market in Bangkok, Thailand, in 2021. Plant samples were identified according to their botanical characteristics described in Flora of Thailand volume 14, part 2, 2019[8] (Voucher No. N. Chongwilaikasem 001). Plant voucher specimen was kept in Thai Traditional Medicine Herbarium, Department of Thai Traditional and Alternative Medicine, Thailand (Voucher No. TTM-c No. 1000792). Plant samples were cleaned and dried in a hot air oven at 60 °C for 2 hours and powdered using electric grinder (500 g multifunction disintegrator WF-10B, Thailand). Roselle powder was kept in desiccator at room temperature until used.

2.2. Preparation of roselle extracts

Roselle powders were extracted in 80% ethanol with 1% hydrochloric acid (HCl) using ultrasonic extraction machine with 60 minutes extraction time, ultrasonic power of 280 W and the frequency of 37 kHz. Plant/solvent ratio was 1:10 w/v, and the extraction temperature was 40°C. The extraction process was repeated 3 times. The extract solution was filtered through Whatman No.1, then the filtrates were combined and evaporated by a rotary evaporator (BUCHI, France) and lyophilized using a freeze-dryer (SciQuip Ltd., UK). The percentage yield of the extract (%yield) was calculated. The extract was kept at -20 °C until use.

2.3. Phytochemical analysis of roselle calyx extracts

2.3.1. Determination of total anthocyanin content by standard calculation

The total anthocyanin content of roselle calyx extract was determined using the pH-differential method according to the guidelines of AOAC[9]. The experiment was analyzed in triplicate. The total anthocyanin was calculated as cyanidin 3-glucoside equivalent as follows:

$$A = (A_{520nm} - A_{700nm})pH\ 1.0 - (A_{520nm} - A_{700nm})pH\ 4.5$$

Where A = the absorbance measured by the pH differential method that was used to calculate total anthocyanin from the equation below

A_{520nm} = the absorbance measured at 520 nm

A_{700nm} = the absorbance measured at 700 nm

Anthocyanin content (cyanidin-3-glucoside equivalents, mg/L) = (A x MW x DF x 1000)/(εx1)

Where MW (molecular weight) = 449.2 g/mol for cyanidin-3-glucoside (C3G)

DF = dilution factor

l = pathlength in cm

ε = 26,900 molar extinction coefficient, in L x mol^{-1} x cm^{-1}, for C3G

10^3 = factor for conversion from g to mg

The average result and standard deviation of total anthocyanin content was calculated.

2.3.2. Thin layer chromatographic (TLC) analysis

Thin layer chromatographic (TLC) analysis of roselle calyx extract was evaluated using the analytical condition as following. TLC system[10]:

- Stationary phase: silica gel 60 F254
- Mobile phase: ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:26 v/v/v/v)
- Detector: UV cabinet under 254 and 366 nm
- Spray reagent: natural product/polyethylene glycol spray reagent (NP/PEG) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) spray reagent

2.4. Determination of in vitro antibacterial activity

2.4.1. Disk diffusion method

The disk diffusion method was used for evaluation of antibacterial effects of roselle calyx extract according to Clinical and Laboratory Standards Institute (CLSI) guidelines[11]. The assay was performed against 2 clinically pathogenic zoonotic bacteria including *Staphylococcus aureus* and *Staphylococcus intermedius*. The bacteria strains were obtained from the Microbiological Laboratory, Veterinary Diagnostic Center, Faculty of Veterinary Medicine, Kasetsart University, Nakhon Pathom, Thailand and were isolated and characterized using differential bacterial culture and biochemical assays for clinical samples according to the standard method of Baron et al.[12]. Standard antibiotic disc; amoxycillin 10 µg (AML10), gentamicin 10 µg (CN10) and doxycycline 30 µg (DO30) (Oxoid, USA) were used as positive controls. All tests were conducted in triplicate and the average zone of inhibition was calculated with the standard deviation.

2.4.2. Agar well diffusion method

The agar well diffusion method was used for evaluation of antibacterial effects of roselle ointment according to the clinical and laboratory standards institute (CLSI) guidelines[11] The agar well diffusion method was used to determine the diameter of the inhibition zone of ointment and ointment base against *S. aureus* and *S. intermedius*. A hole with a diameter of 6 mm was punched with a sterile cork plunger then added 100 mg of ointment and ointment base into the well. The plate was incubated at 37 °C for 18-24 hours and the diameter of the clear zone was recorded. Standard amoxycillin (AML10), gentamicin (CN10) and doxycycline (DO30) were used as positive controls while ointment base was used as negative controls. All tests were conducted in triplicate and the average zone of inhibition was calculated with the standard deviation.

