

## Effect of *Aucklandia lappa* Decne Extract on Antioxidant

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**Abstract** : In the present study, we investigated the antioxidant activity of *Aucklandia lappa* Decne (AL). Cell viability was measured in an MTT assay. Antioxidant effects were evaluated based on total polyphenol/flavonoid contents, ABTS radical scavenging activity, DPPH radical scavenging activity, SOD activity, and ROS content. AL was found not to be toxic at concentrations of 1  $\mu\text{g/mL}$ , 10  $\mu\text{g/mL}$ , and 100  $\mu\text{g/mL}$ , respectively. The phenolic content was higher in AL-D than in AL-E, while the flavonoid content was higher in AL-E than in AL-D. AL-E exhibited higher ABTS radical scavenging activity than AL-D, and the  $\text{EC}_{50}$  values for BHA were 217.1  $\mu\text{g/mL}$  in AL-D and 180.5  $\mu\text{g/mL}$  in AL-E. AL-E also showed the highest DPPH radical scavenging activity.  $\text{EC}_{50}$  values for BHA were 114.2  $\mu\text{g/mL}$  in AL-D and 95.8  $\mu\text{g/mL}$  in AL-E. The SOD-like activity of AL-E was higher than that of AL-D. The  $\text{EC}_{50}$  values for ascorbic acid were 48.5  $\mu\text{g/mL}$  in AL-D and 72.9  $\mu\text{g/mL}$  in AL-E, indicating that both AL extracts have a SOD activity higher than that of ascorbic acid. AL-E reduced relatively more ROS than AL-D. With 100  $\mu\text{g/mL}$  AL-E, the reduction level was almost similar to that of dexamethasone. Our results demonstrate that AL have antioxidant effects, and we believe that they could be very valuable as raw materials for anti-aging products, based on their antioxidant activity.

**Keywords** : *Aucklandia lappa* Decne, Antioxidant, raw material, Medicinal herb, Natural products analysis

### 1. Introduction

Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism. Free radicals have a effect on growth and differentiation of cell in the processes of respiration and metabolism. And it defends body from antigen. The free radicals produced

in the body is decomposed into water and  $\text{CO}_2$  on normal procedure to finally discharge. However, free radicals of about 2-4% remain harmful free radicals which have strong reactivity[1]. Free radical such as  $\text{H}_2\text{O}_2$ ,  $\text{O}_2^-$ , hydroxyl radical and  $\cdot\text{OH}$  cause oxidative stress[1-3].

Oxidative stress is caused by oxidative damage occurring from an imbalance between pro-oxidants and antioxidants in the cell. Oxidative stress can cause impaired bio-membrane ion-transport and protein degradation[4], serious abnormalities of cellular

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metabolism including lipid peroxidation[5], liver cirrhosis, fatty liver, arteriosclerosis, cardiovascular diseases[6], cancer[7], aging[8] and DNA damage[9]. Oxidative stress also triggers the release of inflammatory factors[10].

To cope with oxidative stress, the body generates antioxidants enzymes such as SOD, catalase, glutathion peroxidase, NAD(P)H: Quinone reductase and the thioredoxin system which are called detoxication enzymes. However, when excess amounts of reactive oxygen are produced and these enzymes are limited, supplementary antioxidants have to be provided to prevent or remediate oxidative stress[11–13]. Synthetic antioxidants such as BHA and BHT have been developed as food supplements, cosmetics, or drugs to prevent oxidation. With regard to food additives, there are strict regulations on the target food and the amount of antioxidant that can be included because of safety disputes[14–16]. Synthetic antioxidants that act directly on the skin can cause a variety of side effects such as cancer, chromosomal abnormality, up-regulation of cholesterol and symptom of calcium-scarcity[17–18]. Thus, the quality and safety of this natural antioxidant is very important [17–18]. The various functional cosmetics are developed on natural materials with antioxidant effects[8,10,14,17–18].

