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Antimicrobial and Antioxidative Effects of Roselle (*Hibiscus sabdariffa* L.) Flower Extract and Its Fractions on Skin Microorganisms and Oxidation

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Abstract Antimicrobial and antioxidative effects of 14 different herbal flower extracts on skin microorganisms and oxidation were tested in this research. Herbal flower extracts were prepared with 70% ethanol. Among the herbal flower extracts, roselle (*Hibiscus sabdariffa* L.) flower extract showed the highest antimicrobial activity against *Staphylococcus epidermidis* as determined by a paper disc method. The seventy % ethanol extract of roselle flower was fractionated by sequential hexane, chloroform, ethyl acetate, *n*-butanol, and water fractionation. The growth of *S. epidermidis, Streptomyces collinus, Streptomyces coeruleoprunus, Salmonella enteritidis, Vibrio parahaemolyticus,* and *Malassezia pachydermatis* was most efficiently inhibited by ethyl acetate fraction of roselle flower extract as determined by a paper disc method and growth inhibition curves. In addition, the ethyl acetate fraction, water fraction and butanol fraction showed free radical scavenging and DNA cleavage inhibition activities. These results demonstrate that roselle flowers hold antimicrobial and antioxidative activities against skin microorganisms and oxidants.

Keywords: roselle (*Hibiscus sabdariffa* L.), antimicrobial and antioxidative effect, DNA single-strand cleavage, 2,2-diphenyl-1-picrylhydrazyl (DPPH)

Introduction

Current trends in the manufacture of Asian and natural cosmetic and skin care products favor the use of natural materials due to the negative image of synthetic ingredients and preservatives. These kinds of cosmetics have the advantages of being generally good for the skin and have few side effects even after long-term use (1).

Adult pimple refers to a pimple that occurs after 25 years of age. It is due to unstable secretion of androgen hormone and excessive secretion of sebum. It is characterized by a high rate of recurrence as a symptom that accompanies the aging of the skin, and is difficult to treat with only simple skincare measures (2). Skin microbiota break down the sebum in pores into isolated fatty acids, which induce inflammation in the skin and form flare and pus; in the most severe cases it may form a bladder and leave a scar on the skin by intruding into the deep layers of the skin (3).

The main environmental causes of aging are ultraviolet rays, smoking, excessive drinking, air pollution, fatty and high-calorie food, and mental stress. Especially, 90% of premature skin aging in young people originates from free radicals produced by ultraviolet rays. Among free radicals, superoxide radical (O_2^-), hydroxyl radical (OH^-), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) show very high reactivity (4) that can progress to fatal oxygen toxicity, and bring about decomposition of cell walls, decomposition of protein, oxidation of lipid, and denaturalization of DNA. As a result, they are believed to cause dysfunction of cells, skin cancer, brain diseases like stroke, and Parkinson's disease, cardiac disorder, arteriosclerosis, inflammation, aging, and autoimmune disease (5-7). Especially, accumulated lipid peroxides produced by oxidative damage of unsaturated fatty acids in biological membranes, results in the decline of biological function, aging, or adult disease (8). Therefore, substances that inhibit the growth of skin microorganisms and have antioxidative activity are of great interest since they may cure or prevent various diseases related to the effects of free radicals and may be useful in the treatment of adult pimple (9). This study investigated the antimicrobial and antioxidative effects by using different kinds of natural herbal extracts, and paved the way for the development of new cosmetic ingredients derived from roselle (*Hibiscus sabdariffa* L.) flower extract, which showed the strongest activities.

Materials and Methods

Herbs and chemicals Fourteen different herbs; roselle (*Hibiscus sabdariffa* L.), thyme (*Thymus* spp.), fennel (*Foeniculum vulgare* M.), lavender (*Lavandula* spp.), lemon balm (*Melissa officinalis* L.), rosehip (*Rosa canina* L.), rosemary (*Rosmarinus officinalis* L.), lemongrass (*Cymbopogon citratus*), jasmine (*Jasminium officinale*), peper mint (*Mentha piperita* L.), blue mallow (*Malva sylvestris* L.), orange passion flower, rose flower, and chamomile (*Anthemis nobilis* L.) were screened, and roselle was selected as a main sample material for this study. While ampicillin, 1,1-diphenyl-2-picrylhydrazyl (DPPH), cytochrome *c*, and diethylenetriaminepenta-acetic acid (DTPA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA), all other reagents were of the highest grade available and were obtained from

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commercial suppliers.

