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Antioxidant Activities and Cytotoxicity Effect on Normal Human Dermal Fibroblasts of Roselle (*Hibiscus sabdariffa* L.) Calyces Extracts and W/O/W Emulsion Loaded Extract for Cosmetic Applications

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Abstract

The objective of this study was to evaluate the antioxidant activities and cytotoxicity effect on normal human dermal fibroblasts of roselle (*Hibiscus sabdariffa* L.) calyx extracts and to load with the roselle extract in the inner phase of water in oil in water multiple emulsion for application in facial cosmetic. The extracts of dried roselle calyces were prepared by a maceration technique using ethanol/water at concentrations of 30%, 70%, 95% and 0% (100% DI water). Obtained results showed that the 30% ethanolic extract had a higher amount of total phenolic and anthocyanin content than other extracts. According to the DPPH and ABTS assays, the 30% ethanolic extract exhibited dose-dependent antioxidant activity higher than other extracts with an IC50 value of 0.432±0.001 mg/mL and 0.0855±0.01 mg/mL, respectively. The formulation of water in oil in water multiple emulsion containing the roselle calyx extract was prepared by a beaker method with two-step emulsification. The prepared multiple emulsion showed a light pink color with homogeneously formula. The physiochemical stability of the prepared formula was evaluated at accelerated conditions; room temperature (30±5°C), low temperature (4±1°C) and high temperature (40±1°C) for 30 days and 6 cycles of freeze (-25°C, 24 h)-thaw (4°C, 24 h). Results found that the tested formula showed a good physicochemical stability compared to the initial condition. All finding suggests possible application for the 30% ethanolic extract of roselle calyces as a natural active ingredient in skin cosmetic products.

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Introduction

In recent years, the natural substances have traditionally been used in skin care products due to being considered safer than synthetic substances. The term of natural is defined as an ingredient that is produced by nature or found in nature and is directly extracted from plants or animal products. The herbs, fruits, flowers, leaves, minerals, water and land can be used as sources of natural ingredients (Ribeiro et al., 2015). The bioactive compounds in a natural product can enhance skin health as well as protect the skin against various damaging factors, including ultraviolet radiation (UVR) and free radicals. Free radicals are unstable and highly reactive molecules with a neighboring molecule can increase stability. As a chain reaction, the neighboring molecules which lose or accept electron to then becomes a new free radical. These free radicals significantly contribute to skin damage and accelerate ageing especially reactive oxygen species (ROS) (Michalak, 2022). Antioxidant are substances which can donate an electron to reactive species resulting in the prevention of the radical chain reaction. Prior studies reported that the phenolic-containing extracts exhibited the antioxidant properties (Dudonné et al., 2009). Nowadays, the phenolic compounds which derived the plant widely used in cosmetic products and may eventually replace the use of synthetic antioxidants (Przybylska-Balcerek & Stuper-Szablewska, 2019).

Hibiscus sabdariffa L. known as Roselle and it belongs to the Malvaceae family. Roselle is a medicinal plant which grows in subtropical and tropical regions such as Africa, Egypt, Guatemala, India and Thailand (Juhari et al., 2018). The essential part of roselle is calyces. Roselle calyx is rich in bioactive compounds such as vitamins (e.g., ascorbic acid), anthocyanins (e.g., delphinidin-3-O-sambubioside and cyanidin-3-O-sambubioside) flavonoids (e.g., quercetin, kaempferol, luteolin and apigenin), phenolic (e.g., chlorogenic acid and protocatechuic acid) and organic acids (e.g., citric acid and hibiscus acid) (Riaz & Chopra, 2018). These compounds in rosella calyx extract are contributed to having biological activities such as antimicrobial (Jung et al., 2013), antioxidation (Villalobos-Vega et al., 2023), anti-Inflammatory (Ariyabukalakorn et al., 2019). In addition, hibiscus acid isolated from roselle calyx showed skin aging potential and no cytotoxicity to human dermal fibroblast (Wang et al., 2022). Roselle extract is one plant extract that has been used in cosmetics, such as skin toner/astringents and anti-aging skin care products (Pinsuwan et al., 2010). For example, roselle calyx extract is used as an active ingredient in anti-aging cream (single emulsion) yet nowadays, cream or lotion as multiple emulsion formation containing roselle extract has not been reported in the market. According to scientific reports, roselle calyx extract contains several polar and acidic compounds, so that, their chemical properties have induced skin irritation and low skin permeation (Hjorth, 1969). The incorporation of roselle extract into a cosmetic dosage form has been improved as well as protection against environmental and to preserve their stable properties over time such as emulsion, nano-emulsion, liposome (Bevan et al., 2023). Among the dosage forms, emulsion technology has attracted much attention because it involves simple processing, low energy cost and easy application; moreover, it is widely used in food, pharmaceutical and cosmetic products.