2.5. Development of roselle ointment

Roselle ointment was prepared by infusing roselle calyx extract to an ointment base. The roselle calyx extract was dissolved in diluent then it was sonicated and warmed using a water bath. Then the base was prepared by melting the ointment base on a water bath, then moisturizing agent was added. The ointment base was then the stirred until it completely dissolved then added emulsifying agents and antioxidant which were previously warmed. Then the roselle extract portion that was previously warmed was added into the warm ointment base. The mixture was stirred until it was set.

2.5.1. Quality control of pharmaceutical products

The developed roselle ointment was quality controlled for their physical, chemical, and biological properties as following;

1) Physical property

- Organoleptic characteristics

The organoleptic characteristics of roselle ointment including color, odor, texture and homogeneity were manually examined and recorded.

- Viscosity

The viscosity of roselle ointment was measured using Hakke rotational plate viscometer with sensor C35,2. The measurement was recorded for 3 times then the average and the standard deviation of the results were calculated.

- pH

The pH of roselle ointment was determined using a pH meter. The measurement was recorded for 3 times then the average and the standard deviation of the results were calculated.

- Microbial content

The amount of microbial contamination including *Staphylococcus aureus, Pseudomonas aeruginosa*, and *Clostridium spp*. present in roselle ointment were determined by microbial enumeration tests and test for specified microorganisms Volume 138, Special part 290 D, Page 23[13].

2) Chemical property

- Sample preparation

Roselle ointment (400 mg) was accurately weighed into a centrifuge tube then 2 mL of deionized water was added. The extraction was performed in room temperature using the mixer vortex for 5 minutes, then sonication for 5 minutes. After that, the roselle ointment was warmed on a water bath at 50 °C for 5 minutes. Then, it was centrifuged at 6000 rpm and 5 °C for 5 minutes and the filtrate was collected. The extraction was repeated 2 times. The combined filtrate was centrifuged at 6000 rpm and 5 °C for 10 minutes. Then it was filtrated through 0.45 µm and 0.22 µm, PTFE syringe filter.

- Determination of total anthocyanin content

Total anthocyanin content in roselle ointment were determined by determination of total anthocyanin content by pH differential standard calculation method as described in 2.3.1.

3) Biological property

Antibacterial activity of roselle ointment was evaluated by agar well diffusion method as described in 2.4.2.

2.5.2. Stability of the pharmaceutical product

Roselle ointment (7 g) was kept in wide-mouthed plastic container with inner and outer caps a

the accelerated (40 ± 2 °C, 75% ± 5% relative humidity (RH)), the real time (30 ± 2 °C, 75% ± 5% RH) and in the refrigerator (2 – 8 °C) storage conditions as described in ASEAN guideline on stability study and shelf-life of traditional medicines, 2013[14].

The sampling of roselle ointment was conducted at 0, 1, 2, 3 and 4 weeks for products kept in the real time and refrigerator conditions while the products kept in accelerated conditions were sampled after they were kept for 4 weeks. The stability tests including evaluation of physical, chemical, and biological properties were then performed as mentioned in 2.5.1. For microbial contamination, roselle ointment stored in refrigerator for 4 weeks was selected to determine microbial contamination test.

3. RESULTS AND DISCUSSION

3.1. Preparation of roselle extracts

Roselle extract appeared as dark red powder. The yield of the extract was 57.69 %w/w of dry roselle powders.

3.2. Phytochemical analysis of roselle calyx extracts

3.2.1. Determination of total anthocyanin content by standard calculation

The total anthocyanin contents of roselle extract obtained from standard calculation method was 0.23 ± 0.00 g cyanidin 3-glucoside equivalent in 100 g of extract (g% C3GE).

3.2.2. Thin layer chromatographic (TLC) analysis

Roselle extract was evaluated for chromatographic fingerprints using TLC technique with white light, UV 254 nm, UV 366 nm, NP/PEG and DPPH spray reagents as detectors. As shown in Figure 1, roselle extract showed specific chromatographic fingerprints with some chromatographic bands corresponding to standard anthocyanins including delphinidin-3-sambubioside, cyanidin-3-sambubioside, delphinidin-3-glucoside and cyanidin-3-glucoside which appeared as violet and purple bands under white light at Rf values of 0.15, 0.20, 0.29 and 0.33, respectively (Figure 1 A). After detected under UV 254 nm, there were dark quenching bands on a green background suggested the presences of substances with chromophores in the roselle extract. All standard anthocyanins which appeared as dark quenching bands were found in roselle extract (Figure 1 B). Detection under UV 366 nm showed the fluorescence chromatographic bands suggested the presences of compounds with some fluorescence functional groups such as conjugated double bonds, all standard anthocyanins which appeared as dark bands were found in roselle extract. Moreover, there were some blue fluorescence bands with the major bands at Rf values of 0.50, 0.90 and 0.93. (Figure 1 C). After spraying with NP/PEG spray reagent and detect under UV 366 nm, there were bright blue fluorescence chromatographic bands suggesting the presences of phenolics in roselle extract, all standard anthocyanins which appeared as dark bands were found in roselle extract. There were also some blue fluorescence bands at Rf values of 0.50, 0.90 and 0.93 suggested that they are phenolics (Figure 1 D). After spraying with DPPH spray reagent and detected under white light, some chromatographic bands were positive to this spray reagent and appeared as yellow bands on purple background suggesting the compounds with DPPH scavenging activities. All standard anthocyanins and the blue major phenolic bands which were found in roselle extract were positive to DPPH spray reagent (Figure 1 E). The results from TLC analysis, suggested that roselle extract majorly contained anthocyanins, include delphinidin-3-sambubioside, cyanidin-3-sambubioside, delphinidin-3-glucoside and cyanidin-3-glucoside and some phenolic compounds which possessed DPPH scavenging effects.