*Aucklandia lappa* Decne (AL) is included in the “National Standard of Traditional Medicinal (Herbal and Botanical) Materials” as it is used to treat various diseases including indigestion, abdominal inflation, diarrhea and dysentery[19]. Morphologically, AL is characterized by its conical shape, with a length of 10~30 cm and a diameter of 1~3 cm. It is dark brown/brown in color, has many wrinkles, and has soft/tough texture[20].

AL grows abundantly in Yunnan and Gamsuk of China. AL contain sesquiterpene and sesquiterpene lacton. In addition to polyene alcohols, triterpene, lignans, amino acid-sesquiterpene, alkaloids, tannins and other components have also been reported[21].

Costunolide and dehydrocostus lactone are best known for therapeutic components of AL. These compounds are effective against gastrointestinal hyperkinesis[22] and have antiulcer and cholagogue activities[23]. In oriental medicine, AL are used for pain relief, to treat weakened spleens, to aid in food digestion, to treat acute diarrhea, or to help loss of appetite[24]. A number of studies investigated the component/pattern analysis of AL[25], their toxicity on A549 cells[26], lipid induction[27], anti-cancer activities[28], prostate cancer cell growth inhibition[29], insulin resistance[24] and hepatocellular protection[30]. In this study, we investigated the effect of AL on antioxidant activities to evaluate its possible use in the care of skin.

## 2. Materials and Methods

### 2.1. *Aucklandia lappa* Decne extract

*Aucklandia lappa* Decne was purchased from an Omniherb (Hallym Farm, Daegu, Korea). *Aucklandia lappa* Decne was used in manner of the reflux extraction in 80% ethanol (C<sub>2</sub>H<sub>5</sub>OH) and DW at 80°C for 3 hours, followed by filtration over paper (No. 2, Whatman, USA). The filtrate was evaporated by rotary vacuum evaporator. After the remains was freeze-dried in freeze dryer. Dried extract was dissolved in DW at a concentration of 20 mg/mL and then stored at -70°C until use. The dried extract yields are each 16.80% in AL-E (80% ethanol extraction) and 16.40% in AL-D (AL water extraction).

### 2.2. HPLC pattern analysis

We carried out HPLC (Shimadzu, Japan) pattern analysis on the AL formulation to evaluate consistency. The AL extracts were analyzed by HPLC under the conditions listed in Table 1.

Table 1. HPLC conditions

Column	: Agilent Eclipse C18 (250×4.6) mm, 5 $\mu$ m					UV	: 215 nm
Flow	: 1.0 mL/min					Inj. Vol.	: 30 $\mu$ L
Temp.	: 40° C					Eluent	: A (DW), B (Acetonitrile)
Time (min)	0	20	25	40	50		
A (%)	80	10	10	0	0		
B (%)	20	90	90	100	100		

### 2.3. Cell culture

Mouse origin Raw 264.7 cells (Korea Cell Line Bank, Seoul, Korea) were grown at 37°C in a humidified 5% CO<sub>2</sub> incubator (Forma scientific, USA) in Dulbecco's modified eagle's medium (DMEM; Gibco BRL Co., USA), containing 10% FBS (Gibco BRL Co., USA) and 1% antibiotic-antimycotic (A/A; Gibco BRL Co., USA) until 85% confluence.

### 2.4. MTT Assay

Raw 264.7 cell viability was assessed in an MTT assay. Cells were seeded in a 96 well plate ( $2 \times 10^4$  cells/well) and grown at 37°C in a humidified 5% CO<sub>2</sub> incubator in DMEM containing 10% FBS and 1% A/A for 24h. Cells were incubated with AL at 1  $\mu$ g/mL, 10  $\mu$ g/mL, or 100  $\mu$ g/mL. After 1 day, 10  $\mu$ L of MTT solution (Daeil Lab service, Korea) was added to each well. After incubating for 30 min, absorbance was measured at 450 nm using a microplate reader (Molecular Devices, USA).