A type of emulsion is double emulsions also called multiple emulsions, which consist of two types: water-in oil-in-water ($W_1/O/W_2$) emulsions and oil-in-water-in-oil ($O_1/W/O_2$) emulsions, mainly depending on the loaded active agents. (Lamba et al., 2015). In $W_1/O/W_2$, water is first dispersed in oil which is then dispersed in another water phase, similarly for $O_1/W/O_2$, oil is first dispersed in water and then this emulsion is dispersed in a second oil phase. Multiple emulsions provide protective encapsulation to bioactive substances (hydrophilic and hydrophobic) in the inner phase droplets (Wang et al., 2017; Niknam et al., 2020). The $W_1/O/W_2$ emulsions have been investigated for loading the hydrophilic active agents (e.g., polyphenols, phenolics) better than O/W emulsions, because the release of hydrophilic compounds can be prolonged and better controlled (McClements, 2015). However, the emulsifier is very important to obtain emulsion because it can effectively protect the double emulsion droplets against flocculation, creaming and coalescence, thus contributing to the multiple emulsion stability. In addition, emulsifier must be biodegradable and nontoxic.

In this study, the roselle calyx extract was prepared using a maceration technique at room temperature to treat the bioactive compound in the extract. Then, the antioxidant activities and cytotoxicity effect on normal human dermal fibroblasts of roselle calyx extract were investigated. This extract was loaded into the internal phase of $W_1/O/W_2$ emulsion which was formulated by two-step emulsification method. The physiochemical characterization of prepared multiple emulsion and their stability were analyzed.

Materials and methods

1. Raw materials

The dried red calyx of roselle (*Hibiscus sabdariffa* L.) was purchased from Chao Krom Poe dispensary pharmacy, Samphanthawong, Bangkok, Thailand. 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), Folin-Ciocalteu reagent, Gallic acid (HPLC grade), Quercetin (HPLC grade) and Trolox were purchased from Sigma-Aldrich (USA). Ethanol (99%, AR grade) was purchased from Labscan (Thailand). Ethanol (Commercial grade) and deionized water were purchased from S.N.P. General Trading (Thailand). Sodium carbonate (Na_2CO_3, AR grade), Aluminium chloride ($AlCl_3$, AR grade), Sodium acetate (AR grade), Potassium chloride (KCl, AR grade) and Sodium chloride (NaCl, AR grade) were purchased from Univar Ajax Finechem (New Zealand). Mineral oil, Sorbitane monooleate, Polysorbate 80, Phenoxyethanol and Triethanolamine were supplied by Namsiang (Thailand).

2. Preparation of roselle calyx extracts

The red calyx of roselle is the type most commonly used (green, red and dark red) because of their high content of anthocyanin and acids (Wong et al., 2002; Peredo Pozos et al., 2020). An extract of red calyx of roselle was produced by a maceration technique as previously studied (Kusnadi & Purgiyanti, 2021), with modification. Dried calyx was ground into powder using a grinder and then the fine powder was kept in a sealed container protected from light at 4°C in refrigerator until usage. The extract of roselle calyx was macerated at room temperature (30±5°C) in various concentrations of ethanol including 30%, 70%, 95% and 0% (100% deionized water). The extraction was done in the ratio of dried roselle and solvent 1:10 w/v. Briefly, 10 g of dried calyx powder was macerated in 100 mL of each solvent and then kept at room temperature for 24 h. The resulting solvent was filtered through a Whatman No.1 filter paper. The marc was re-extracted again in the same method and done in triplicates. The pooled filtrate was concentrated using a vacuum rotary evaporator (Buchi, R-205/v, Switzerland) at 45°C. Each crude extract was weighed and kept in a tight container protected from light at 0°C in refrigerator. The yield of each extract was calculated using the following equation:

$$\text{Yield (\%)} = (W_1 \times 100)/(W_2) \quad [1]$$

where W1 is the weight of roselle calyx extract and W2 is the weight of the dried roselle calyx powder.

3. The quantitative analysis

3.1 Total phenolic content (TPC)

Total phenolic content of roselle calyx extracts was analyzed by a Folin-Ciocalteu method of previous research (Mohd-Esa et al., 2010) with some modifications. Briefly, 0.2 mL of each extract solution (1.0 mg/mL) was mixed with 1.0 mL of Folin-Ciocalteu reagent (10% v/v) and 0.8 mL of sodium carbonate (Na_2CO_3) (7.5% w/v) was added and vortex. After incubation for 30 min at room temperature, the absorbance of the incubated solution was measured at 765 nm, using a UV-visible spectrophotometer (Shimadzu, UV-2401PC, Japan). The amount of total phenolic was expressed as milligram of gallic acid equivalents per gram of extract (mgGAE/g extract) from the calibration curve. This assay was performed in triplicate.

3.2 Total flavonoid content (TFC)

Total flavonoid content of roselle calyx extracts was determined based on the formation of a complex flavonoid aluminium as previously studied (Meda et al., 2005), with slightly modification. Briefly, 100 µL of aluminium chloride ($AlCl_3$) (2.0 %w/v) was mixed with the same volume of each extract solution (1.0 mg/mL) and allowed to stand at room temperature for 10 min. The absorbance was measured at 415 nm using a microplate reader (Biochrom, EZ Read 2000, England). The total flavonoid content was expressed as milligram of quercetin equivalent per gram of extract (mgQE/g extract) from the calibration curve. This assay was done in triplicate.