3.4 Determination of in vitro antibacterial activity

3.4.1. Disk diffusion method

The roselle extract was tested for inhibitory effects against 2 clinically pathogenic bacteria including *Staphylococcus aureus* and *Staphylococcus intermedius* using disk diffusion method. As shown in Table 1, at the concentration of 3.13 mg/disc the extract promoted inhibitory effects against tested bacteria with the inhibition zone of 9.0 ± 0.00 and 9.7 ± 0.52 mm, respectively.

Previous study reported that roselle ethanol extracts showed antibacterial agents against some bacteria, especially *Staphylococcus aureus* and *Micrococcus luteus*. However, they were reported to promote less inhibitory effects to *Escherichia coli* and *Salmonella enteritidis*[15].

3.5. Development of roselle ointment

3.5.1. Quality control of pharmaceutical products

Roselle ointment was developed with good physical characteristics. The physical, chemical and biological properties of roselle ointment and ointment base after preparation (Day 0) were as following;

1) Physical property
   - Organoleptic characteristics

Roselle ointment appeared as homogeneous,

smooth texture, dark red semi-solid with unique odor while ointment base appeared as homogeneous, smooth texture, white semi-solid with unique odor (Figure 2).

- Viscosity

The viscosity of roselle ointment and ointment base using shear rate (ẏ) 100.00 1/s were 773.00 ± 10.58 and 837.00 ± 7.55 centipoise, respectively (Table 4).

- pH

The pH of roselle ointment and ointment base were 3.81 ± 0.02 and 8.95 ± 0.06, respectively (Table 4). The pH of roselle ointment was close to the pH of the native povidone-iodine (4.0 and 3.2-4.9)[16-17] and was within the range of the pH of povidone-iodine according to USP 42 and BP 2019[18-19]. Moreover, the pH was adjusted to be in the range of 1-3 to maintain the structure of anthocyanins in roselle ointment to be in the active flavylium cation form[20]. However, to adjust the pH of the roselle ointment to be not too low, the pH adjuster such as triethanolamine, sodium hydroxide and sodium bicarbonate could be added into the ointment formulation to increase the pH[21-23].

- Microbial content

Microbial content in roselle ointment in room temperature was determined by Microbial enumeration tests and test for specified microorganisms[13]. The amount of microbial contamination including *Staphylococcus aureus, Pseudomonas aeruginosa*, and *Clostridium* spp. were absent in 1 g. Total aerobic microbial count (TAMC) was not more than 2 x 10^2 cfu/g and total yeast and mold count (TYMC) was not more than 2 x 10 cfu/g present in roselle ointment. The results suggested that roselle ointment passed the criteria according to Notification of the Ministry of Public Health 2021 Volume 138, Special part 290 D, Page 23[13].

2) Chemical property

Total anthocyanin content in roselle ointment was determined by pH differential standard calculation method as described in 2.3.1.

Total anthocyanin content in roselle ointment was found to be 7.50 ± 0.00 mg% C3GE of the ointment. While total anthocyanin content in roselle ointment base cannot be detected (Table 5).

3) Biological property

Antibacterial activity of roselle ointment was evaluated by agar well diffusion method as described in 2.4.2. Roselle ointment and ointment base promoted inhibitory effects against *Staphylococcus aureus* with the inhibition zone of 14.31 ± 0.13 and 10.47 ± 0.21 mm, respectively (Table 6).

Roselle ointment and ointment base promoted inhibitory effects against *Staphylococcus intermedius* with the inhibition zone of 10.56 ± 0.13 and 12.39 ± 0.21 mm, respectively (Table 6).

3.5.2 Stability of the pharmaceutical product

The stability test of the roselle ointment was conducted according to ASEAN guideline on stability study and shelf-life of traditional medicine (2013)[14]. Roselle ointments in wide-mouthed plastic container with inner and outer caps and stored in the refrigerator 2 - 8 °C and the real time (30 ± 2 °C, 75% ± 5% RH) storage conditions were sampled at 0, 1, 2, 3 and 4 weeks while roselle ointments stored in the accelerated condition (40 ± 2 °C, 75% ± 5% RH) were sampled only after 4 weeks. The stability tests including evaluation of physical, chemical, and biological properties were then performed.

