International Journal of ChemTech Research
CODEN (USA): IJCRGG, ISSN: 0974-4290, ISSN(Online):2455-9555
Vol.9, No.08 pp 541-547, 2016

In vitro screening of Korean halophytes for cosmeceutical ingredients

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Abstract :

Salt-tolerant halophyte plants have various beneficial health effects, but their effects on skin health are largely unknown. To identify novel cosmeceutical ingredients, we screen ed 23 parts of 21 Korean halophyte plant species collected from the Jeju Biodiversity Research Institute (JBRI) in Jeju Island,the southemmost island of the Korean Peninsula. Total flavonoid and phenolic contents as well as 2.2 diphenyl-1-picrylhydrazyl (DPPH) a nd 2,2'-azubi-bis(3-ethlbenzothiazoline-6-sulphonic acid(ABTS)scavenging activities weree valuated in the halophyte plants. In the ABTS assay, we observed significantly greater s cavenging activity for Rumex acetosella [half-maximal inhibitory concentration (IC_{50}) <1 00 ug/ml] than the other plants. R acetosella also had the highest total flavonoid and p henolic contents (40.6 and 63.6 mg, respectively). However,the halophyte plants in thisst udy showed low elastase and no tyrosinase inhibition activities.We investigated the anti- inflammatory effects of these halophyte plants for potential use in skin products andpre parations. Specifically, they were screened for inhibitory effects on the proinflammatory mediator nitric oxide (NO) in lipopolysaccharide (LPS)- stimulated macrophage RAW 26 4.7 cells. Aretemisia fukudo aerial parts and Aster spathulifolitus whole plants strongly i nhibited LPS-stimulated NO production in a concentration-dependent manner, with IC_{50} v alues of 35.6 and 81.7 ug/ml respectively. These results suggest that these halophyte pla nts possess several biological activities that confer potent inhibition of skin aging and i nflammation. Further investigations will focus on cell-based invitro asssays and the identitication of the major active compents mediating anti-aging and anti inflammation.
Keywords : ABTS, DPPH, Cosmetic, Elastase, Halophytes, Tyrosinase.

Introduction

Skin aging is a complex phenomenon that includes age-dependent/chronological aging and premature aging/photoaging. The former describes changes in elasticity of the skin over time, while the latter is predominately a result of exposure to UV radiation, which is one of the most significant external stress-inducing factors. Irrespective of aging category, skin wrinkles are associated with excessive reactive oxygen species (ROS) and inflammatory responses[1]. Although the skin possesses elaborate defense mechanisms that interact with ROS to eliminate their poisonous effects, excessive and chronic exposure to UV can counteract these defense mechanisms, leading to oxidative stress and damage, resulting in photoaging[2]. Excessive ROS accumulation can also cause a number of deleterious effects on the skin via the activation of skin aging-related enzymes, such as tyrosinase, elastase, and inducible nitric oxide synthase[3]. Tyrosinase is a rate-limiting enzyme

that catalyzes the first two steps in mammalian melanogenesis[4]. Elastase is a proteolytic enzyme involved in the degradation of elastin, leading to skin aging[5]. Inducible nitric oxide synthase (iNOS) also is a key enzyme that generates nitric oxide (NO); it plays an important role in numerous physiological and pathophysiological conditions, e.g., skin inflammation[6,7]. Therefore, ROS and free radicals are among the most important factors triggering the skin aging process.

Antioxidants include a broad range of substances that have the ability to protect living cells from damage caused by ROS-induced oxidative stress by donating one of their electrons. They function as ROS scavengers and block the generation of ROS. Many naturally occurring antioxidants are used in cosmetics owing to their potential to promote skin health, safety, and consumer acceptability. The commercial development of natural antioxidants to enhance skin health is of current interest owing to the relationship between antioxidants and skin aging[8].

To screen anti-wrinkle materials, methods based on a single relatively stable reagent, such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radicals, are most common owing to their simple set-up and ease of control[9]. Previous research has demonstrated that DPPH and ABTS methods could be used to determine antioxidant activity in many plants extracts. Despite extensive research on the antioxidant activity and phytochemical composition of common and medicinal plants, little is known about these properties in halophyte plants in Korea.