3.3 Total anthocyanin content (TAC)

Total anthocyanin content of roselle calyx extracts was determined using the pH differential method of previous research (Wu et al., 2018) with some modifications. This pH differential method is based in the change of color of anthocyanin with pH: at pH 1.0 colored oxonium ions are formed, whereas at pH 4.5 predominates the colorless hemiketal form. The difference in the absorbance of the pigments at 520 nm is proportional to the pigment concentration. Briefly, two buffer solutions were prepared at pH 1.0 (0.025 M of potassium chloride (KCl)) and pH 4.5 (0.4 M of Sodium acetate (CH_3COONa). The extract solution (1.0 mL) was mixed with each buffer solution (2.0 mL) and then shaken. Absorption measuring at 517 and 700 nm was read using a microplate reader (Biochrom, EZ Read 2000, England) after incubation for 15 min in the dark at room temperature. Total

anthocyanin value was expressed as milligram of cyanidin-3-glucoside equivalents per gram of extract according to the following equations:

$$\text{Absorbance (A)} = (A_{517} - A_{700})\ \text{pH 1.0} - (A_{517} - A_{700})\ \text{pH 4.5} \quad [2]$$

$$\text{TAC (mg/L)} = (A \times MW \times DF \times 1000)/(\varepsilon \times l) \quad [3]$$

where MW is molecular mass of cyanidin-3 glucoside (449.2 g/mol), DF is the dilution factor (1.0), 1000 is the gram to milligram conversion coefficient, ε is the molar absorptivity, calculated as cyanidin-3-glucoside (26,900 L/mol/cm) and l is the cuvette radius (1.0 cm).

4. *In vitro* antioxidant activities

4.1 DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

The free radical scavenging activity of roselle calyx extracts was determined by using DPPH assay according to the method described by Yakaew et al. (2016) with slightly modification. Briefly, 150 µL of DPPH ethanolic solution (0.2 mM) was then mixed with 75 µL of various concentrations of each extract solution. The mixture was incubated at room temperature for 30 min in the dark. After incubation, absorbance was measured at 515 nm and measured using a microplate reader (Biochrom, EZ Read 2000, England). This study was compared with the positive control, Trolox. The radical scavenging activity was calculated as a percentage of DPPH discoloration using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = [1 - (A_S/A_B)] \times 100 \quad [4]$$

where AS is an absorbance of DPPH with the tested sample and AB is an absorbance of DPPH without the tested sample. The concentration providing 50% inhibition (IC50) was calculated from the graph of inhibition percentage plotted against the sample concentration. The study was run in triplicate.

4.2 ABTS+ [(2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)] assay

The free radical scavenging capacity of $ABTS^+$ was assayed as previously studied (Wu et al., 2018), with some modification. Briefly, to prepare an $ABTS^{\bullet+}$ radical, the $ABTS^{\bullet+}$ solution (7.0 mM) was mixed with potassium persulphate (2.45 mM) at a ratio of 1:1 (v/v). After that, the mixture was kept in the dark at room temperature for 12-16 h. The working reagent was prepared by mixing the $ABTS^{\bullet+}$ solution with ethanol to an absorbance of 0.70±0.02 at 734 nm using a microplate reader. Then, to determine the $ABTS^{\bullet+}$ radical scavenging activity, 100 µL of various concentrations of the extract solution was added to 2.0 mL of $ABTS^{\bullet+}$ solution. The mixture was well mixed and left to stand for 10 min in the dark place at room temperature, The absorbance was taken at 734 nm using a microplate reader (Biochrom, EZ Read 2000, England). Trolox was used as a positive compound. The $ABTS^{\bullet+}$ scavenging activity was calculated in the same way as DPPH radical scavenging activity.

5. Cytotoxicity to normal human dermal fibroblasts

Cytotoxicity assay was also done to determine cell viability of the cultured fibroblast cells using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay according to the method described by Ala AA, et al (2018) with modification. Briefly, normal human dermal fibroblast (NHDF) cell was used as a cell model to study the cytotoxicity. The NHDF (ATCC# PCS-201-010) cells was trypsinized and seeded at approximately $8x10^3$ cells/well into 96-well plate and cultured in the complete growth media of DMEM-F12 supplemented with 10%FBS and 1% v/v Penicillin/Streptomycin. NHDF cells were maintained at 37°C with 5%CO_2 for 24 h. Then, the culture media was removed and washed with sterile phosphate buffer saline (PBS). The NHDF cells underwent treatment with different concentration of crude extracts comparison with the control. After 24 h, the media containing the samples was discarded carefully and washed with sterile PBS. Then, NHDF cells were incubated with MTT solution and incubated at 37°C in the dark for 2 h. The medium was removed and the formazan crystals was dissolved with dimethyl sulfoxide (DMSO). The absorbance of each well was read on microplate reader at 570 nm. The % cell viability was calculated by the below equation:

$$\text{Cell viability (\%)} = (A_S/A_C) \times 100 \quad [5]$$

where AS is an absorbance of the tested sample and AC is an absorbance of control (cell untreated with the tested sample).

6. Preparation of water in oil in water emulsion

Water in oil in water containing roselle calyx extract was prepared using beaker method with two-step emulsification as previously studied (Mahmood et al., 2014) with modification. The extract of roselle calyx was loaded in the inner phase (primary water in oil emulsion) of water in oil in water multiple emulsion. The concentration of roselle calyx extract was considered from the obtained result of antioxidant activity. The

compositions and their functions of water in oil in water formula is shown in Table 1.