1) Physical property

- Organoleptic characteristics

The organoleptic characteristics of roselle ointment and ointment base are shown in Table 2 and 3.

After 2 weeks, roselle ointments stored in the refrigerator (2 - 8 °C) maintained the same organoleptic characteristics as initial day (Day 0) including homogeneous, smooth texture, dark red semi-solid with unique odor. However, at the third week, the organoleptic characteristics of the roselle ointment had changed compared to Day 0. It became non-homogeneous, the texture was not smooth, there was slight phase separation, and the color had changed to a dark reddish-brown, although it remained the unique odor. These organoleptic changes remained until the end of week 4.

After 1 week in real-time storage conditions (30 ± 2 °C, 75% ± 5% RH), the organoleptic characteristics of the roselle ointment had changed compared to the initial day (Day 0). It became non-homogeneous, the texture was not smooth, there was slight phase separation, and the color had changed to a dark reddish-brown, although it remained the unique odor. These changes remained until the end of week 4. The same organoleptic changes were observed in the ointments stored under accelerated conditions (40 ± 2 °C, 75% ± 5% RH) after 4 weeks of the storage.

After 2 weeks, ointment base stored in the refrigerator (2 - 8 °C) maintained the same organoleptic characteristics as initial day (Day 0) including homogeneous, smooth texture, white semi-solid with unique odor. However, by the third week, the organoleptic characteristics of the ointment base had changed compared to Day 0. It became non-homogeneous, the texture was not smooth, there was slight phase separation, although it remained white color and unique odor. These changes remained until the end of week 4.

After 1 week in real-time storage conditions (30 ± 2 °C, 75% ± 5% RH), the organoleptic characteristics

of the ointment base had changed compared to the initial day (Day 0). It became non-homogeneous, the texture was not smooth, there was slight phase separation, although it remained white color and unique odor. Similar changes in organoleptic changes were observed in the ointment base stored in accelerated conditions (40 ± 2 °C, 75% ± 5% RH) after 4 weeks.

The results suggested that for the organoleptic properties including homogeneity, texture, the roselle ointment and ointment base should be kept in the refrigerator (2 - 8 °C) which could maintain the stability for 2 weeks.

- Viscosity

The viscosity of roselle ointment and ointment base are shown in Table 4. After 4 weeks in all storage conditions, the viscosity of the roselle ointment increased from 773.00 ± 10.58 centipoise (initial value) to the range of 852.00 ± 16.09 to 1057.00 ± 7.94 centipoise (approximately 5 - 35% from the initial value). Moreover, in all storage conditions, the viscosity of the ointment base increased from 837.00 ± 7.55 centipoise (initial value) to the range of 877.33 ± 12.34 to 1037.67 ± 36.56 centipoise (approximately 5 - 34% from the initial value) after 4 weeks. The results showed that the storage time increased, the viscosity of roselle ointment and ointment base increased. The viscosity of roselle ointment and ointment base met the acceptance criteria of the viscosity using shear rate (ẏ) 100.00 1/s in the range of 100 – 10,000 centipoise[24].

- pH

The pH of roselle ointment and ointment base are shown in Table 4. After 4 weeks in all storage conditions, the pH of the ointment increased from 3.81 ± 0.02 (initial value) to the range of 3.96 ± 0.08 to 4.67 ± 0.11 (approximately 5 - 23 % from the initial value). The results showed that the storage time increased the pH of the roselle ointment. Meanwhile, the pH of the ointment base decreased from 8.95 ± 0.06 (initial value) to the range of 8.07 ± 0.08 to 8.46 ± 0.03 (approximately 5 - 10 % from the initial value) suggested that the storage time slightly decreased the pH value of the ointment base.

- Microbial content

In this study, the ointments that were tested for microbial contamination after they were stored for 4 weeks, were selected from their physical and chemical properties. Since it was found that all roselle ointments stored in refrigerator, the real time and the accelerated conditions were not physically stable after 4 weeks. Therefore, only the roselle ointment stored in the refrigerator which had more chemical stability according to total anthocyanin content after 4 weeks than the roselle ointments store in other two conditions (20% remaining), was tested for microbial contamination.

The amount of microbial contamination including *Staphylococcus aureus, Pseudomonas aeruginosa*, and *Clostridium spp*. were absent in 1 g. Total aerobic microbial count (TAMC) was not more than 2 x 10^2 cfu/g and total yeast and mold count (TYMC) was not more than 2 x 10 cfu/g in roselle ointment. The results suggested that roselle ointment kept in the refrigerator for 4 weeks passed the criteria according to Notification of the Ministry of Public Health 2021 Volume 138, Special part 290 D, Page 23[13].

2) Chemical property

Total anthocyanin content in roselle ointment kept in all storage condition for 4 weeks was determined by pH differential standard calculation method as described in 2.3.1.

