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추출방법에 따른 두충 껍질 추출물의 항산화, 항염증 활성 비교

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Antioxidative, Anti-inflammatory, and DNA Damage Protective Effect in Cortex Extracts of *Eucommia ulmoides* by Roasting

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ABSTRACT

Background: *Eucommia ulmoides* has long been used as an herbal medicine for the treatment of diabetes, hypertension and other diseases in many Asian countries.
Methods and Results: This study aimed at evaluating the antioxidant and anti-inflammatory properties of its water (EU-DW, and REU-DW) and ethanol (EU-EtOH, and REU-EtOH) extracts, as well as those of non-roasted *E. ulmoides* (EU) and roasted EU (REU) cortex. The following were assessed in each extract: total phenolic and flavonoid contents, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and nitrite radical scavenging activities, reducing power, DNA damage prevention activity, and nitric oxide (NO) inhibition activity. Both EU and REU extracts showed high phenolic and flavonoid content, dose-dependent DPPH radical scavenging capacity, high reducing activity, and considerable DNA damage prevention activity. EU extracts showed remarkable ABTS free radicals scavenging capacity. REU extracts showed a higher radical scavenging capacity and played an important role in inhibiting NO production.
Conclusions: The results of this study suggested that aqueous and ethanol extracts of EU and REU possess antioxidant capacities, and prevent oxidative damage to DNA, probably owing to their phenolic and flavonoid content. Therefore, EU and REU could be candidates antioxidant supplements.

***Key Words*:** *Eucommia ulmoides*, Antioxidative Effect, Anti-inflammatory Effect, Nitric Oxide, Oxidative DNA Damage, Roasting

INTRODUCTION

Antioxidants play a crucial role in mitigating chronic diseases by reducing the oxidative damage caused by reactive oxygen species (ROS) to cellular components (Peterson *et al*., 2002). ROS consist of both free radicals, such as superoxide anion radicals ($O_2\cdot$) and hydroxyl radicals, and non-free radical species such as singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2). Free radicals are produced in living organisms, and are one of the leading causes of damage to cell structure, DNA, lipids, and proteins. They also increase the risk for more than 30 different diseases (Ali *et al*., 2008).

These free radicals and ROS are produced inside the body owing to different metabolic processes, including metabolic respiration and cell-mediated immune responses (Chang *et al*., 2012). Since they contain unstable electron configurations, free radicals are very reactive (Clarkson and Thompson, 2000), and cause cell death and tissue damage.

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Free radicals or ROS can also be introduced into the body by different environmental pollutants, such as radiation, pesticides, and cigarette smoke (Masoko and Eloff, 2008). Therefore, the continuous and uncontrolled accumulation of ROS inside the body causes many chronic diseases including cancer, cardiovascular disease, neurodegenerative disorders, aging, and inflammation (Aruoma, 1998). Free radicals also play a crucial role in oxidative stress (Gülçin, 2006). Because of redox stress, activated immune cells release pro-inflammatory cytokines, and reactive oxygen and nitrogen species, leading to biological molecule damage, including imbalances in physiological and pathological pathways (Lonkar and Dedon, 2011).

Antioxidants are believed to play a key role in minimizing or preventing the formation of free radicals and stopping cell damage through their capacity for donating electrons or hydrogen ions (Rice-Evans *et al*., 1997). There are many known antioxidants, but much attention has been given to phenolic compounds. The phenolic compounds are known to have antioxidant activities owing to their redox properties, which permit them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers, and also help in metal chelation.

Various synthetic antioxidants, such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and propyl gallate (PG), are commonly used in the food industry. However, using these synthetic antioxidants is not safe because they may cause toxicity issues (Erkan *et al*., 2008).

Therefore, over the last few years, attention in the food, cosmetic, and pharmaceutical industries to naturally occurring antioxidants has gradually increased. As such, the free radical scavenging activity of medicinal plants may have great relevance to the prevention of diseases.