Table 1 The compositions and their functions of water in oil in water formula

Ingredients	Quantity (%w/w)	Functions
First step: Primary W/O emulsion		
Deionized water	qs to 100	Diluent
Sodium chloride (NaCI)	0.3	Inner phase checking
Mineral oil	20.0	Emollient
Sorbitane monooleate (Span 80, HLB=4.3)	4.0	Lipophilic emulsifier
Triethnolamine (TEA)	0.2	pH adjuster to 5.0-5.5
Phenoxyethanol	1.0	Preservative
Second step: W/O/W multiple emulsion		
Primary w/o emulsion	80.0	Inner phase of multiple
Polysorbate 80 (Tween 80. HLB=15)	6.0	emulsion Hydrophilic
Deionized water	14.0	emulsifier Diluent

Remark: Q.S.is quantum satis (as much as is enough), HLB is hydrophile-lipophile balance

Briefly, primary W/O emulsion prepared by emulsifying the oil phase (mineral oil and sorbitan monooleate) with the aqueous phase (deionized water and sodium chloride) in the presence of lipophilic surfactant while heating both phases at 75°C. The oil phase and aqueous phase was mixed by homogenizer (Silverson L5M, England) at 1500 rpm for 30 min and the temperature of this mixture was maintained at 70-75°C. Preservative and pH adjuster and/or roselle extract added to the mixture at 40°C, then stirred to emulsion formation.

For the second stage emulsification, the primary W/O emulsion was added slowly to a secondary aqueous phase containing hydrophilic emulsifier (deionized and tween 80) at stirring speed of 700 rpm for 15 min using a homogenizer (Silverson L5M, England) and the formation of multiple emulsion was confirmed by microscopic analysis.

7. Evaluation of physicochemical characterization

7.1 The appearance, homogeneously formula, phase separation and color were determined by visualization.

7.2 The morphology was investigated using a microscope (MLB 3200, Kruss, Germany). Observations were made at 40X magnification after diluting the multiple emulsions.

7.3 Electrical conductivity value of the multiple emulsion was determined with an electrical conductivity probe of pH/mV/EC/TDS/NaCl/Temp Starter B100 (Ohaus, Ohaus Instruments, Shanghai, China).

7.4 pH value was determined using pH meter (Starter B100, Ohaus Instruments, Shanghai, China) which was calibrated using standard buffer solution.

7.5 The viscosity was determined by Brookfield viscometer (DV-I Prime, Brookfield Ametex, USA) at 50 rpm using a spindle no.63.

7.6 The L* a* b* color value was measured using a Chroma meter (CR-400, Centasia, Konica Minolta, Thailand)

8. Investigation of physiochemical stability

The multiple emulsion loaded roselle calyx extract was kept in air-tight containers under accelerated condition using 6 cycles of alternative freeze-thaw (-25°C for 24 h followed by 4°C for 24 h as 1 cycle), low temperature (4±1°C) for 30 days, high temperature (40±1°C) for 30 days and room temperature (30±5°C) for 30 days. The samples were removed from the storage conditions and allowed to achieve room temperature prior to the evaluation of the physicochemical characteristics compared with initial condition.

9. Statistical analysis

Statistical analysis of the data was analyzed by performing a one-way analysis of variance (ANOVA) and Duncan post-hoc tests using SPSS program (SPSS ver. 22.0 for Windows, SPSS Inc., Chicago, IL, USA) and the statistical significance was $p < 0.05$.

Results and discussion

1. Roselle calyx extracts and quantification of antioxidant compounds in the extracts

The effective extraction and proper assessment of antioxidants from herbals are crucial when exploring the potential antioxidant sources and promote the application in the cosmetic field. Extraction is the first step in the isolation of phenolic compounds from plant materials by traditional methods such as maceration and soxhlet extractions, which have been used for many decades. In this present study, obtained extracts from roselle calyx was produced using a maceration method with different concentrations of water and ethanol; aqueous (100% water), 30% ethanol, 70% ethanol and 95% ethanol. The extraction results found that the maximum yield value was obtained extract by maceration with 30% (v/v) ethanol which was 50.67% (w/w). The yield then tended to decrease when the ethanol concentration was 70% and 95%, respectively (Table 1). This might be due to the combination between water and ethanol which serves the extraction of compounds that are soluble in water and ethanol in roselle calyx such as phenolic acid, flavonoid and anthocyanin. Other compounds may have been extracted and contribute to higher yield.

Presented in Fig. 1, the physical appearance of the crude extracts from roselle calyx is visually evaluated and shows that both extracts from 30% ethanol and aqueous gave a red powder and a red-brown powder for 70% ethanolic extract whereas the 95% ethanolic extract shows a dark brown viscous liquid. All crude extracts were kept in tight container at 0°C for further studies.