No total anthocyanin content could be detected in ointment base. However, total anthocyanin content in roselle ointment stored in all storage condition decreased by the storage time had passed.

As shown in Table 5, Total anthocyanin contents in roselle ointment kept in the refrigerator (2 – 8 °C) decreased from 7.50 ± 0.00 mg% C3GE to 3.00 ± 0.01 mg% C3GE (40.00% of initial amount) after 2 weeks of storage and decreased to 1.50 ± 0.00 mg% C3GE (20.00% of initial amount) after 4 weeks storage. In real time storage condition (30 ± 2 °C, 75% ± 5% RH), total anthocyanin contents in the roselle ointment only lasted for 2 weeks which the content decreased to 1.50 ± 0.00 mg% C3GE (20.00% of initial amount) and could not be detected from the third week onwards.

Moreover, in accelerated condition (40 ± 2°C, 75% ± 5% RH), no total anthocyanin content could be detected in 4 weeks of storage.

From the results, roselle ointments kept in the refrigerator provided the highest %remaining of total anthocyanin contents for 4 weeks. Therefore, roselle ointment should be kept in the refrigerator to maintain anthocyanin content for 4 weeks.

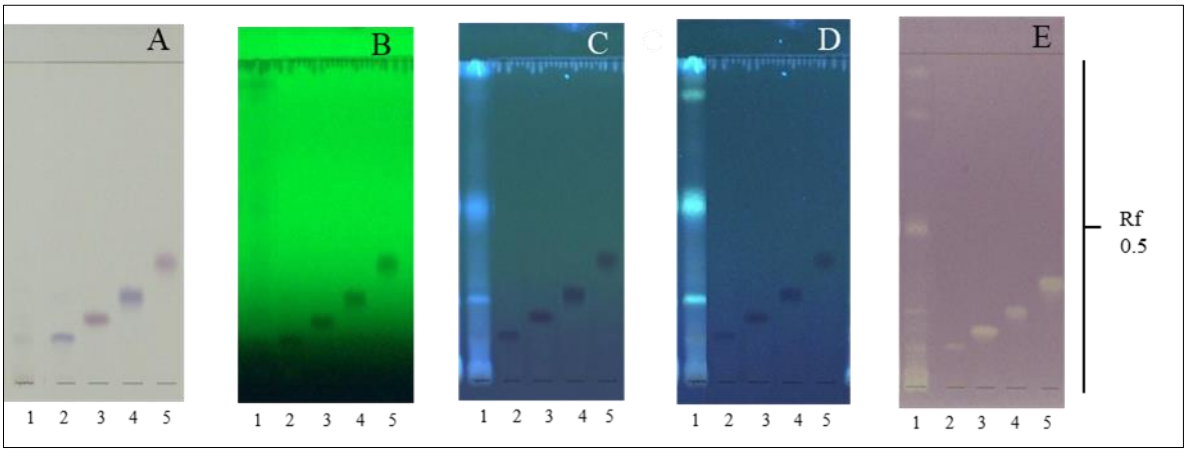

Figure 1. TLC chromatograms of roselle extract. Tracks 1 = roselle extract, 2 = delphinidin-3-sambubioside, 3 = cyanidin-3-sambubioside, 4 = delphinidin-3-glucoside, 5 = cyanidin-3-glucosid. Adsorbent: Silica gel GF254. Solvent system = ethyl acetate: glacial acetic acid: formic acid: water (100:11:11:26 v/v/v/v). Detectors: (A) white light; (B) UV 254 nm; (C) UV 366 nm; (D) NP/PEG spray reagent under UV 366 nm; (E) DPPH spray reagent under white light.

Table 1. Antibacterial activity of the roselle extract against 2 clinically isolated bacteria tested by the disk diffusion method.

Sample	Concentration (mg/disc)	Zone of inhibition (mm)	
		Staphylococcus aureus	***Staphylococcus intermedius***
Roselle extract	3.13	9 ± 0.00	9.7 ± 0.52
AML10	0.01	42 ± 0.00	8 ± 0.00
CN10	0.01	25.5 ± 0.45	15 ± 0.00
DO30	0.01	18 ± 0.00	13.5 ± 0.00