Bacterial strains *Staphylococcus epidermidis* KCTC 1917, *Staphylococcus aureus* subsp. *aureus (S. aureus)* KCCM 11335, *Propionibacterium acnes* KCCM 41747, *Pseudomonas aeruginosa* KCCM 11804, *Vibrio parahaemolyticus* KCCM 11965, *Salmonella enteritidis* KCCM 12021, *Candida albicans (C. albicans)* KCCM 11282, *Streptomyces collinus* KCCM 40498, *Streptomyces coeruleoprunus* KCCM 41264, and *Malassezia pachydermatis* KCCM 50031 were purchased from the Korean Culture Center of Microorganisms (KCCM, Seoul, Korea), and the Biological Resource Center (KCTC, Daejeon, Korea) (Table 1).

Preparation of herbal extracts Herbal extracts were prepared as described (10). Dried herbal flowers (150 g) were mixed, at the rate of 1:10 w/v, with 70% ethanol, and shaken at 150 rpm and 50°C for 48 hr. Plant ingredients were then removed with gauze and the liquid extract concentrated in a rotary vacuum evaporator (N-1000; Eyela, Tokyo, Japan) (11). Following freeze-drying (Ilshin, Korea), we added distilled water to the powder (500 mg/mL) and serially extracted the ingredients with (in that order) hexane, chloroform, ethyl acetate, *n*-butyl alcohol, and water as described (10). Each fraction was lyophilized by concentrating and freeze-drying. Double-distilled water was added (100 mg/mL) and all the samples were filtered through 0.45 μm PVDF filter (Millipore, Milford, MA, USA). The extracts, including fractions, were designated as follows: 70% ethanol extract, EE; hexane fraction, HF; chloroform fraction, CF; ethyl acetate fraction, EAF; *n*-butyl alcohol fraction, BF; water fraction; WF.

Antimicrobial tests and inhibitory effects Procedures to test antimicrobial activities were as described by Kang *et al.* (10). After culturing overnight, each tested microorganism strain was inoculated in 100 μL of the appropriate agar media. Three mg of herbal extract were loaded onto 10-mm sterilized paper discs (Advantech, Tokyo, Japan) in close contact with growth media and incubated for 18 hr at a cultivating temperature adjusted to meet each test strain needs (Table 1). *P. acnes* (KCCM 41747) was cultivated anaerobically (Anoxomat™ Mark-II, Mart, Netherlands) (12, 13). To quantify the degree of growth inhibition, each strain was grown in appropriate media (O.D. at 660 nm = 0.04) and subjected to the contents of each fraction at 1 mg/mL, after which the $O.D._{660}$ was measured again (Mini Photo 518 Spectrophotometer; Taitec, Saitama, Japan). *P. acnes* (KCCM 41747) was incubated at the surface culture in liquid media.

Free radical scavenging activity Two mL of each sample containing various concentrations of EE, HF, CF, EAF, BF, and WF was mixed with 1 mL of 0.2 mM DPPH in the ethanol. After 30 min at room temperature, the absorbance at 517 nm was recorded (UV-1610PC; Shimadzu, Kyoto, Japan). Free radical scavenging activities were expressed as electron donating abilities (EDA) which express a decrement rate of absorbance with and without a sample. EDA (%) was calculated as follows: EDA (%) = $[1-(S/B)]\times 100$, where S, absorbance at 517 nm in the presence of a sample; B, absorbance at 517 nm in the absence of sample (14, 15).

Plasmid DNA isolation *Escherichia coli* UT481 was transformed by plasmid pBR322 (4361 bp) and stored in a cryogenic freezer (-70°C), in the laboratory. Competent cell (16), transformation, and plasmid isolation were done as described by the method of Park *et al.*(17).