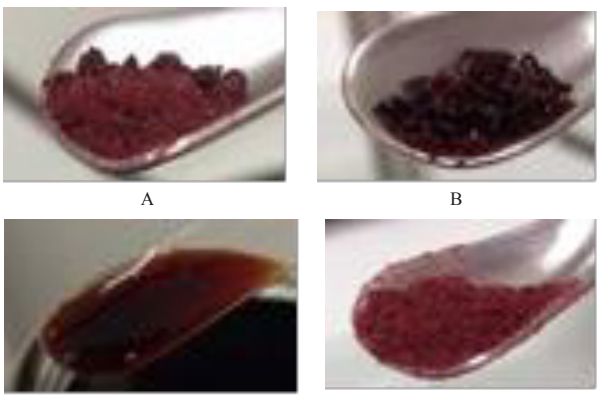

Fig. 1 Visual appearance of the crude extracts from roselle calyx prepared by various concentrations of ethanol;
(A) 30% ethanol, (B) 70% ethanol, (C) 95% ethanol and (D) 100% aqueous (0% ethanol)

The content of total phenolic, total flavonoid and total anthocyanin in each extract are shown in Table 2. The content of total phenolic compounds in the roselle calyx extracts were calculated with the calibration curve equation of gallic acid (0.0625-1.0 mg/mL, Y= 1.005X + 0.0272, R^2 = 0.999) based on Folin-Ciocalteu assay. The total phenolic content of 30% ethanolic extract had no significantly difference ($p>0.05$) higher than 70% ethanolic extract and the lowest content was extracted with 95% ethanol; this means that ethanol is not a good solvent for extracting phenolic compounds. This demonstrated that the total phenolic content in roselle calyx extract might contain mainly the protocatechuic acid (PCA) and chlorogenic acid (Riaz & Chopra, 2018). The results were similar to previously research (Villalobos-Vega et al., 2023) which showed that phenolic compounds are soluble in hydroalcoholic mixtures containing equal amounts of ethanol and water.

The amount of total flavonoid compounds of roselle calyx extracts were calculated with the calibration curve equation of quercetin (0.039-0.625 mg/ml, Y=13.925X + 0.1051, R^2 = 0.9995) which found that 95% ethanolic extract had significantly ($p<0.05$) higher amount of total flavonoid and followed by 30% ethanolic extract, 70% ethanolic extract and aqueous extract, respectively. The finding imply that the ethanol-water (5%) solvent could extract the flavonoid compounds from roselle calyx such as quercetin, kaempferol, luteolin and apigenin more than other solvents which contain higher percentage of water.

According to the pH-differential method, the total anthocyanin content of roselle calyx extract showed no significantly differences ($p>0.05$) in the higher content of 30% ethanolic extract which was 321.73±26.72 mg Cyanidin-3-glucoside equivalents/g extract than aqueous extract (274.60±23.79 mg Cyanidin-3-glucoside equivalents/g extract. This study confirms that roselle calyx extract displayed high content in anthocyanins. Prior research reported that anthocyanins had been identified in the calyx of roselle including delphinidin-3-O-sambubioside and cyanidin-3-O-sambubioside (Piovesana, Rodrigues & Zapata Noreña, 2019). The determination showed lower content of both total phenolic and total anthocyanin which were extracted with 95% ethanol and results were similar to prior reports (Villalobos-Vega et al., 2023).

Table 2 Yield value and content of total phenolic, total flavonoid and total anthocyanin in roselle calyx extracts

Roselle calyx extracts	Yield (%w/w)	Total phenolic content (mgGAE/g extract)	Total flavonoid content (mgQE/g extract)	Total anthocyanin content (mg Cyanidin-3-glucoside equivalents/g extract)
30% ethanolic extract	50.67	48.78±0.83^a	6.18±0.91^b	321.73±26.72^a
70% ethanolic extract	29.77	47.45±0.12^a	3.46±0.17^c	66.80±11.05^c
95% ethanolic extract	23.43	32.72±0.76^c	14.98±1.47^a	39.89±10.07^d
Aqueous extract	39.96	44.80±1.10^b	1.67±0.25^d	274.60±23.79^b

Remark: The content of total phenolic, total flavonoid and total anthocyanin are given as mean ± SD (n=3). The different superscript letter in the same column represents significant differences when compared with each extract at $p<0.05$

2. *In vitro* antioxidant activities

The antioxidant activities of roselle calyx extracts were determined by measuring its DPPH and $ABTS^+$ radicals scavenging activities. The concentration providing 50% inhibition (IC_{50}) of roselle calyx extracts and positive control (Trolox) are presented in Table 3.

Table 3 Antioxidant activities of roselle calyx extracts

Samples	IC_{50} (mg/mL)	
	DPPH radical	$ABTS^+$ radical
30% ethanolic extract	0.43±0.001^d	0.08±0.001^d
70% ethanolic extract	0.51±0.014^c	0.10±0.001^c
95% ethanolic extract	1.62±0.037^a	0.11±0.008^c
Aqueous extract	1.04±0.003^b	0.27±0.003^a
Trolox	0.04±0.001^e	0.25±0.001^b

Remark: Values are given as mean ± S.D of triplicate. The different superscript letter in the same column represents significant differences at $p<0.05$

The antioxidant activity was carried out using a radical scavenging assay including DPPH and $ABTS^+$ methods. A DPPH assay was used to assess nitrogen radicals including reactive nitrogen species (RNS) that are well-known to be pro-inflammatory mediators, whereas ABTS+ radical assay was used to target oxygen radicals for estimating neutralization of reactive oxygen species (ROS). The IC_{50} value is a parameter widely used to measure free radical scavenging activity. A smaller IC50 value corresponds to a higher antioxidant activity. Our results found that the 30% ethanolic extract showed significantly ($p<0.05$) exhibited DPPH and $ABTS^+$ radical scavenging activity higher than other extracts. This demonstrated that antioxidant activities result of 30% ethanolic extract are related to both total phenolic and total anthocyanin contents. The free radical scavenging activity of the extract may result from high phenolic and anthocyanin contents. Thus, the antioxidant properties of this extract may be attributed to the both phenolic and anthocyanin which may act as an antioxidant. Our results were similar to prior reports (Wu et al., 2018).