+ AML10 = amoxycillin 10 µg, CN10 = gentamicin 10 µg, DO30 = doxycycline 30 µg

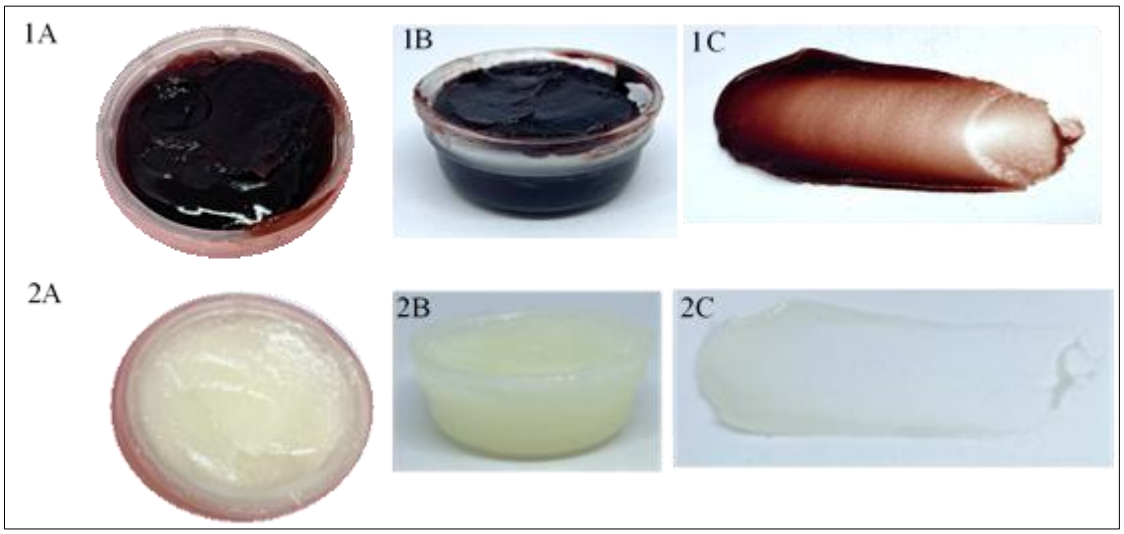

Figure 2. Physical characteristics of roselle ointment and ointment base (Day 0), 1A = top view of ointment in container, 1B = side view of ointment in container, 1C = spreading of ointment, 2A = top view of ointment base in container, 2B = side view of ointment base in container, 2C = spreading of ointment base.

Table 2. Organoleptic characteristics of roselle ointment in stability test.

Sample	Storage condition	Storage time	Physical characteristic
Ointment	RT	Day 0	- Homogeneous - Smooth texture - Dark red semi-solid - Unique odor
	RF	Week 1	The same to day 0
		Week 2	The same to day 0
		Week 3	- Non-homogeneous - Slightly phase separation - Not smooth texture - Dark reddish brown semi-solid - Unique odor
		Week 4	The same to week 3
	RTC	Week 1	- Non-homogeneous - Slightly phase separation - Not smooth texture - Dark reddish brown semi-solid - Unique odor
		Week 2	The same to week 1
		Week 3	The same to week 2
		Week 4	The same to week 3
	AC	Week 4	- Non-homogeneous - Slightly phase separation - Not smooth texture - Dark reddish brown semi-solid - Unique odor

+ RT = room temperature, RF = in the refrigerator (2 – 8 °C), RTC = the real time (30 ± 2 °C, 75% ± 5% RH) and, AC = the accelerated (40 ± 2 °C, 75% ± 5% RH)

Table 3. Organoleptic characteristics of roselle ointment base in stability test.

Sample	Storage condition	Storage time	Physical characteristic
Ointment base	RT	Day 0	- Homogeneous - Smooth texture - White semi-solid - Unique odor
	RF	Week 1	The same to day 0
		Week 2	The same to day 0
		Week 3	- Non-homogeneous - Slightly phase separation - Not smooth texture - Unique odor
		Week 4	The same to week 3
	RTC	Week 1	- Non-homogeneous - Slightly phase separation - Not smooth texture - Unique odor
		Week 2	The same to week 1
		Week 3	The same to week 2
		Week 4	The same to week 3
	AC	Week 4	- Non-homogeneous - Slightly phase separation - Not smooth texture - Unique odor

+ RT = room temperature, RF = in the refrigerator (2 – 8 °C), RTC = the real time (30 ± 2 °C, 75% ± 5% RH) and, AC = the accelerated (40 ± 2 °C, 75% ± 5% RH)

Table 4. The pH and viscosity of roselle ointment and ointment base in stability test.