DNA single-strand cleavage by hydrogen peroxide and cytochrome c pBR322 DNA is a supercoiled (SC) double-strand circular DNA. Single-strand cleavage was detected by observing the change in the SC form to the open circular (OC) form, a relaxed circular form. The SC DNA (1.0 μg/μL) was treated with 20 μM DTPA, 21.85 μM cytochrome *c* (Fe^{2+}), and 858 μM hydrogen peroxide. Different fractions and contents such as EE, HF, CF, EAF, BF, and WF (10, 100, and 1,000 ppm) were added to the DNA solution (18). The final volume of the reaction

Table 1. Microbial strains, media, and incubation temperatures used for the antimicrobial tests

Microorganisms		Media used[1)]	Incubation Temp.(°C)
Gram(+)			
S. epidermidis	KCTC 1917	NB & NA	37
S. aureus	KCCM 11335	TSB & TSA	37
P. acnes	KCCM 41747	RCMB & RCMA	37
S. collinus	KCCM 40498	YMEB &YMEA	28
S. coeruleoprunus	KCCM 41264	YMEB &YMEA	26
Gram(-)			
P. aeruginosa	KCCM 11804	TSB & TSA	37
V. parahaemolyticus	KCCM 11965	NB+3%NaCl&NA+3%NaCl	37
S. enteritidis	KCCM 12021	NB & NA	37
Yeast			
C. albicans	KCCM 11282	YMB & YMA	25
M. pachydermatis	KCCM 50031	YMB & YMA	28

[1)]All from Difco laboratories, Detroit, MI, USA: NB, Nutrient broth; NA, nutrient agar; TSB, trypticase soy broth; TSA, trypticase soy agar; RCMB, reinforced clostridial broth; RCMA, reinforced clostridial agar; YMEB, yeast malt extract broth; YMEA, yeast malt extract agar; NB+3% NaCl, nutrient broth with 3% NaCl; NA+3% NaCl, nutrient agar with 3% NaCl; YMB, TM broth; YMA, YM agar.

Table 2. Antimicrobial activity of herbal flower extracts (70% ethanol)[1)]

	S. epidermidis
Hibiscus sabdariffa L.	+++++[2)]
Thymus spp.	-
Foeniculum vulgare M.	
Lavandula spp.	-
Melissa officinalis L.	-
Rosa canina L.	-
Rosmarinus officinalis L.	+++
Cymbopogon citratus	-
Jasminium officinale	+
Mentha piperita L.	-
Malva sylvestris L.	-
Orange Passion flower	-
Rose flower	-
Anthemis nobilis L.	-

[1)]Each strain was inoculated onto an agar plate and 3 mg of herb extract was loaded on a 10-mm sterilized paper disc (Advantech).
[2)] +, Degree of antimicrobial activity; -, no activity.

mixture was 50 μL. It was incubated at 37°C for 30 min then separated using 1.0% agarose gel electrophoresis (100 V for 30 min).

Results and Discussion

Recovery of extract and fractions Ethanol extract obtained from the 150 g of roselle flower was 66.6 g (recovery 44.4%). Fractions of hexane, chloroform, ethyl acetate, *n*-butyl alcohol, and water obtained by the sequential fractionation of the ethanol extract (30 g) were 0.29 (recovery 0.97%), 0.19 (recovery 0.63%), 2.50 (recovery 8.3%), 3.48 (recovery 11.6%), and 19.20 g (recovery 64.0%), respectively.

Identification of herb extract with antimicrobial activity against *S. epidermidis* Antimicrobial activity of the ethanol extract (EE) from 14 different herbs was tested against *S. epidermidis* KCTC 1917. Roselle flower extract showed the highest antimicrobial activity, while the extracts from rosemary and jasmine were less effective (Table 2). Other extracts did not show activity. Clear zone formation by the roselle EE reached almost 99% of the ampicillin control (3 mg/disc, Fig. 1). This is consistent with the previous studies, reporting the EE from dried roselle leaves and petals reducing aflatoxin formation (19) and food-borne microorganism growth (10). This data may suggest that roselle flowers possess an antimicrobial activity against skin microorganisms.