3. Cytotoxicity effect on normal human dermal fibroblasts of roselle extracts

Cell viability is one of the criteria for evaluation of cytotoxicity test of crude extracts. Here, the cytotoxic effect of roselle calyx extract on normal human dermal fibroblast (NHDF) cells was evaluated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The obtained results are expressed as percentage of cell viability and concentration of extracts are shown in Fig. 2. The percentage of cell viability after exposure to 5-250 ug/mL of all roselle calyx extracts was significantly decreased ($p<0.05$) when compared with control cell (0 ug/mL, untreated the extracts) of each extract. However, the results showed that the percentage of cell viability was more than 70% after treating with all concentration of each extract. It demonstrated that all extracts from roselle calyx was not cytotoxic to NHDF cells. These results were similar to prior reports (Wang et al., 2022) which found that the ethanolic extract of roselle calyx did not show a cytotoxicity effect on human dermal fibroblasts.

4. Physicochemical characterizations of water-in-oil-in-water emulsion

Water in oil in water (W/O/W) emulsion was prepared using beaker method with two-step emulsification. Roselle calyx extract was produced by 30% ethanol and selected for loading in the inner phase of W/O/W multiple emulsion. The physicochemical data of W/O/W emulsion base (no extract) and W/O/W emulsion loaded extract including physical appearance, color value (L*, a*, b*), electrical conductivity, pH value and viscosity value are presented in Table 4.

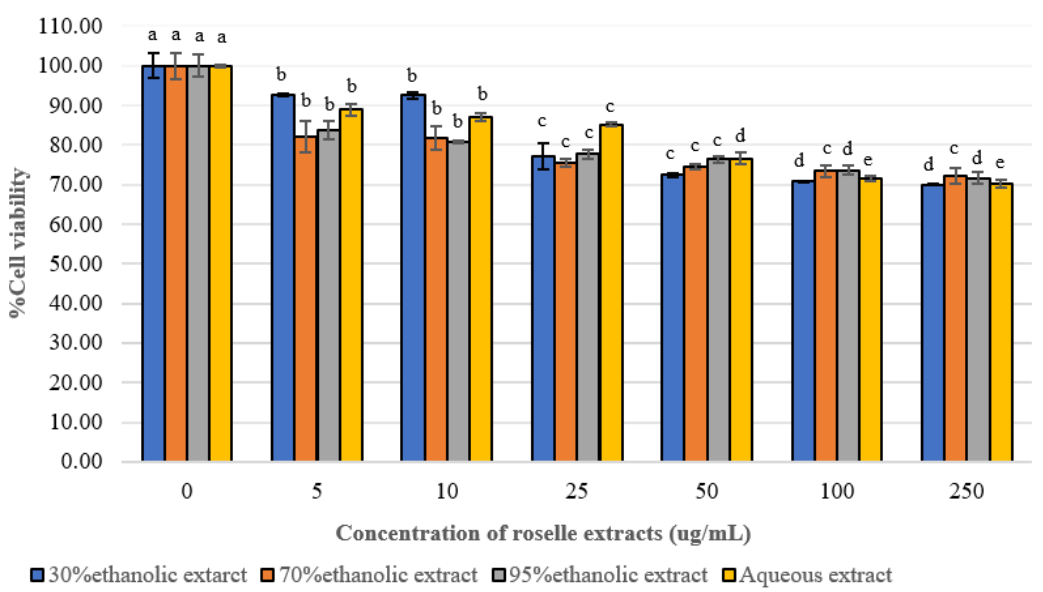

Fig. 2 The percentages of NHDF cell viability after exposure to roselle calyx extracts. Each bar represents mean ± S.D. of triplicate. The different subscript letters on the chart in each extract represents significantly different when compared with each concentration in the same extract at $p<0.05$

Table 4 The physicochemical data of W/O/W emulsion base (no extract) and W/O/W emulsion loaded extract

Formulations	Physical appearance	Electrical conductivity (μS/cm)	pH	Viscosity (cP)	L*, a*, b* value
W/O/W emulsion base (no extract)	Homogeneously Non-separation Non- flocculation White color	+83.23±1.11[a]	6.56±0.03[a]	12.5±0.17[a]	L* = 65.12±1.21[a] a* = -2.64±0.22[a] b* =2.62±0.39[a]
W/O/W emulsion loaded extract	Homogeneously Non-separation Non-flocculation Light pink color	+82.67±1.15[a]	5.59±0.04[b]	11.7±1.08[a]	L* = 53.47±0.22[b] a* = 0.32±0.04[b] b* = 7.24±0.04[b]

Remark: Values are given as mean ± S.D of triplicate. The different superscript letter in the same column represents significant differences at $p<0.05$

The electrical conductivity of multiple emulsion was evaluated. The basic principle of this test is that water is a good conductor of electricity. In this study, the electrical conductivity of multiple emulsion base (no extract) and multiple emulsion loaded extract showed positive charge. This test found to be positive as water which is the continuous phase. And in the formulations added sodium chloride (NaCl) to the aqueous phase used as electrolyte, thus, its conductivity increases greatly. Obtained result showed that the electrical conductivity of multiple emulsion loaded extract and multiple emulsion base (no extract) was not significantly ($p>0.05$) decreased. This implied that the increasing of electrical conductivity values may be due to the transfer of NaCl which was entrapped in the internal aqueous phase of the multiple emulsion to the external aqueous phase. While the decrease may be attributed to the transfer of NaCl lost into external aqueous phase during the process of manufacturing towards the internal aqueous phase (Jiao & Burgess, 2003).