Eucommia ulmoides belongs to the monotypic family Eucommiaceae. *E. ulmoides* is a species of small tree native to China and its common name is “Du-zhong”. It is widely cultivated in China as its bark has been used as a traditional medicine for a long time. Furthermore, in Korea, Japan and China, the aqueous leaf extract Du-zhong tea is used to improve hypertension, and has also received attention as a functional food (Takeshi *et al*., 2001). Moreover, it is also used in Korea to treat diabetes. It could be found many phytochemicals in Du-zhong, including the polyphenolics pyrogallol, chlorogenic acid, flavonoids, astragalin, coumaric acid, and triterpenes (Kawasaki *et al*., 2000).

Some previous studies (Kawasaki *et al*., 2000; Takeshi *et al*., 2001; Kim *et al*., 2012) also support that the extract of EU cortex is a high free radical scavenger and it has the capacity to inhibit NO production. Kim *et al*. (2012) demonstrated that EU extracts help to inhibit NO production in lipopolysaccharides (LPS) stimulated RAW264.7 cells as dose dependent manner.

Therefore, we extracted using water and ethanol with EU and REU, and tried to find out the antioxidant effect of extracts, the ability to prevent DNA damage, and the anti-inflammatory effect of NO release.

MATERIALS AND METHODS

1. Reagents and chemicals

Folin-ciocalteau (FC reagent), galicacid, 2,2-azino-bis (3-ethylbenzo thiazoline-6-sulfonic acid) diammonium salt (ABTS), sodium nitrite, sulfanilic acid, acetic acid, sodium citrate, ascorbic acid, ferrous sulfate heptahydrate ($FeSO_4$) were obtain from Sigma-Aldrich (St. Louis, MO, USA).

1,1-Dipheny-2-picrylhydrazyl (DPPH) was obtained from Wako Pure ChemicalIndustries (Osaka, Japan). D-catechin was purchased from MP Biomedicals (Santa Ana, CA, USA). Hydrogen peroxide 30% was obtained from Daejung Chemicals and Metals (Siheung, Korea). The pBR322 DNA AND 6X DNA loading dyes were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

RAW264.7 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Dulbeco’s Modified Eagle’s Media (DMEM), penicillin-streptomycin, fetal bovine serum (FBS) have been bought from GIBCO BRL (Life Technologies Co., Carlsbad, CA, USA). All chemicals have been used without any further purification.

2. Preparation of extracts

Eucommia ulmoides (EU) cortex prepared respectively four samples, EU and roasted EU, each using ethanol (EU-EtOH and REU-EtOH) and water (EU-DW and REU-DW). EU cortex and roasted EU cortex was bought from Chungbuk herb farming association (Jechon, Korea). Roasted EU was roasted in cylindrical roaster at 150℃ for 30 min.

EU has been botanically identified by Korea medicine herbal association and deposited in Bio-food and drug material lab in Konkuk University, South Korea.

The water extract was prepared by using 100 g of the EU cortex and extracted with 1 ℓ distilled water for 2 h at 50℃. The procedure of water extraction has been repeated for three

times. For ethanol extract, 100 g of EU cortex were immersed in 1 ℓ, 75% ethanol and stored at room temperature (RT) for 7 days. Next, Whatman No. 1 filter paper was used to filter the extracts and then the extracts have been evaporated under reduced pressure at 50℃ in a vacuum rotary evaporator (Eyela, Bohemia, NY, USA).

The concentrated mass was freeze-dried, weighed and stored in a refrigerator at -20℃ until use. The yields have been calculated using the following equation:

Y (%)
=(total extracted sample weight/total dry sample weight)×100.

3. Determination of total phenolic content

As in our previous studies (Lee *et al*., 2017), Folin-Ciocalteu (FC) method was used to determine the total phenolic content of the extracts with little modification. Firstly, each sample solution (10 ㎎/㎖) was made by dissolving the sample in distilled water and ethanol and various concentrations of gallic acid (5 - 100 ㎍/㎖) were prepared in distilled water.