Table 3. Antimicrobial activity of fractions obtained from roselle flower extracts[1)]

Strians	Clear zone (mm)[2)]				
	CF	HF	EAF	BF	WF
Gram(+)					
S. epidermidis	-[3)]	-	25.3	22.8	16.5
S. aureus	-	11.9	15.3	14.6	12.5
P. acnes	-	-	-	-	-
S. collinus	-	-	30.7	26.7	25.5
S. coeruleoprunus	17.0	-	37.6	34.0	34.0
Listeria monocytogenes	-	-	11.2	11.2	11.2
Gram(-)					
P. aeruginosa	-	11.9	11.4	-	-
V. parahaemolyticus	15.2	-	29.45	26.3	21.7
S. enteritidis	-	-	19.0	11.6	11.2
Yeast					
C. albicans	-	-	-	-	-
M. pachydermatis	16.5	-	38.1	29.7	34.7

[1)]Each strain was inoculated on an agar plate and 3 mg of herb extract was loaded on a 10-mm sterilized paper disc (Advantech).
[2)]Clear zone diameter.
[3)]No inhibitory zone was formed.

Antimicrobial activity of herb fractions To further examine the antimicrobial activity, we fractionated the EE with several solvents serially, in the order of hexane, chloroform, ethyl acetate, *n*-butyl alcohol, and water as described (10). The hexane fraction (HF) and chloroform fraction (CF) showed low antimicrobial activity against Gram-negative or Gram-positive bacteria, but the ethyl acetate fraction (EAF), butanol fraction (BF), and water fraction (WF) all exhibited relatively high activities against both Gram-negative and Gram-positive microorganisms

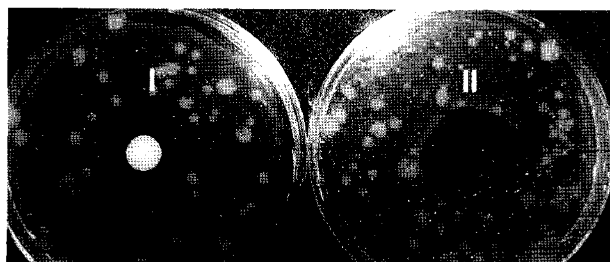

Fig. 1. Comparison of the antimicrobial activity of roselle flower extract and ampicillin. *S. epidermidis* KCTC 1917 was inoculated in agar media. I, ampicillin; II, EE

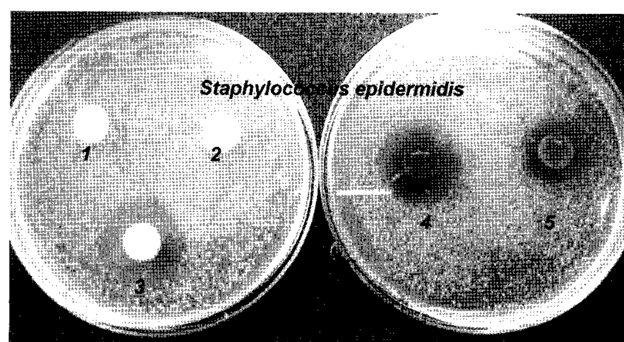

Fig. 2. Comparison of the antimicrobial activity of roselle flower fractions and ampicillin. *S. epidermidis* KCTC 1917 was inoculated in agar media. 1. CF; 2. HF; 3. EAF; 4. BF; 5. WF

(Fig. 2, Table 3). The EAF was most the effective inhibitor of microbial growth, based on clear zone formation (Fig. 2, Table 3). EAF, BF, and WF, in order of confirmed antimicrobial activity, were added to growth media (1 mg/mL) and the inhibitory effects were measured (Fig. 3). *S. epidermidis* incubated with BF and WF showed gradual elevation of optical density after 6 hr of incubation; however, with the addition of EAF, growth inhibition of *S. epidermidis* was maintained throughout almost 24 hr of incubation (Fig. 3A). *V. parahaemolyticus* with WF exhibited an elevation in optical density after 4 hr of incubation. BF and EAF maintained dramatic growth inhibition through the entire 24 hr of incubation (Fig. 3B). *S. enteritidis* incubated with the BF and WF fractions accumulated rapidly from 6 to 16 hr of incubation and growth continued with little change until 24 hr (Fig. 3C). The addition of EAF, however, resulted in visible growth inhibition (Fig. 3C). *M. pachydermatis, S. collinus,* and *S. coeruleoprunus* with WF, BF, and EAF maintained dramatic growth inhibition through the entire 24 hr of incubation (Fig. 3D, E, F). As observed from clear zone formation experiments (Table 3), the growths of *C. albicans* and *P. acnes* were not inhibited by the HF, CF, BF, WF, and EAF fractions. The reason for the ineffectiveness of the fractions on the growth inhibition of *C. albicans* and *P. acnes* is unclear. Previously, it was reported that an ethanol extract from dried roselle leaves was shown to have an *in vitro* inhibitory effect against some fungi (20), but it was found to be ineffective against *Lumbricus terrestries* (19). It was also reported that oil extracted from roselle seeds inhibited the growth of *Bacillus anthracis*, but not *Proteus vulgaris* and *P. aeruginosa* (21). Presently, we are attempting to isolate the active antimicrobial constituent from the fractions of roselle flower. Overall, these results show that roselle flowers harbor some antimicrobial activity with a varied antimicrobial spectrum of effectiveness, and that the critical ingredient is highly enriched in the EAF of the extract.