The pH value of multiple emulsion loaded extract was 5.59±0.04 which was significantly ($p<0.05$) decreased compared with multiple emulsion base (no extract). The decreasing in pH value of multiple emulsion loaded extract was probably due to the production of highly acidic of the extract which had a high content of phenolic compound. Both formulations were similar to prior reports that the topical products should be acidified and possess pH in the range of 4 to 6 (Lukić, Pantelić, & Savić, 2021).

The viscosity of multiple emulsion loaded extract and multiple emulsion base (no extract) was not significantly ($p>0.05$) decreased. The viscosity of multiple emulsion may be due to the adding of sorbitane monooleate (Span 80) in the oil phase of water in oil primary emulsion and incorporated NaCl salt in the internal aqueous phase. The decreasing of viscosity in the multiple emulsion loaded extract may be due to a gradual permeation of salt through the oil layer to the external continuous aqueous phase. Our study was similar to prior reports (Jiao & Burgess, 2003).

The color of the prepared multiple emulsion was measured in terms of the L*, a*, b* color space system. In this color space, L* represents the lightness and a and b are color coordinates: where +a is the red direction, -a is the green direction, +b is the yellow direction and -b is the blue direction. The resulting showed L* a* b* color value of multiple emulsion base (no extract) and multiple emulsion loaded extract were significantly ($p<0.05$) different. The color value of multiple emulsion loaded extract was L*=53.47±0.22, a*=0.32±0.04 and b*=7.24±0.04 which showed creamy light pink in color which may be caused by the color of 30% ethanolic extract. This is similar to visual investigation.

The morphology of prepared multiple emulsion was investigated using an optical microscope. The physical appearance and micrograph images of W/O/W emulsion base (no extract) and W/O/W loaded roselle calyx extract are shown in Fig.3. Physical appearance of W/O/W emulsion base (no extract) and W/O/W emulsion loaded extract provided the good physical characteristics including homogeneously, non-separation or non-flocculation occurrence. According to color appearance, multiple emulsion base (no extract) showed a white color and light pink color for multiple emulsion loaded extract, determined by visualization (Fig.3A and 3B). The shape of the W/O/W droplet in emulsion base (no extract) (Fig.3C) and emulsion loaded roselle calyx extract (Fig.3D) appeared to be multiple globules droplets showing inter and external phases of water and oil phase in the middle and both micrographs showed the same in behaviour of W/O/W emulsion structure.

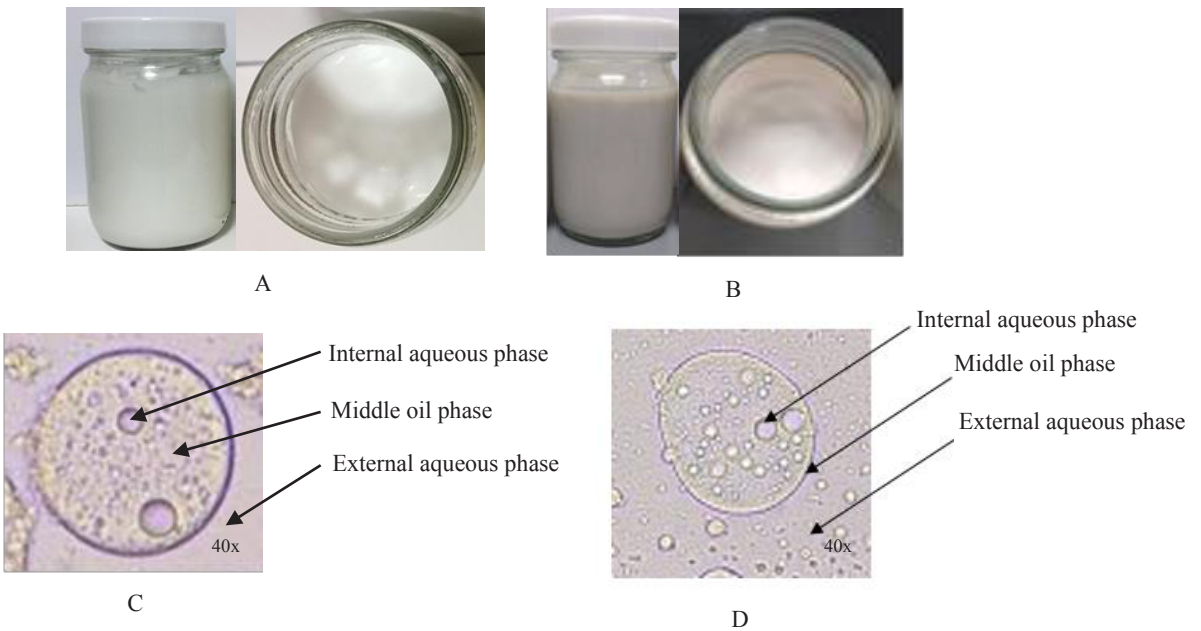

Fig. 3 The physical appearance of (A) water in oil in water emulsion base and (B) water in oil in water emulsion loaded roselle calyx extract and a typical photomicrograph of (C) water in oil in water emulsion base and loaded roselle calyx extract in the internal aqueous phase (D)