### 2.5. Total polyphenol content analysis

Total phenolics were determined using FCR as described by Velioglu *et al.* (1998)[31] with slight modifications. Briefly, AL extracts dissolved in DW (10 mg/mL) was mixed with 500  $\mu$ L of Foiln-Ciocalteu's phenol reagent, ethanol (FCR; Sigma Co., USA) and allowed to sit at room temperature for 3 min. Then, 1 mL of Na<sub>2</sub>CO<sub>3</sub> solution and 7.5 mL of DW were added to the mixture. After 40 min, absorbance was measured at 760 nm. Results were expressed as gallic acid (GAE; Sigma

Co., USA) per gram.

### 2.6. Total flavonoid content analysis

The flavonoid content was determined according to the method described by Kumaran and Karunakaran (2006)[32] with slight modifications. Briefly, 500  $\mu$ L of extract (10 mg/mL) in ethanol (diluted 10-fold) was mixed with 100  $\mu$ L of 10% aluminum nitrate (Sigma Co., USA), 100  $\mu$ L of 1 M potassium acetate (Sigma Co., USA) and 4.3 mL of 80% ethanol. Absorbance was measured at 415 nm after 40 min. The amount of flavonoids in the extract in AL was expressed in terms of quercetin (Sigma Co., USA) ( $\mu$ g/mL).

### 2.7. ABTS radical scavenging assay

The scavenging activity of the extract on ABTS (Sigma Co., USA) free radicals was assessed according to the method reported by Arnao *et al.* (2001)[33] with slight modifications. Briefly, a 5  $\mu$ L solution of the extract at different concentrations (1  $\mu$ g/mL, 10  $\mu$ g/mL, or 100  $\mu$ g/mL) was mixed with 95  $\mu$ L of 7.4 mM ABTS in PBS. The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 10 min. Blank solutions were prepared for each test sample solution (5  $\mu$ L) and 95  $\mu$ L of PBS while the negative control was 95  $\mu$ L of 7.4 mM ABTS solution plus 5  $\mu$ L of DW. BHA (Sigma Co., USA) was used as the positive control. The absorbance (optical density, or OD) of the assay mixtures was measured at 732 nm. ABTS radical inhibition was calculated using the equation: ABTS radical

scavenging activity (%) = (Blank OD-Sample OD)/Blank OD×100

### 2.8. DPPH radical scavenging assay

The scavenging activity of the extract on DPPH (Sigma Co., USA) free radicals was assessed according to the method reported by Gyamfi *et al.* (1999)[34] with slight modifications. Briefly, a 100  $\mu$ L solution of the extract at different concentrations (1  $\mu$ g/mL, 10  $\mu$ g/mL, or 100  $\mu$ g/mL) was mixed with 150  $\mu$ L of 0.5 mM DPPH in ethanol. The mixture was shaken vigorously and allowed to stand at 37°C in the dark for 30 min. Blank solutions were prepared with each test sample solution (100  $\mu$ L) and 150  $\mu$ L of ethanol while the negative control was 150  $\mu$ L of 0.5 mM DPPH solution plus 100  $\mu$ L of DW. BHA was used as the positive control. The absorbance of the assay mixture was measured at 517 nm. DPPH radical inhibition was calculated using the equation: DPPH radical scavenging activity (%) = (Blank OD-Sample OD)/Blank OD×100

### 2.9. SOD-like activity assay

The SOD (Sigma Co., USA) activity of the extract was assessed according to the method reported by Martin *et al.* (1987)[35] with slight modifications. Briefly, a 200  $\mu$ L solution of the extract at different concentrations (1  $\mu$ g/mL, 10  $\mu$ g/mL, or 100  $\mu$ g/mL) was mixed with 2.6 mL of 50 mM Tris-HCl buffer (Sigma Co., USA) and 0.2 mL of pyrogallol (Sigma Co., USA). The mixture was shaken vigorously and allowed to stand at 37°C in the dark for 10 min. Subsequently, 100  $\mu$ L of 1 N HCl was added to the mixture and the absorbance of the assay mixture was measured at 420 nm. L-ascorbic acid (vitamin C) (Sigma Co., USA) was used as the positive control. SOD activity was calculated using the equation: SOD activity (%) = (Blank OD-Sample OD)/Blank OD×100