Quenching of DPPH Quenching of DPPH free radical scavenging activities in the herb extract and fractions were also tested, focusing on their ability to bleach the stable radical DPPH (22). Because of its odd electron, DPPH gives a strong absorption band at 517 nm in visible spectroscopy. As this electron becomes paired off in the presence of a free radical scavenger, the absorption vanishes, and the resulting decolorization is stoichiometric with respect to the number of electrons taken up (23). The standard curve of ascorbic acid was measured at the concentration range from 1 to 5 μg/mL (Fig. 4 in set). Based on the standard curve, the effectively-concentrated 50% (EC_{50}) value of ascorbic acid was 2.97±0.10 μg/mL. The samples of EE, CF, EAF, BF, and WF with different concentrations (25 to 225 μg/mL) showed a dose dependent DPPH quenching capacity (Fig. 4). However, HF showed no DPPH quenching capacity. EC_{50} of EAF, BF, and WF were 76.81±0.36, 132.07±6.67, and 171.17±4.40 μg/mL respectively. Previously, it has been reported that the scavenging effects (EC_{50}) of DPPH radical in extracts or fractions vary depending on the sources of medicinal plants or herbs. For example, the EC_{50} for DPPH radical by methanol extracts of *Cornus officinalis S. et Z* (Japanese Cornel Dogwood) leaves and *Lagerstroemia indica* L. (Lythraceae) flowers were 44.4 and 29.6 μg/mL respectively (24). EC_{50} in the ethanol extracts of *Ganoderma lucidum* Krast (Ganodermataceae) was 412 μg/mL (25). Researchers have reported that there is a positive correlation between free radical scavenging activity and total phenolic compounds (26, 27). Phenolic compounds are effective hydrogen donors, which make them good antioxidants (28, 29). Furthermore, they