Next, each solution of sample / standard (40 ㎕) was taken in a 96 well plate and then, added 20 ㎕ of 1 N FC reagent and 60 ㎕ of 20% sodium carbonate solution. After that, the mixture was left for incubation in the dark at RT for 30 min.

Lastly, a UV-visible spectrophotometer (Sunrise, Tecan Austria GmbH, Grodig, Austria) has been used to take the absorbance at 700 ㎚ wavelength. The total phenolic content was measured from the standard calibration curve of gallic acid. Results were expressed as ㎎ gallic acid equivalents (GAE) per 100 g of dry mass.

4. Determination of total flavonoid content

Aluminum chloride colorimetric assay was applied to determine the total flavonoid content (Samad *et al*., 2014) whereas catechin was used as standard. The concentration of the sample and standard were 2.5 - 25.0 ㎍/㎖ and 10.0 ㎎/㎖ respectively.

Initially, 25 ㎕ of each sample and standard reagent were taken in 96 well plate and then 125 ㎕ of distilled water (DW) and 8 ㎕ of 5% sodium nitrite solution were added. After the completion of 5 min incubation, 15 ㎕ of 10% aluminum chloride was added to the mixture and again left for 6 min incubation at RT. After that, 50 ㎕ of sodium hydroxide (1 M) and 27 ㎕ of DW were subjected in the mixture. Finally, the absorbance was taken at 517 ㎚ wavelength using a UV-visible spectrophotometer.

Total flavonoid content was measured as ㎎ of catechin equivalent (CE) per 100 g of dry mass with the help of standard calibration curve.

5. DPPH radical scavenging activity

DPPH radical scavenging activities of ethanol and water extracts were determined according to the method of our previous study (Lee *et al*., 2017).

Different concentrations (0.25 to 4 ㎎/㎖) of sample and BHT (as positive control) were prepared. Then, each of sample /standard solution (80 ㎕) was mixed with 80 ㎕ of DPPH solution. Next, the mixture was shaken in the dark at RT for 30 min. Finally, the absorbance was taken at 517 ㎚ wavelength. The same procedure has been followed to prepare the control (instead of sample and standard). The following equation has been used to evaluate the DPPH radical scavenging activity:

DPPH radical scavenging activity (%)
= [(absorbance of the control – absorbance of the sample)
/absorbance of the control] × 100

6. ABTS radical scavenging activity

ABTS radical scavenging activity was determined by the method of Re *et al*. (1999) with little modification.

First of all, 7 mM ABTS solution was made by dissolving in water and then added 2.45 mM potassium persulfate solution to form $ABTS^{+}$, and left the mixture in the dark for 12 h - 16 h at RT to complete the reaction. Freshly prepared $ABTS^{+}$ solution was diluted with 0.1M phosphate buffer saline (PBS, pH 7.4) to adjust its absorbance within 0.70 ± 0.02 at 734 ㎚ wavelength. Next, 0.2 ㎖ of various concentrations of the sample and ascorbic acid (0.25 - 4 ㎎/㎖) were mixed with 0.8 ㎖ of $ABTS^{+}$ solution. Lastly, the mixture left for 5 min incubation at room temperature and absorbance was taken at 734 ㎚ wavelength. The scavenging activity of ABTS free radical was calculated using the following equation:

ABTS scavenging activity (%)
= [(absorbance of the control – absorbance of the sample)
/absorbance of the control] × 100

7. Ferric reducing power

Reducing power activities of the extracts were evaluated according to the Hasnat *et al*. (2014) with little modification.

Initially, 0.5 ㎖ of each sample or standard reagent (ascorbic acid as positive control) at different concentration (0.25 - 4 ㎎/㎖) was made. Then, 0.5 ㎖ of 0.2 M sodium phosphate buffer (pH 6.6) and 0.5 ㎖ of potassium ferricyanide (10 ㎎/㎖) were mixed with different concentrations and allowed the mixture for incubation in a water bath at 40℃ for 20 min. After incubation 1 ㎖ of each sample or standard (0.25 - 4 ㎎/㎖) were transferred in new e-tube and added 0.5 ㎖ of 10% trichloro acetic acid.