5. Physicochemical stability study of W/O/W emulsion loaded roselle calyx extract

The physicochemical stability of prepared multiple emulsion was determined under accelerated conditions including freeze-thaw (-25°C for 24 h followed by 4°C for 24 h as 1 cycle) for 6 cycles, low temperature (LT) (4±1°C) for 30 days, high temperature (HT) (40 ±1°C) for 30 days and room temperature (RT) (30±5°C) for 30 days, results are shown in Table 5. The physical appearance of prepared multiple emulsion was determined in the terms of color, odor and homogeneity, according to visual investigation compared with initial condition. Color had little changes in color of samples kept at high temperature whereas others conditions were not different. Odor did not change at all by conditions tested. The homogeneity of the remaining formation and the phase separation or flocculation did not occur, results are shown in Fig 4. This demonstrates that the concentration of sorbitane monooleate (Span 80) in the oil phase and polysorbate 80 (Tween 80) in the continuous phase had enhanced multiple emulsion stability. In addition, sodium chloride (NaCl) salt has a complex effect on the stability of W/O/W multiple emulsion. It stabilizes the inner droplet (Jiao & Burgess, 2003).

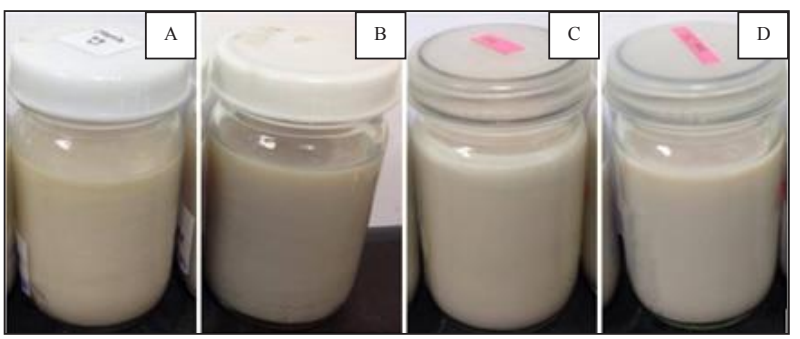

Fig. 4 The physical appearance of W/O/W emulsion loaded extract after storage at (A) room temperature (RT), (B) high temperature (HT) (40 ±1°C), (C) low temperature (LT) (4±1°C) and (D) freeze-thaw cycle

The electrical conductivity and viscosity were not significantly ($p>0.05$) changed compared with the initial condition. The electrical conductivity and viscosity values slightly changed indicating a small net water diffusion through the oil layer during storage. Previously revealed pH of skin range of 4 to 6 are considered to be average pH of the skin. Results of pH value analysis found that the multiple emulsion under the room temperature condition did not significantly ($p>0.05$) change compared with the initial condition. This indicated that that primary emulsion which was entrapped in the extract was stable after storage at room temperature. However, other conditions, the pH value did significantly ($p<0.05$) change which was due to the production of highly acidic of the extract which had high content of phenolic compound. For the colorimetry detection, there was little change in L* a* b* color value under all condition tests and these results were similar to evaluating by visual.

Table 5 Physicochemical stability of W/O/W emulsion loaded roselle calyx extract

Conditions	Electrical conductivity (μS/cm)	Viscosity (cP)	pH	Color		
				L*	a*	b*
Initial	+82.67±1.15a	11.7±1.08a	5.59±0.04a	53.47±0.22a	0.32±0.04a	7.24±0.04a
RT	+82.33±2.08a	12.7±0.60a	5.49±0.06a	50.33±0.44b	0.41±0.07a	5.62±0.24b
HT	+83.33±0.58a	11.5±1.05a	5.34±0.15b	57.86±2.08c	0.15±0.02b	6.52±0.40c
LT	+83.00±1.00a	10.8±0.60a	5.40±0.10c	56.38±1.05d	0.39±0.02a	7.46±0.65a
Freeze-thaw cycles	+82.00±2.65a	13.5±1.20a	5.75±0.07d	55.24±2.54a	0.96±0.03c	5.51±0.17d

Remark: Values are given as mean ± S.D of triplicate. The different superscript letter in the same column represents significant differences when compared with initial condition at $p<0.05$. RT: room temperature; LT: low temperature; HT: high temperature

Conclusion

The crude extract from roselle calyx which was extracted by 30% ethanol was found to have high content of phenolic and anthocyanin contents which possessed DPPH and ABTS+ radical scavenging activities. Additionally, all crude extract from roselle calyx showed no toxicity effect on normal human dermal fibroblast (NHDF) cell. The W/O/W emulsion which loaded roselle calyx extract into the internal aqueous phase of primary water in oil was formulated by beaker method with two-step emulsification. Prepared formular showed a good physicochemical stability after storage under all conditions tested when compared with the initial conditions. All findings indicated that the 30% ethanolic extract of roselle calyx can be used as a natural antioxidant for an active ingredient in skin cosmetic products.

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