Sample	Temperature (°C)	Time	pH	%	Viscosity (cP)	%
Ointment	RT	Day 0	3.81 ± 0.02	100	773.00 ± 10.58	100
	RF	1 week	3.96 ± 0.08	104.11	964.67 ± 35.08	124.67
		2 weeks	4.02 ± 0.12	105.51	1038.67 ± 31.34	134.15
		3 weeks	4.12 ± 0.13	108.14	1057.00 ± 7.94	126.23
		4 weeks	4.56 ± 0.10	119.79	1037.33 ± 11.59	123.88
	RTC	1 week	4.01 ± 0.04	105.34	915.00 ± 12.00	118.36
		2 weeks	4.58 ± 0.05	120.23	965.33 ± 23.46	124.77
		3 weeks	4.67 ± 0.11	122.68	891.00 ± 5.00	115.27
		4 weeks	4.62 ± 0.07	121.36	852.00 ± 16.09	110.19
	AC	4 weeks	4.54 ± 0.13	119.17	879.33 ± 5.13	105.07
Ointment base	RT	Day 0	8.95 ± 0.06	100.00	837.00 ± 7.55	100
	RF	1 week	8.46 ± 0.03	94.56	912.00 ± 2.65	108.97
		2 weeks	8.11 ± 0.14	90.61	1037.67 ± 36.56	123.90
		3 weeks	8.28 ± 0.06	92.52	1031.00 ± 19.08	133.29
		4 weeks	8.25 ± 0.15	92.18	1037.33 ± 11.59	126.55
	RTC	1 week	8.17 ± 0.06	91.32	877.33 ± 12.34	104.77
		2 weeks	8.36 ± 0.04	93.37	954.67 ± 10.69	114.07
		3 weeks	8.18 ± 0.03	91.36	952.67 ± 8.08	113.80
		4 weeks	8.07 ± 0.08	90.14	944.33 ± 49.24	112.79
	AC	4 weeks	8.33 ± 0.02	93.04	944.33 ± 14.36	122.14

+ RT = room temperature, RF = in the refrigerator (2 – 8 °C), RTC = the real time (30 ± 2 °C, 75% ± 5% RH) and, AC = the accelerated (40 ± 2 °C, 75% ± 5% RH), cP = centipoise

Table 5. Total anthocyanin contents of roselle ointment and ointment base in stability test.

Sample	Temperature (°C)	Time	Total anthocyanin contents (mg% C3GE)	%remaining
Ointment	RT	Day 0	7.50 ± 0.00	100
	RF	1 week	3.60 ± 0.01	48.00
		2 weeks	3.00 ± 0.01	40.00
		3 weeks	2.40 ± 0.00	32.00
		4 weeks	1.50 ± 0.00	20.00
	RTC	1 week	1.80 ± 0.00	24.00
		2 weeks	1.50 ± 0.00	20.00
		3 weeks	CD	CD
		4 weeks	CD	CD
	AC	4 weeks	CD	CD
Ointment base	RT	Day 0	CD	CD
	RF	1 week	CD	CD
		2 weeks	CD	CD
		3 weeks	CD	CD
		4 weeks	CD	CD
	RTC	1 week	CD	CD
		2 weeks	CD	CD
		3 weeks	CD	CD
		4 weeks	CD	CD
	AC	4 weeks	CD	CD

+ RT = room temperature, RF = in the refrigerator (2 – 8 °C), RTC = the real time (30 ± 2 °C, 75% ± 5% RH) and, AC = the accelerated (40 ± 2 °C, 75% ± 5% RH), CD = cannot be detected, mg% C3GE = mg% cyanidin-3-glucoside equivalent of the ointment

Table 6. Antibacterial activity of roselle ointment and ointment base in stability test against 2 clinically isolated bacteria by the agar well diffusion method.

Bacteria	Sample	Temperature (°C)	Time	Zone of inhibition (mm)
Staphylococcus aureus	Ointment	RT	Day 0	14.31 ± 0.13
		RF	4 weeks	11.97 ± 0.05
		RTC	4 weeks	11.00 ± 0.30
		AC	4 weeks	9.72 ± 0.09
	Ointment Base	RT	Day 0	10.47 ± 0.21
		RF	4 weeks	9.89 ± 0.26
		RTC	4 weeks	9.53 ± 0.19
		AC	4 weeks	9.39 ± 0.05
	Amoxycillin 10 µg			40.00 ± 0.00
	Gentamicin 10 µg			25.17 ± 0.29
	Doxycycline 30 µg			18.50 ± 0.00
Staphylococcus intermedius	Ointment	RT	Day 0	10.56 ± 0.13
		RF	4 weeks	9.86 ± 0.05
		RTC	4 weeks	9.67 ± 0.00
		AC	4 weeks	9.61 ± 0.10
	Ointment Base	RT	Day 0	12.39 ± 0.21
		RF	4 weeks	11.00 ± 0.22
		RTC	4 weeks	10.78 ± 0.05
		AC	4 weeks	10.78 ± 0.13
	Amoxycillin 10 µg			9.00 ± 0.00
	Gentamicin 10 µg			15.50 ± 0.00
	Doxycycline 30 µg			16.50 ± 0.00

+ RT = room temperature, RF = in the refrigerator (2 – 8 °C), RTC = the real time (30 ± 2 °C, 75% ± 5% RH) and, AC = the accelerated (40 ± 2 °C, 75% ± 5% RH)

3) Biological property

Antibacterial activities of roselle ointment and ointment base in stability test determined against 2 clinically isolated bacteria by the agar well diffusion method are shown in Table 6.