Afterward, the mixture was then centrifuged at 3,000 rpm in a refrigerated centrifuge machine for 10 min, maintaining the temperature 4℃. Then, 0.5 ㎖ of the upper portion of the mixture was isolated and mixed 0.5 ㎖ of distilled water and 0.1 ㎖ of $FeCl_3$ solution (1 ㎎/㎖).

The absorbance was taken at 700 ㎚ wavelength. The half maximal inhibitory concentration (IC_{50}) of each sample was also evaluated in this study.

8. Nitrite scavenging activity

Nitrite scavenging activity was evaluated as described in Choi *et al*. (2008). Initially 0.5 ㎖ of sample or standard at various concentrations (0.25 - 4 ㎎/㎖) were mixed with 0.5 ㎖ of 1 mM $NaNO_2$ and 4 ㎖ of 0.2 M citrate buffer (pH 3). Then the mixture was subjected to incubation in a water bath for 1 h at 40℃. After incubation, 0.4 ㎖ of the mixture was separated and added 0.8 ㎖ of acetic acid and 80 ㎕ of Griess A reagent [n-(1-napthyl) ethylenediamine dihydrochloride] and 80 ㎕ of Griess B reagent (sulfanilic acid 250 ㎎ + 5% phosphoric acid) and left the mixture for 15 min to complete the reaction.

Ascorbic acid was used as a positive control. Finally, the absorbance was taken at 520 ㎚ wavelength. The scavenging activity of each sample/standard solution was calculated using the following equation:

Scavenging activity (%)
= [(absorbance of the control – absorbance of the sample)
/absorbance of the control] × 100

9. Determination of half maximal inhibitory concentration (IC_{50}) and antiradical power (ARP)

IC_{50} values were evaluated as followed in Plazza *et al*. (2014). Extracts that exhibited more inhibition percentage than 50% were tested at various concentrations (0.03, 0.06, 0.12, 0.25, 0.5, 1, 2 and 4 ㎎/㎖).

Then, the IC_{50} was obtained by linear regression analysis of dose response curve plotting between % inhibition and concentration. IC_{50} is the concentration of sample that required scavenging free radical by 50%.

Antiradical power expressed as $1/IC_{50}$ or $ARP = 1/IC_{50}$.

10. Prevention of oxidative DNA damage

The DNA protective effect of EU and REU extracts were performed according to the Debnath *et al*. (2013) with minor modification.

Initially, 5 ㎕ of distilled water, 2 ㎕ of different concentration of sample (0.1, 0.5, and 1 ㎎/㎖), 2 ㎕ $FeSO_4$ (0.08 mM), 3 ㎕ of 30% H_2O_2 and 1 ㎕ of plasmid pBR 322 DNA are mixed in a test tube. Then, the mixture was incubated for 1 h at 37℃ and after incubation, 2 ㎕ of 6 × DNA loading dye was added to the mixture. Finally, the mixture was subjected to 0.8% agarose gel electrophoresis.

The three forms of DNA bands (supercoiled, linear and open circular) were stained with ethidium bromide. The increase or lose percentage of supercoiled monomer was compared with control value to determine the antioxidant effects on DNA.

11. Cell culture

The murine macrophage RAW264.7 cell was purchased from the Korean Cell Line Bank (Seoul, Korea) and cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units/㎖) and streptomycin (100 ㎍/㎖). The cells arestored in a humid atmosphere at 37℃ in 5% CO_2 (Sanyo, MCO-15AC, Tokyo, Japan).

12. Nitric oxide determination

NO production by the macrophages was determined with the cell supernatant by using the Greiss reagent. RAW264.7 cells (5×10^4 cells/well) were seeded in a 96-well plate and incubated for one day.

Then the media was removed and added 100 ㎕ of fresh media containing a different concentration of extracts. Next, after 1 h the cells were stimulated with LPS at a concentration of 1 ㎍/ for 24 h. After that, 80 ㎕ supernatant of the mixture mixed with equal amount of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthyl-ethylenediamine dihydrochloride in water). Lastly, the absorbance was measured at 540 ㎚ wavelength.