### 2.10. ROS assay

ROS reduction by the extract was assessed according to the method reported by Lee *et al.* (2014)[10] with slight modifications. Raw 264.7 cells were seeded in a 12 well plate ( $2 \times 10^5$  cells/well) and grown at 37°C in a humidified 5% CO<sub>2</sub> incubator in DMEM containing 10% FBS and 1% antibiotic-antimycotic for 24 h. Cells were incubated with extract at a concentration of 1  $\mu$ g/mL, 10  $\mu$ g/mL, or 100  $\mu$ g/mL and LPS (Sigma Co., USA) at a concentration of 1  $\mu$ g/mL. After 1 day, 100  $\mu$ L of 10  $\mu$ M DCF-DA (Sigma Co., USA) was added to the pellet and the mixture was incubated at 37°C in the dark for 15 min. ROS levels were measured by flow cytometry (Becton Dickinson, USA). ROS inhibition was calculated using the equation: ROS inhibition (%) = Sample FI/Control FI×100

### 2.11. Analysis

Results are expressed as mean±standard deviation. Statistical significance was evaluated with a one-way analysis of variance using SPSS ver. 18.0 (Chicago, IL, USA).

## 3. Results and Discussion

### 3.1. HPLC pattern analysis of AL extracts

Analysis result of AL extract were able to identify that costunolide was eluted at 22.62 min. Dehydrocostus lactone was eluted at 21.11 min (Fig. 1-A, B). Also, pattern of AL extracts shows a difference in the content of the two representative compounds (costunolide and dehydrocostus lactone) according to the solvent (Fig. 1-C, D). The content of costunolide and dehydrocostus lactone was each 28.256 mg/L or 21.169 mg/L in AL-E (Fig. 1-C). AL-D was each 1.449 mg/L or 3.009 mg/L (Fig. 1-D). The result was higher in AL-E than in AL-D (Fig. 1-C, D). Costunolide and dehydrocostus lactone are the

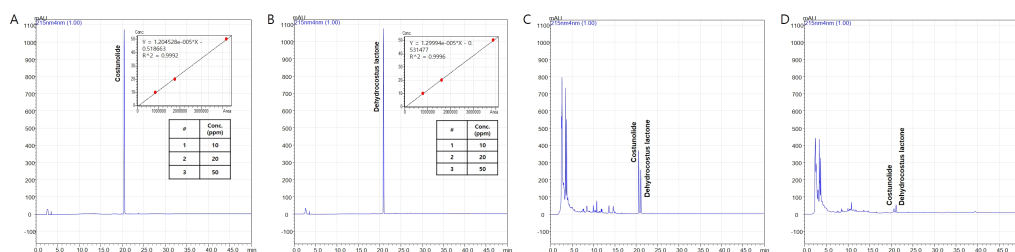


Fig. 1. HPLC pattern analysis of AL extracts. Costunolide (A), dehydrocostus lactone (B). AL 80% ethanol extraction (C) and AL water extraction (D).