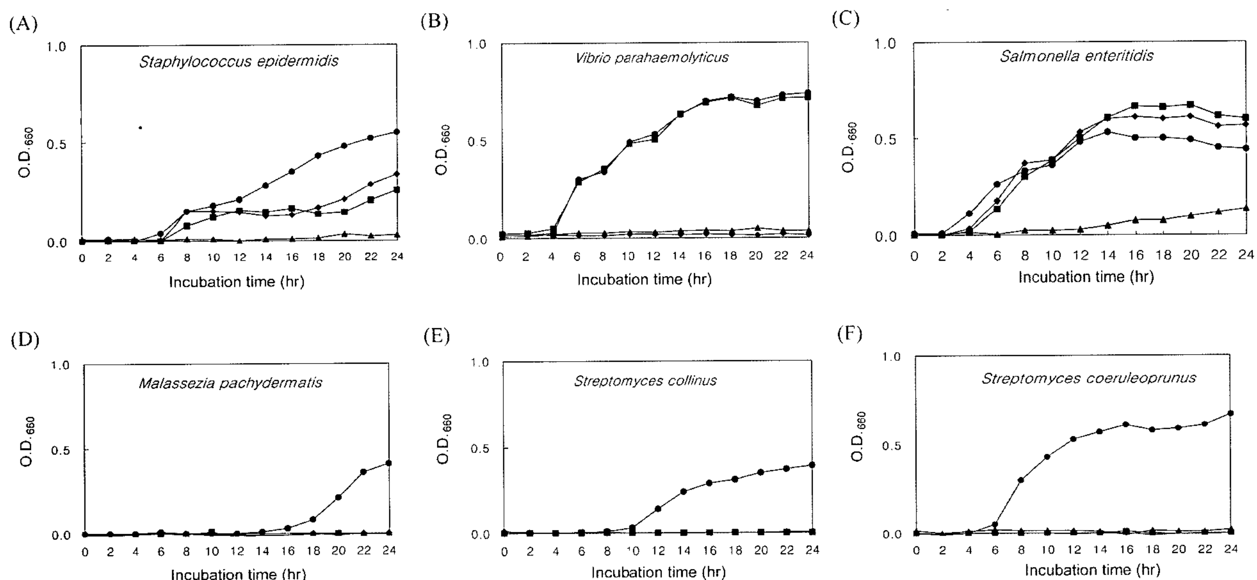

Fig. 3. Inhibitory effects of roselle fractions against skin microorganisms during a 24-hr culturing period. (A) *Staphylococcus epidermidis*; (B) *Vibrio parahaemolyticus*; (C) *Salmonella enteritidis*; (D) *Malassezia pachydermatis*; (E) *Streptomyces collinus*; (F) *Streptomyces coeruleoprunus*. ●, Control cells (no fraction added); ▲, EAF; ■, WF; ◆, BF.

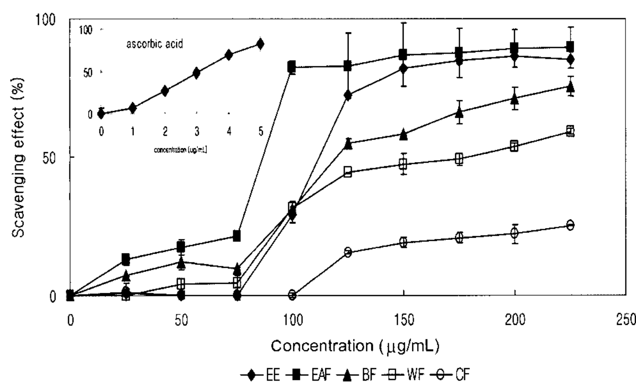

Fig. 4. Concentration-dependent scavenging activity on DPPH radical of 70% ethanol extract and fractions. EE, 70% ethanol extract; EAF, ethyl acetate fraction; BF, butyl alcohol fraction; WF, water fraction; CF, chloroform fraction. Inset: concentration-response curve for DPPH scavenging activity of standard ascorbic acid solution. Data shown are mean values of 6 complete sets of experiments (n=4).