13. Statistical analysis

All experiments were performed in triplicate and all data were expressed as mean with standard deviation (SD). The correlation coefficient (r^2) between the parameters tested was established by regression analysis. Statistical analyses were performed with Graphpad Prism 5.Ink (GraphPad Software, San Diego, CA, USA). The observed differences were analyzed for statistical significance by two-way ANOVA analysis of variance with Duncan's Multiple Range Test (DMRT).

Differences of $p < 0.05$ were considered as significant. Pearson's bivariate correlation test was performed to calculate linear correlation coefficient between the total phenolic and flavonoid content of each extract and antioxidant activity in terms of ARP.

RESULTS AND DISCUSSION

In this study, the water extract and ethanol extracts of EU and REU prepared and the effects of those extracts on antioxidant and anti-inflammatory activity were performed.

The evaluation of the total phenolic and flavonoid contents, synthetic free radical (DPPH, ABTS) scavenging capacity, reducing power, nitrite scavenging capacity, DNA damage prevention activity, and inhibition of NO have been done. BHT and ascorbic acid were used as a positive control to compare antioxidant activities due to a well-known antioxidant properties.

1. Total polyphenol and flavonoid contents and extraction yield

Phenolic compounds are considered to be the most important antioxidative plant components (Elzaawely *et al*., 2005). Due to the presence of redox properties, phenolic compounds are potent in adsorbing and neutralizing free radicals, quenching singlet-triplet oxygen, or decomposing peroxide (Adedapo *et al*., 2009).

Phenolic compounds stop the lipid peroxidation chain reaction by donating hydrogen ions to free radicals, acting as an antioxidant (Samad *et al*., 2014). Therefore, total phenolic content can be used as an indicator of antioxidant potential (Jiang *et al*., 2012). However, it is known that flavonoids are the most common group of polyphenolic compounds in the human diet and exist ubiquitously in plants.

In this study, the yields were 5.75% and 10.57% for EU water and ethanol extracts, respectively and 5.91% and 4.3% for REU water and ethanol extract, respectively. In the present study, total phenols were calculated on the basis of a standard curve of gallic acid and expressed as ㎎ gallic acid equivalents (GAE) per 100 g of dry mass. Table 1 describes the total phenolic content for all four extracts.

The phenolic contents were 29.8 ㎎ and 13.3 ㎎ GAE per 100 g of dry mass for water and ethanol EU extracts respectively. Both REU extracts contained higher phenolic contents than EU extracts. The measured phenolic contents for REU water and ethanol extracts were 101.49 ㎎ and 74.87 ㎎ GAE per 100 g of dry mass respectively.

Quantitative determinatin of total flavonoids was done on the basis of catechin standard curve and expressed as ㎎ catechin equivalents (CE) per 100 g of dry mass. The flavonoid content of water and ethanol EU extracts were 56.6 ㎎ and 85.8 ㎎ CE per 100 g of dry mass respectively, and for water and ethanol REU extracts were 110.1 ㎎ and 121.0 ㎎ CE per 100 g of dry mass respectively (Table 1).

These results indicated that phenolic and flavonoid antioxidant compounds of EU were increased by roasting. Additional, when comparing DW extraction with EtOH extraction, it can be seen that yield increases in EtOH extraction. It is well known that the higher the polarity of the solvent, the higher the yield of the fraction extraction (Lee *et al*., 2006). Therefore, this extract would also have improved yields in EtOH extraction methods (Table 1).

2. DPPH radical-scavenging activity

DPPH is a stable free radical and is used widely to determine radical scavenging capacity. Generally, DPPH is

Table 1. The total phenolic and flavonoid content of water and ethanol extracts of non-roasted *E. ulmoides* (EU) and roasted EU (REU) cortex.