Halophytes naturally survive in saline environments. Many people believe that they are a source of valuable natural products with economic potential[10]. Recent studies have reinforced the notion that salt-tolerant plants exert beneficial health effects; for example, the dichloromethane extract of *Oenotheralaciniata* and *Artemisia fukudo* essential oil prevent lipopolysaccharide (LPS)-induced inflammation[11,12]. Bae et al. (2016) also reported that *Limoniumtetragonum* extract could be a useful source of anti-MMP agents[13]. However, apart from these studies, little is known about the beneficial effects of halophyte plants on skin health[14,15]. Accordingly, in the present study, total polyphenolic content, antioxidant activity, and inhibitory activity against tyrosinase, elastase, and NO production were examined in 21 halophyte plants grown on Korean seashores.

Materials and Methods

Halophyte plant materials and extract preparation

Halophyte plants were collected from the Jeju Biodiversity Research Institute (JBRI) on Jeju Island in 2008. Voucher specimens were deposited at the Jeju Biodiversity Research Institute (JBRI) and the identity of the vouchers and fresh plants was verified by Dr. Gwanpil Song.

Total flavonoid and phenol contents

The total flavonoid and polyphenol contents were determined using the Folin–Ciocalteu assay. Quercetin and gallic acid were used as standards, and the flavonoid and total polyphenol contents of each sample were expressed in milligrams of quercetin (mg QUE/g) and gallic acid (mg GAE/g) equivalent per gram of extract, respectively.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay

DPPH is a stable free radical that is widely used to examine free radical-scavenging ability by reactions with natural extracts and their compounds to yield 1,1-diphenyl-2-picrylhydrazine (DPPH-H). The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of the halophyte plant extracts was estimated according to a previously reported procedure, with some modifications[14]. Briefly, 180 μL of DPPH solution (0.2 mM in ethanol) was mixed with various concentrations (10–2000 μg/mL) of halophyte extracts, and incubated in the dark at 25°C for 10 min. Then, absorbance was measured at 517 nm. Absorbance was also estimated for a negative control consisting of only ethanol. The DPPH radical scavenging activity of the sample was calculated using the following equation:

DPPH scavenging activity = $[(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$.

ABTS Radical Scavenging Assay

ABTS is a blue chromophore produced by the reaction between ABTS and potassium persulfate. It is characterized by a specific absorbance at 734 nm, which decreases with the scavenging of the proton radicals. ABTS assays are the most widely used spectrophotometric assays; they are used to assess both hydrophilic and lipophilic antioxidants,unlike other antioxidant assays. The ABTS radical scavenging activity of the halophyte plant extracts was measured as previously described, with minor modifications[14]. Briefly, 7 mM ABTS and 2.45 mM potassium persulfate were mixed and then incubated in the dark at 25°C for 16 h. The ABTS solution was diluted with ethanol to obtain the working solution with an absorbance at 700 nm (Abs_{700}) of 0.78 ± 0.02. Then, various concentrations (10–2000 μg/mL) of the halophyte plant extracts were mixed with 180 μL of the ABTS solution, incubated in the dark at 25°C for 10 min, and Abs_{sample} and $Abs_{control}$ (ethanol only) were measured. The ABTS radical scavenging activity was calculated using the following equation:

ABTS radical scavenging activity (%) = $[(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$.

Tyrosinase inhibition assay

The mushroom tyrosinase inhibition activity was performed using the methods described by Kim et al. (2016), with slight modifications[14]. Briefly, 70 μL of halophyte extract was combined with 30 μL of mushroom tyrosinase (500 Units/mL in sodium phosphate buffer) in triplicate. After preincubation of the test mixture (100 μL) at 37°C for 5 min, 110 μL of substrate (2 mML-tyrosine) was added to each well. The test mixture (200 μL) was incubated for 10 min at 37°C, and the absorption due to the formation of dopa-chrome was measured at 475 nm. A similar mixture without the plant extract and a solution of hydroquinone-*O*-β-glucopyranoside (arbutin) were used as the vehicle and positive control, respectively. Each treatment was replicated three times, and the percent inhibition of the tyrosinase activity was calculated as follows:

inhibition (%) = $[1 - (Abs_{sample} - Abs_{blank})/Abs_{control}] \times 100$,

whereAbs_{blank} is the absorbance of the blank and the half maximal inhibitory concentration (IC_{50}) indicated the concentration of the halophyte plant extracts that inhibited tyrosinase activity by 50%, as determined by linear curve fitting.