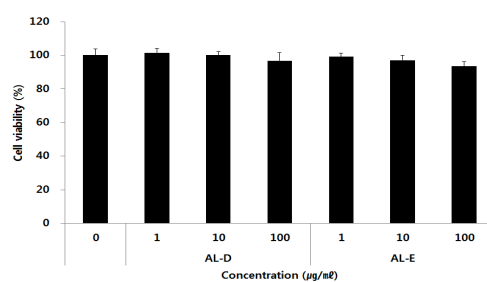


Fig. 2. Cell viability of Raw 264.7 cells after AL extracts treatment. Cell viability was measured in an MTT assay. The results are expressed as the mean  $\pm$  standard deviation of three independent experiments.

representative components of AL. The compound analysis by HPLC corroborates previous reports on AL[25,36]. Costunolide and dehydrocostus lactone have been suggested to possess various biological activities, including anti-tumor, anti-ulcer, antioxidant and anti-inflammatory effects[37–39]. Their levels were significantly higher in ethanol extracts than in water extracts. In addition, the levels of costunolide were higher than those of dehydrocostus lactone. It is thought that the difference of content in ALE and ALD be able to affect the anti-oxidation.

### 3.2. Cell viability

Treatment with AL extracts at a concentration of 1  $\mu$ g/mL, 10  $\mu$ g/mL, or 100  $\mu$ g/mL caused no cytotoxicity in Raw 264.7 cells (Fig. 2). Therefore, we concluded that the extracts can be used for cellular treatments at the indicated concentrations in our

experiments.

### 3.3. Total polyphenol and flavonoid contents

The total polyphenol contents of AL extracts were measured using gallic acid as reference material. The results are shown in Table 2. The total flavonoid content of AL extracts were measured using gallic acid as a reference material. Results are shown in Table 2. Phenolic component which reacts during the oxidation-reduction as a substrate has the hydroxy (-OH) group in the structure[40–41]. Hydroxy Group has a nature that combine with other compounds easily[42]. Phenolic component is reported to have the antioxidant and anti-microbial effects because of its propensity to combine with protein[40]. Flavonoid is reported that flavonoid degrade production of ROS[43]. Polyphenol and flavonoid are reported effect of antivirus, anti-inflammation and anti-cancer[43]. Especially,

flavonoid is reported that antioxidant properties of flavonoids are effected mainly via scavenging of superoxide anions[44]. So polyphenol and flavonoid-based materials have been generally used to prevent or improve chronic diseases caused by oxidative stress[10]. Our results demonstrate that the phenolic content in AL-D was higher than in AL-E, while the flavonoid content was higher in AL-E than in AL-D. The difference in contents of polyphenol and flavonoid between the two extracts was assumed to have an effect on the antioxidant, effects.

### 3.4. ABTS and DPPH radical scavenging activity

The ABTS radical scavenging activities of AL extracts at a concentration of 1  $\mu\text{g/mL}$ , 10  $\mu\text{g/mL}$  and 100  $\mu\text{g/mL}$  were measured. AL-D extracts exhibited  $0.6 \pm 1.5\%$ ,  $2.1 \pm 0.8\%$  and  $38.5 \pm 3.4\%$  ABTS radical scavenging activity at the respective concentrations (Fig. 3-A). For AL-E the respective activities were  $0.7 \pm 0.5\%$ ,  $9.1 \pm 1.1\%$  and  $47.7 \pm 2.8\%$  (Fig. 3-A). The DPPH radical scavenging activities of AL extracts at a concentration of 1  $\mu\text{g/mL}$ , 10  $\mu\text{g/mL}$  and 100  $\mu\text{g/mL}$  were measured. AL-D