Extract	Total phenolic (㎎·GAE/100g of dry mass)	Total flavonoid (㎎·CE/100g of dry mass)	Yield (%)
EU-DW[1)]	29.80±0.11^{c}	56.60±1.12^{d}	5.75
EU-EtOH[2)]	13.30±1.01^{d}	85.80±2.58^{c}	10.57
[3)]REU-DW	101.50±1.94^{a,***}	110.10±0.40^{b,**}	5.91
REU-EtOH	74.90±3.18^{b,***}	121.00±0.59^{a,**}	4.30

All data are expresses as means ± standard deviation (n = 3). GAE; Gallic acid equivalent, CE; Catechin equivalent. [1)]EU-DW; *E. ulmoides* water extract, [2)]EU-EtOH; *E. ulmoides* ethanol extract, [3)]REU-DW; roasted *E. ulmoides* water extract, [4)]REU-EtOH; roasted *E. ulmoides* ethanol extract. Statistically significant difference between four extracts was obtained by One-way ANOVA followed by Duncan's Multiple Range Test (DMRT, DW vs EtOH, $^{**}p < 0.01$, $^{***}p < 0.001$).

deep violet in color, but changes to yellow when it reacts with any antioxidant and receives hydrogen ions to form α-diphenyl-β-picrylhydrazine (Babu *et al*., 2013).

Fig. 1 represents the percentage of DPPH radical scavenging activity where BHT was used as a positive control. It has been found that all the four extracts scavenged DPPH radicals in a dose-dependent manner. The DPPH radical scavenging capacity ranged from 8.48% to 69.10% and from 15.36% to 79.02% for water and ethanol EU extracts respectively, whereas the scavenging activity was 42.16% - 93.09% and 50.02% - 94.02% for water and ethanol REU extracts respectively, at concentrations of 0.25 - 4 ㎎/㎖.

The IC_{50} (half-maximal inhibitory concentration) values are shown in Table 2, and show very prominently that REU extracts were more efficient on DPPH radical scavenging activity than EU extracts. The IC_{50} values of EU were found to be 1.53 ± 0.35 ㎎/㎖ for the water and 1.42 ± 0.04 ㎎/㎖ for the ethanol extract. Moreover, the REU extracts showed a lower IC_{50} value (0.320 ± 0.008 ㎎/㎖ and 0.246 ± 0.007 ㎎/㎖ for water and ethanol extracts respectively (Table 2). REU extracts had a similar DPPH radical scavenging activity to that of BHT. DPPH radical scavenging activity of both REU extracts was not significant difference ($p < 0.05$) from that of BHT that was used a positive control due to its antioxidant activity.

These results indicated that all the extracts increased DPPH radical scavenging activity in a dose-dependent manner. In addition, water and ethanol based REU extracts showed higher scavenging activities among the four extracts, and their inhibiting potency was almost similar to that of BHT, especially at the 1, 2, and 4 ㎎/㎖ concentrations. Hence, the extracts helped to inhibit DPPH free radical activity through hydrogen donation ability, and can be used as a natural antioxidant.