Porcine pancreatic elastase inhibition assay

The porcine pancreatic elastase (PPE) inhibitory activity in the halophyte extracts were was assayed using *N*-Succ-$(Ala)_3$-*p*-nitroanilide (SANA) as the substrate, and the release of ρ-nitroanilide was monitored following the methods described by Kim et al. (2016) [14]. Briefly, 10 μL of PPE (0.1 mg/mL) and 5 μL of SANA (6.5 mM) were added to 165 μL of Tris-hydrochloride (HCl) buffer (0.2 M) containing the halophyte plant extracts. The test mixture (0.2 mL) was incubated for 15 min at 25°C, and the absorption due to the formation of ρ-nitroaniline was measured at 405 nm. Then, the same mixtures without the halophyte plant extracts and a solution of oleanolic acid were used as the vehicle and positive controls, respectively. Each treatment was replicated three times, and the percentage (%) of PPE inhibition was calculated as follows:

inhibition (%) = $[1 - (Abs_{sample} - Abs_{blank})/Abs_{control}] \times 100$.

Cell culture and nitric oxide determination

RAW 264.7 murine macrophages were purchased from the Korean Cell Line Bank (Seoul, Korea) and were maintained at sub-confluence in a 5% CO_2 humidified atmosphere at 37°C. For the routine subculture, Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 μg/mL) was used. The nitrite concentration of the cell culture medium was measured as an indicator of NO production according to the Griess reaction method[6]. In brief, RAW 264.7 cells (1.8×10^5 cells/mL) were plated in 24-well plates, incubated for 24 h, pretreated with the indicated concentrations of the halophyte extracts for 2 h, and then challenged with LPS (1 μg/mL) for an additional 18 h. Then, equal volumes of the culture medium and Griess reagent (1% sulfanilamide and 0.1% *N*-(1-naphthyl)-ethylenediaminedihydrochloride in 5% phosphoric acid) were mixed for 10 min, and absorbance was measured at 540 nm.

MTT assay for cell viability

Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The RAW 264.7 cells (2.5×10^5 cells/mL) were plated in 24-well plates, preincubated for 18 h, and then treated with the indicated concentrations of the halophyte plant extracts and LPS for 24 h. The supernatant was removed and formazan crystals were dissolved in DMSO. Absorbance was measured at 540 nm, and the percentage of the cells showing cytotoxicity was determined relative to the control group with LPS only.

Data analysis

All data are expressed as means ± standard deviation (SD) of at least triplicates experiments. Student's *t*-tests and a one-way analysis of variance (ANOVA) were used for the statistical analyses, and $P < 0.005$ and $P < 0.001$ were considered significant.

Results and Discussion

The flavonoid and phenolic contents

Many studies have reported that flavonoid and polyphenolic compounds are effective cosmeceutical ingredients owing to their beneficial biological properties. Flavonoid- and/or polyphenol-rich plants are effective in the prevention of facial wrinkles, melanin accumulation, and skin inflammation. Therefore, the total flavonoid and phenol contents of halophyte plant extracts were assessed, and the results are presented in Table 1. The highest total flavonoid and phenolic contents were found in *Rumexacetosella*(40.6 QUE/g and 63.6 mgGAE/g, respectively) followed by the aerial parts of *Artemisia fukudo*(39.7 QUE/g and 64.0 mg GAE/g, respectively).

Antioxidant activity

The antioxidant activity of a substance depends on the availability of electrons to neutralize free radicals[16]. In this study, the antioxidant activity of halophyte plant extracts was evaluated using DPPH and $ABTS^+$ radical scavenging assays. The results of the radical scavenging assays for all extracts are presented in Table 1 as the IC_{50} value (mg/mL), which indicates the concentration of the test solution that efficiently scavenged half of the DPPH free radicals. The best results were obtained for *Euphorbia helioscopia*(whole plant)*, Artemisia fukudo*(aerial parts), and *Rumexacetosa*(whole plant), with IC_{50} values of 172.8, 191.9, and 204.8 μg/mL, respectively. Accordingly, these active extracts may contain constituents with strong proton-donating (DPPH-H) abilities[17].