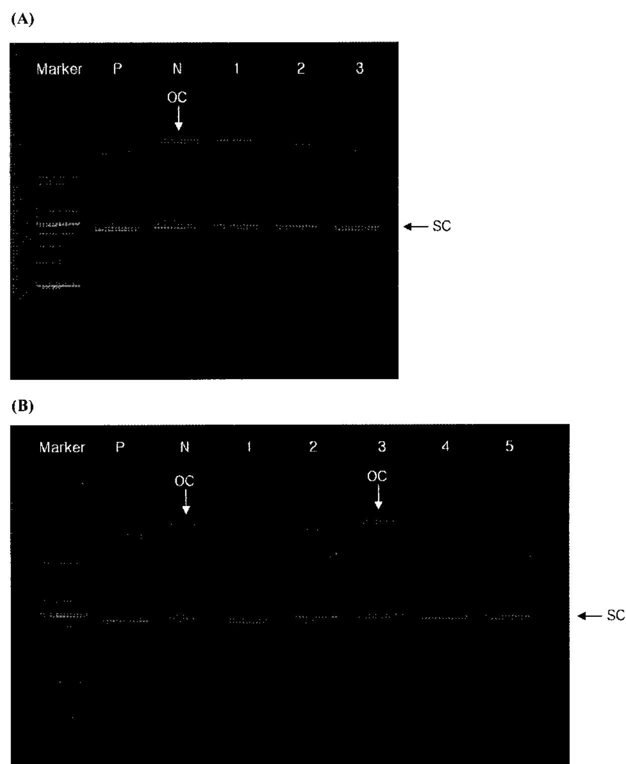

Fig. 5. Inhibitory effects of roselle extract and fractions on DNA single-strand cleavage induced by hydrogen peroxide and cytochrome *c*. (A) Marker (1 kb ladder, iNtRON, Korea); lane P, positive control (pBR322+DTPA); lane N, negative control (pBR322+DTPA+H_2O_2+cytochrome *c*); lane 1, pBR322+DTPA+ H_2O_2+cytochrome *c*+10 ppm WF; lane 2, pBR322+DTPA+H_2O_2+ cytochrome *c*+100 ppm WF; lane 3, pBR322+DTPA+H_2O_2+ cytochrome *c*+1,000 ppm WF. (B) Marker (1 kb ladder, iNtRON, Korea); lane P, positive control (pBR322+ DTPA); lane N, negative control (pBR322+DTPA+H_2O_2+cytochrome *c*); lane 1, pBR322+ DTPA+H_2O_2+cytochrome *c*+1,000 ppm EE; lane 2; pBR322+ DTPA+H_2O_2+cytochrome *c*+ 1,000 ppm CF; lane 3, pBR322+ DTPA+H_2O_2+cytochrome *c*+1,000 ppm HF; lane 4, pBR322+ DTPA+H_2O_2+cytochrome *c*+1,000 ppm EAF; lane 5, pBR322+ DTPA+H_2O_2+cytochrome *c*+1,000 ppm BF. SC and OC in the figure indicate the position of the supercoiled and open circular forms of DNA, respectively.

possess ideal structural properties for free radical scavenging activities (30). Previously, it was reported that roselle flower extracts contain phenolic compounds exerting antioxidative activities (19, 31). Further analyses are required to study the major constituents of the roselle flower extract and fractions exerting the antioxidative activities.

DNA single-strand cleavage by hydrogen peroxide and cytochrome *c* When plasmid pBR322 DNA was treated with H_2O_2 in the presence of cytochrome *c*, DNA single-strand cleavage was observed (32). Therefore, we examined the inhibition of DNA cleavage by roselle flower extract and fractions. Figure 4 shows the result of the agarose gel electrophoresis. pBR322 DNA (positive DNA, lane P) shows two major bands with sizes of 4.3 and over 10 kb, and shows a minor band at the position of 6 kb. However, 6 and over 10 kb bands disappeared and another band with a size of far over 10 kb appeared as the result of the H_2O_2 treatment in the presence of cytochrome *c* (Fig. 5, lane N). With the addition of WF, the inhibitions of DNA cleavage, whose effectiveness was concentration-dependent, were observed (Fig. 5A). With the addition of EAF, BF, and EE, the inhibitory activities against the DNA single-strand cleavages were observed, but no inhibitory activity was observed with the HF (Fig. 5B). These results match the herb extract's free radical removal capacity (Table 4), which demonstrates that roselle flower extract has antioxidant properties. Lipid peroxidation is not only problematic in the food industry, but also in human biology. Numerous researchers report that products of lipid peroxidation and reactive oxygen species that cause lipid peroxidation are closely associated with a variety of diseases such as cancer, and also to the aging process (18). It is also reported that hydrogen peroxide and cytochrome c, when in close proximity, can cause lipid peroxidation and destruction of DNA (18, 32). Therefore, the fact that roselle flower extract and fractions can prevent DNA cleavage, which is caused by free radicals and peroxide, implies the possibility of it acting as an antioxidant within cells. Berberine, a component of *Coptis japonica* Makino, and theaflavins, a component of black tea, which showed similar activities to the result above, were reported to diminish hydrogen peroxide induced DNA cleavage (18, 32). These data also suggest that roselle flowers possess antioxidative activity. The roselle flower extract can also be used as an antioxidative additive or pharmaceutical supplement because of its effectiveness against oxidation, although bioactive ingredient(s) exerting antioxidative effects have not been identified. In the future, analyses of roselle flower extract and fractions are need to be undertaken to separate and identify the active constituents that prevents DNA cleavage.

Previously, we showed that roselle petals harbor antimicrobial activity against foodborne and food spoilage microorganisms (10). The calyces of roselle flower are

consumed worldwide as a cold beverage and as a hot drink (sour tea). The red anthocyanin pigments in the calyces are used as food colouring agents (19). The roselle extracts are characterized by a very low degree of toxicity. The LD_{50} of roselle calyx extract in rats was found to be above 5,000 mg/kg (19). This study indicates that roselle flower extract and fractions exhibit antimicrobial activities that inhibit the growth of some skin microorganisms and antioxidative activities against oxidants and oxidatively induced DNA single-strand cleavages. In view of the results of this study and because of its relative safety, roselle flower extracts and fractions could be a source of cosmetically and/or therapeutically useful products.

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