extracts exhibited  $0.4 \pm 1.0\%$ ,  $5.9 \pm 1.5\%$  and  $74.0 \pm 1.1\%$  DPPH radical scavenging activity at the respective concentrations (Fig. 3-B). For AL-E, these values were  $6.6 \pm 1.7\%$ ,  $23.0 \pm 1.9\%$  and  $87.5 \pm 1.0\%$  activity, respectively (Fig. 3-B). Both DPPH radical and ABTS radical play a role as a stabilizer of the unstable and harmful free radicals originated from the internal body by supplying proton ion and stabilizing the unstability of free radical[45]. Therefore, a unknown specific material is used as an index that evaluate the antioxidant function which eliminates the hydroxyl radical or superoxide radical produced by physical or antioxidant function of the body[45]. AL-E showed a relatively higher outcome in terms of DPPH radical scavenging activity, and the  $EC_{50}$  ( $y = 19.013\ln(x) + 10.305$ ,  $R^2 = 0.964$ ) values for each BHA were 114.2  $\mu\text{g/mL}$  in AL-D and 95.8  $\mu\text{g/mL}$  in AL-E. In addition, the ABTS radical scavenging activity was higher for AL-E than for AL-D, and the  $EC_{50}$  ( $y = 16.918\ln(x) + 22.092$ ,  $R^2 = 0.9361$ ) values for each BHA were 225.8  $\mu\text{g/mL}$  in AL-D and 188.2  $\mu\text{g/mL}$  in AL-E. Specifically from the result of the radical scavenging activity, AL-E

Table 2. Total phenolic or flavonoid content of AL extracts

Sample	Total phenolic contents (GAE $\mu\text{g/mL}$ )	Total flavonoid contents (quercetin $\mu\text{g/mL}$ )
AL-D <sup>1</sup>	$342.2 \pm 9.5$	$78.4 \pm 3.5$
AL-E <sup>2</sup>	$301.3 \pm 6.1$	$111.6 \pm 5.0$

<sup>1</sup>AL-D, AL water extraction

<sup>2</sup>AL-E, AL 80% ethanol extraction.

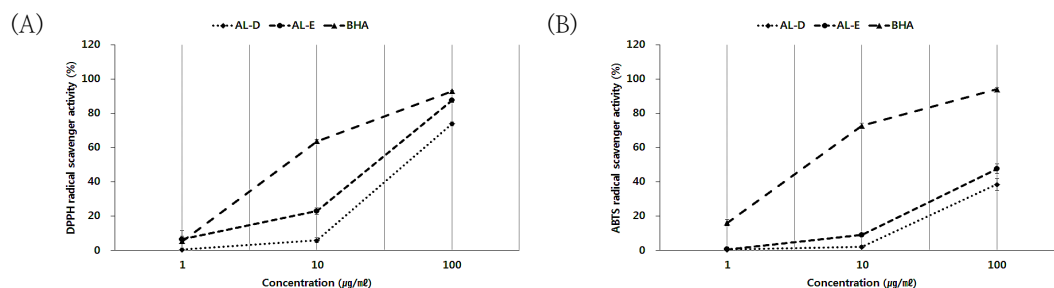


Fig. 3. DPPH (A) and ABTS (B) free radical scavenging activity of AL extracts. The results are expressed as the mean  $\pm$  standard deviation of three independent experiments.

showed similar scavenging activity when compared to the BHA with density of 100  $\mu$ g/mL, on the other hand, AL-D showed lower radical scavenging activity. According to the research reported, the radical scavenging activity of ABTS and DPPH is correlated to the content of flavonoid[42], and the difference between the radical scavenging activities of both AL-E and AL-D is related to the content of flavonoid. Also, Ahn *et al.*, (2011)[46] compared DPPH radical scavenging activities of five types of oriental medicinal herbal oil and supported a point that the AL has the highest radical scavenging activity.

### 3.5. SOD-like activity

The SOD activities of AL extracts at a concentration of 1  $\mu$ g/mL, 10  $\mu$ g/mL and 100  $\mu$ g/mL were measured. AL-D extracts exhibited 11.5 $\pm$ 4.1%, 38.7 $\pm$ 5.0% and 89.6 $\pm$ 5.1% SOD activity at the respective concentrations (Fig. 4). AL-E showed activities of 17.8 $\pm$ 4.4%, 45.5 $\pm$ 3.8% and 90.2 $\pm$ 2.6%, respectively (Fig. 4). SOD catalyzes the hydrolysis of superoxide ions to hydrogen peroxide, which can fundamentally remove free radicals[47]. In this respect, SOD is more effective than other types of antioxidants.