Table 1.Biological activities of Jejuhalophyte plant extracts

Scientific names	Parts	ABTS scavenging activity (IC50, μg/mL)	DPPH scavenging activity (IC50, μg/mL)	Tyrosinase inhibition activity (IC50, μg/mL)	Elastase inhibition activity (IC50, μg/mL)	Total flavonoid contents (mg/g QUE)	Total phenol contents (mg/g GAE)
Sonchus asper	Roots	1342.2	-	-	-	1.5	7.5
Rumexacetosa	Whole plants	106.8	204.8	-	99.3	20.8	57.7
Limoniumtetragonum	Whole plants	187.8	321.2	-	997.7	7.0	32.4
Raphanussativus for. Raphanistroides	Whole plants	1121.4	-	-	-	6.5	9.2
Lathyrusjaponicus	Whole plants	328.3	759.8	-	-	25.7	21.1
Sonchusoleraceus	Aerial parts	322.3	241.4	-	-	35.3	37.8
Sonchusoleraceus	Roots	-	-	-	-	1.4	5.5
Salicornia europaea	Whole plants	-	-	-	-	2.8	5.3
Rumexacetosella	Whole plants	84.7	397.4	-	1232.8	40.6	63.6
Demidoviatetragonoides	Whole plants	1240.0	-	-	4913.5	6.3	7.5
Euphorbia jolkini	Leaves	-	-	-	5387.7	40.9	146.6
Suaedamaritima	Whole plants	-	-	-	-	4.8	4.5
Euphorbia helioscopia	Whole plants	123.0	172.8	-	5000.0	18.9	56.7
Suaeda japonica	Whole plants	181.5	698.0	-	-	24.2	29.7
Aster spathulifolius	Whole plants	359.2	359.0	-	-	27.6	36.5
Artemisia fukudo	Aerial parts	132.0	191.9	-	4275.3	39.7	64.0
Artemisia fukudo	Roots	284.0	446.7	-	-	13.1	29.7
Suaedaaustralis	Whole plants	945.7	-	-	-	1.8	4.2
Suaedaglauca	Whole planst	279.3	976.9	-	-	28.1	33.0
Lysimachiamauritiana	Whole plants	125.2	450.4	-	-	20.9	45.1
Calystegiasoldanella	Whole plants	541.0	531.3	-	3970.4	17.1	34.3
Angelica japonica	Whole plants	427.4	461.8	-	-	35.7	38.5
Salsolakomarovii	Whole plants	1079.7	-	-	-	5.1	6.7
Controls	Quercetin	25.5	22.5				
	Vitamin C	5.2	8.2				
	BHT	22.2	23.0				
	Arbutin			86.5			
	Oleanolic acid				95.0		

ABTS radical cation scavenging activity of halophyte plants and standard butylated hydroxytoluene (BHT) and vitamin C is summarized in Table 1. The highest ABTS radical scavenging activity values were observed in the extracts of *Rumexacetosella, Rumexacetosa, Euphorbia helioscopia, Lysimachiamauritiana, Artemisia fukudo, Suaeda japonica*, and *Limoniumtetragonum*, with IC_{50} values of 84.7, 106.8, 123.0, 125.2, 132.0, 181.5, and 187.8 μg/mL, respectively. Their activities were greater than or comparable to those of butylated hydroxytoluene (BHT) and vitamin C (IC_{50} values, 23.0 and 8.2 μg/mL, respectively). In these extracts, the high ABTS radical scavenging activity indicated high total flavonoid and phenolic contents.

Tyrosinase and elastase inhibition capacity

The capacity capacities of the halophyte plant extracts to inhibit two key enzymes involved in skin aging are presented in Table 1. Among the 23 extracts, *R. acetosa* was the most effective against elastase, with 50% inhibition of the enzyme activity at its highest concentration (which corresponds to 99.3 μg/mL for the final mixture). This extract was significantly ($P < 0.05$) more active against elastase than oleanolic acid (95.0 μg/mL), a positive control.

Anti-inflammation capacity

NO plays a central role in skin inflammation,including atopic dermatitis and acne bulgaris. We evaluated the effects of the 23 halophyte plant extracts on NO synthesis in activated macrophages. We treated cells with increasing concentrations of halophyte extracts and then stimulated them with LPS for 24 h. The unstimulated RAW264.7 cells secreted basal levels of NO, whereas LPS stimulation induced NO production at a significant level. In total, 5 out of 23 halophyte extracts suppressed NO production in a dose-dependent manner. Of the 23 test extracts, four showed a greater than 50% inhibition of NO production at a concentration of 200 μg/mL in the culture media. Furthermore, two of the five extracts (*A. fukudo*aerial partsand *A. spathulifolius*whole plants) showed highly potent, concentration-dependent inhibition, with IC_{50} values of 35.6 and 81.7 μg/mL, respectively (Table 2). In addition, as shown in Table 2, the number of viable activated macrophages was not significantly altered, as determined using MTT assays, indicating that the inhibition of NO synthesis by the 23 halophyte plant extracts was not due to cytotoxic effects alone.