Roselle ointment and ointment base at day 0 promoted inhibitory effects on *Staphylococcus aureus* with the inhibition zone of 14.31 ± 0.13 and 10.47 ± 0.21 mm, respectively. After 4 weeks, roselle ointment and ointment base in all storage conditions showed the decreased inhibitory effects with the inhibition zone ranging from 9.72 ± 0.09 to 11.97 ± 0.05 mm and 9.39 ± 0.05 to 9.89 ± 0.26 mm, respectively.

Roselle ointment and ointment base at day 0 promoted inhibitory effects on Sta*phylococcus intermedius* with the inhibition zone of 10.56 ± 0.13 and 12.39 ± 0.21 mm, respectively. After 4 weeks, roselle ointment and ointment base in all storage conditions showed the decreased inhibitory effects with the inhibition zone ranging from 9.61 ± 0.10 to 9.86 ± 0.05 mm and 10.78 ± 0.05 to 11.00 ± 0.22 mm, respectively.

It was found that roselle ointment exhibited stronger antibacterial effect against *Staphylococcus aureus* than ointment base as it showed higher inhibition zone than that from ointment base. The results suggest that roselle extract in roselle ointment still promoted and was responsible for antibacterial effect of roselle ointment.

The inhibition zone observed with the ointment base at day 0 suggests that the composition of the formulation such as antioxidants could also promote antibacterial activity. Adding the antioxidants to the formulation could also maintain the stability of active compounds in the roselle extracts and prolong antibacterial activities.

The antibacterial activities of roselle ointment in all storage conditions still remained to inhibit *Staphylococcus aureus* and *Staphylococcus intermedius* after 4 weeks storage.

The results suggested that roselle ointment formulation promoted in vitro antibacterial effect against *Staphylococcus aureus* and *Staphylococcus intermedius*. The inhibitory activities still remained after 4 weeks storage in all conditions. Therefore, besides anthocyanins, other chemical constituents in roselle ointment could be responsible for antibacterial effects. These compounds were maybe more stable than anthocyanin and could remain in the ointment for 4 weeks of storage.

Moreover, there were some previous studies reported about antibacterial activities of phenolic compounds found in roselle[25-27]. Gallic acid showed antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Listeria monocytogenes* with MIC values in the range of 500 – 2,000 µg/mL while ferulic acid inhibited the growth of all tested bacterial pathogens with MIC values ranged from 100 to 1250 µg/mL[28]. Chlorogenic acid effectively promoted strong inhibitory effect against

Staphylococcus aureus, *Streptococcus pneumoniae*, *Bacillus subtilis*, *Escherichia coli*, *Shigella dysenteriae* and *Salmonella* Typhimurium with the MIC values ranging from 20 to 80 μg/mL[29].

Moreover, the antibacterial activities of roselle ointment correspond to a previous study reported that roselle ethanolic extract at the concentration of 250 mg/mL exhibits antibacterial effect against wound isolated *Pseudomonas aeruginosa* with wound healing effect through inactivating the TLR4 pathway and antioxidant activity[30]. The crude methanol extract of roselle calyces (10 mg/disc) was reported to has as antibacterial activities against gram-positive and gram-negative bacteria including *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 49461, *Bacillus cereus* ATCC 10876, *Escherichia coli* ATCC 25922, *Salmonella enteric* ATCC 5174, *Klebsiella pneumonia* ATCC 27736, *Proteus vulgaris* ATCC 49132, and *Pseudomonas aeruginosa* ATCC 27853[31]. Another the previous study indicated that hibiscus acid obtained from roselle calyx treatment can effectively reduce extracellular adenosine triphosphate (ATP) secretion and carbonyl protein production, as well as maintain a high level of reduced/oxidized glutathione (GSH/GSSG) in skin cells[32].

4. CONCLUSION

In this study, roselle extract was prepared and quality controlled for chemical and biological properties. Delphinidin-3-sambubioside, cyanidin-3-sambubioside, delphinidin-3-glucoside, and cyanidin-3-glucoside were found to be major anthocyanins in roselle extract along with some phenolic compounds. Roselle extract promoted in vitro inhibitory effects against clinical isolated *Staphylococcus aureus* and *Staphylococcus intermedius*

Roselle ointment was also developed and quality controlled for physical, chemical and biological properties. It promoted good physical and chemical characteristics with in vitro antibacterial effects against clinical isolated *Staphylococcus aureus* and *Staphylococcus intermedius*. Roselle ointment were evaluated for stability according to ASEAN guideline on stability study and shelf-life of traditional medicine (2013)[14] From the results, roselle ointment kept in plastic container with inner and outer caps should be kept in the refrigerator (2 - 8 °C) for 2 weeks to maintain physical and chemical properties. However, the antibacterial effect of roselle ointment had been decreased but still remained after 4 weeks of storage in all storage conditions.

Future studies to improve the stability of roselle formulation should be conducted with more study about other possible active phytochemicals such as phenolic compounds.

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Conflict of interest

The authors declare that they have no conflict of interest.

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