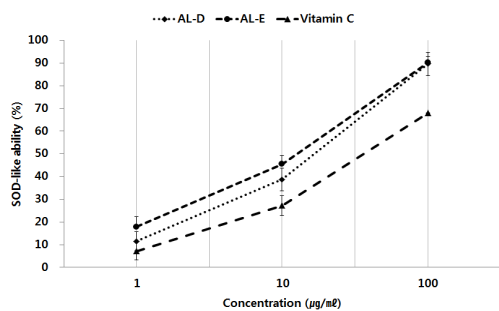


Fig. 4. SOD-like activity of AL extracts. Activities were determined by absorbance measurement at 420 nm. The results are expressed as the mean  $\pm$  standard deviation of three independent experiments.

AL-E has a higher SOD-like activity than AL-D. The  $EC_{50}$  ( $y=13.206\ln(x)+3.6829$ ,  $R^2=0.9628$ ) values for ascorbic acid were 48.5  $\mu$ g/mL in AL-D and 72.9  $\mu$ g/mL in AL-E, showing that both AL have a higher SOD activity than ascorbic acid.

### 3.6. Effect of AL extracts on ROS production

The effect of AL extracts on ROS production was evaluated in Raw 264.7 cells. AL-D treatment at a concentration of 1  $\mu$ g/mL, 10  $\mu$ g/mL and 100  $\mu$ g/mL led to a reduction in ROS, as 88.2 $\pm$ 4.7%, 86.7 $\pm$ 3.5% and 75.1 $\pm$ 4.1%, respectively (Fig. 5, black bars). In the case of AL-E, the relative ROS levels were 84.6 $\pm$ 3.4%, 73.8 $\pm$ 2.5% and 69.6 $\pm$ 1.6%, respectively (Fig. 5, grey bars). The ROS is known as direct cause of oxidative stress and initiates inflammatory reaction[48]. This results showed that AL-E and AL-D reduced ROS production. Also, AL-E was relatively better at reducing ROS, when compared to AL-D. When cells was treated with 100  $\mu$ g/mL AL-E, the reduction level was almost similar to that of 1  $\mu$ g/mL dexamethasone. AL-E had superior antioxidant efficacy when compared to AL-D. By reviewing the overall results of anti-oxidant

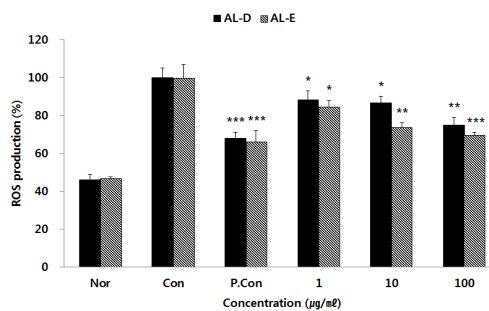


Fig. 5. Effect of AL extracts on ROS production in Raw 264.7 cells. ROS was measured using FACS. The results are expressed as the mean  $\pm$  standard deviation of three independent experiments (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ) for control.

effects, two types of AL abstracts, AL-D and AL-E, both have high marks in the anti-oxidant effect, and AL-E has higher anti-oxidant activity than AL-D.

#### 4. Conclusion

In this study, We compared the efficacies of antioxidant activities of AL. Costunolide and dehydrocostus lactone are the representative components of AL. Their levels were significantly higher in ethanol extracts. AL was not toxic in Raw 264.7 cells at the tested concentrations. In addition, AL-E had superior ABTS and DPPH radical scavenging activities, SOD activity and ROS reduction efficacy, compared to AL-D. This result is in line with the observed flavonoid contents for both types of AL. However, more in-depth studies are required to determine the synergistic effects between the different biological activities. Future studies are targeted on the underlying mechanisms and clinical applications of AL's biological functions. Our results demonstrate that AL show potential as raw materials for anti-aging products based on their antioxidant activity, and as supplements for various diseases based on their antioxidant effects.

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