Table 2.Inhibitory effects of halophyte plant extractson nitric oxide (NO) production in RAW 264.7 cells

Scientific names	Parts	Cell viabilities(%)	Nitric oxide inhibitions (%)		
			200 μg/mL	100 μg/mL	50 μg/mL
Sonchus asper	Roots	108.5 ± 8.7	27.0 ± 1.4	25.3 ± 5.5	22.5 ± 7.8
Rumexacetosa	Whole plants	109.6 ± 3.7	24.2 ± 5.7	21.1 ± 2.1	23.0 ± 1.0
Limoniumtetragonum	Whole plants	106.4 ± 7.3	26.4 ± 5.2	19.8 ± 1.9	23.3 ± 3.9
Raphanussativus for. Raphanistroides	Whole plants	101.8 ± 9.5	26.5 ± 3.3	26.2 ± 3.2	21.6 ± 6.4
Lathyrusjaponicus	Whole plants	141.9 ± 9.6	30.0 ± 1.1	22.5 ± 2.1	19.8 ± 4.0
Sonchusoleraceus	Aerial parts	112.5 ± 2.1	62.5 ± 8.8	31.3 ± 2.4	20.5 ± 4.0
Sonchusoleraceus	Roots	112.5 ± 8.2	32.0 ± 2.2	27.7 ± 2.7	27.4 ± 2.5
Salicornia europaea	Whole plants	108.2 ± 2.6	29.6 ± 1.4	22.2 ± 4.9	25.8 ± 4.1
Rumexacetosella	Whole plants	108.1 ± 2.5	82.0 ± 2.1	62.3 ± 1.6	34.4 ± 8.5
Demidoviatetragonoides	Whole plants	103.7 ± 2.7	35.1 ± 4.4	29.4 ± 3.6	26.8 ± 2.3
Euphorbia jolkini	Leaves	108.0 ± 6.5	48.8 ± 3.4	46.7 ± 3.8	44.2 ± 3.3
Suaedamaritima	Whole plants	122.9 ± 2.5	29.1 ± 3.5	23.4 ± 2.7	22.2 ± 1.5
Euphorbia helioscopia	Whole plants	114.6 ± 4.9	46.1 ± 3.9	33.4 ± 1.7	24.3 ± 1.8
Suaeda japonica	Whole plants	103.1 ± 2.7	27.7 ± 1.3	15.8 ± 2.1	13.2 ± 1.9
Aster spathulifolius	Whole plants	106.5 ± 6.7	86.3 ± 2.4	58.4 ± 5.4	43.8 ± 2.4
Artemisia fukudo	Aerial parts	114.7 ± 2.7	80.1 ± 2.0	62.4 ± 4.4	36.4 ± 6.5
Artemisia fukudo	Roots	96.3 ± 3.8	36.5 ± 8.4	24.3 ± 4.9	12.5 ± 3.3
Suaedaaustralis	Whole plants	100.2 ± 9.3	-124.6 ± 9.2	-75.6 ± 8.6	-63.0 ± 8.8
Suaedaglauca	Whole planst	105.8 ± 1.1	30.9 ± 1.8	13.8 ± 0.6	9.8 ± 3.4
Lysimachiamauritiana	Whole plants	110.1 ± 2.7	29.1 ± 3.5	27.7 ± 3.5	19.6 ± 4.6
Calystegiasoldanella	Whole plants	109.9 ± 3.6	24.1 ± 6.5	14.9 ± 2.1	6.7 ± 4.2
Angelica japonica	Whole plants	103.3 ± 4.1	24.5 ± 3.0	10.9 ± 3.1	5.3 ± 4.3
Salsolakomarovii	Whole plants	102.6 ± 5.8	21.6 ± 3.9	4.5 ± 2.7	-1.4 ± 1.4

NO production was assayed in culture medium of lipopolysaccharide (LPS, 1 μg/mL)-stimulated cells (24 h) and treated with extracts (50, 100, and 200 μg/mL). Cell viability was determined by the MTT assay. Values are mean ± standard error of the mean (SEM) of triplicate experiments.

Conclusion

In conclusion, our study results demonstrated that *R. acetosella*whole plants, *S. oleraceus*aerial parts, *A. spathulifolius*whole plants,and *A. fukudo*aerial partsefficiently inhibited LPS-stimulated NO production in a concentration-dependent manner, and scavenged ABTS and DPPH radicals. Accordingly, these and other halophyte plants possess several biologically active components that may be useful cosmeceutical ingredients. However, additional investigations are necessary, including: (1) in vitro cell-based assays to comprehensively elucidate the plant properties, (2) chemical identification of the major active componentsmediating the anti-

aging and anti-inflammatory effects of these halophyte plants, and (3) analyses of their stability in new cosmetic and cosmeceutical formulations.

Acknlwledgements

This work was supported by the "Leaders INdustry-university Cooperation" grant funded by the the Ministry of Education, Science & Technology (MEST) through the Jeju National University.

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