3. ABTS radical-scavenging capacity

ABTS is frequently used to measure the antioxidant capacity

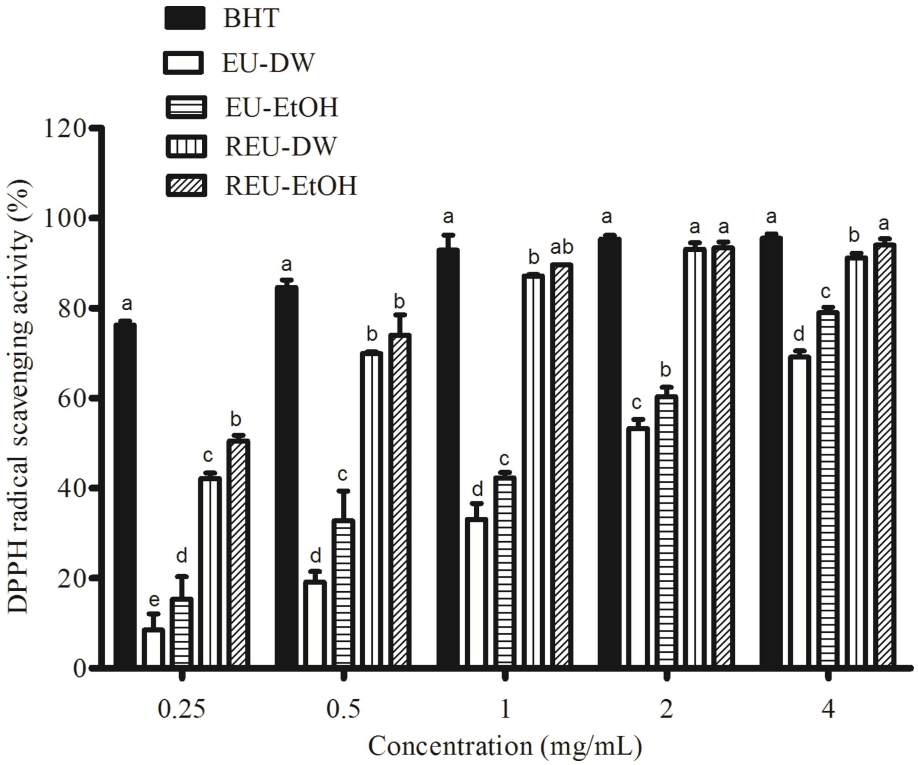

Fig. 1. DPPH radical scavenging activity of the extracts with various concentration in non-roasted *E. ulmoides* (EU) and roasted EU (REU) cortex. BHT was used as a positive control. Each value is expressed as the means ± SD (n = 3). EU-DW; *E. ulmoides* water extract, EU-EtOH; *E. ulmoides* ethanol extract, REU-DW; roasted *E. ulmoides* water extract, REU-EtOH; roasted *E. ulmoides* ethanol extract. *Means not sharing the same letter are significantly different. *p*-values were obtained by One-way ANOVA followed by Duncan's Multiple Range Test (DMRT, $p < 0.05$).

Table 2. DPPH, ABTS and nitrite radical scavenging capacities with IC_{50} values of non-roasted *E. ulmoides* (EU) and roasted EU (REU) cortex.

Extract	DPPH radical		ABTS radical		Nitrite radical	
	Scavenging capacity (%)	IC_{50} (㎎/㎖)	Scavenging capacity (%)	IC_{50} (㎎/㎖)	Scavenging capacity (%)	IC_{50} (㎎/㎖)[1]
EU-DW[3]	53.30±2.02***	1.53±0.04	100.00±0.84***	0.18±0.00	19.60±2.02***	ND[2]
EU-EtOH[4]	60.30±2.06***	1.43±0.05	100.00±0.65	0.16±0.01	18.70±1.31***	ND
REU-DW[5]	93.10±1.44	0.32±0.01	97.10±0.10	0.14±0.00	27.70±0.59***	ND
REU-EtOH[6]	93.40±1.31	0.25±0.01	99.60±1.34	0.12±0.01	25.80±0.58***	ND
Positive control	BHA[7]		As.A		As.A[8]	
	95.80±0.89	0.19±0.02	99.30±1.79	0.01±0.00	54.00±0.91	1.83±0.03

[1]IC_{50} value (㎎/㎖); the concentration in which 50% is inhibited, [2]ND; Not detected, [3]EU-DW; *E. ulmoides* water extract, [4]EU-EtOH; *E. ulmoides* ethanol extract, [5]REU-DW; roasted *E. ulmoides* water extract, [6]REU-EtOH; roasted *E. ulmoides* ethanol extract, [7]BHA; butylated hydroxyanisole, [8]As.A; ascorbic acid. All scavenging capacity tests were performed at the concentration of 2 ㎎/㎖ extract. Statistically significant difference between the each experimental and the positive control group was determined by *t*-test (***$p < 0.001$).

of food. ABTS is converted to its radical cation, which is blue, by the addition of sodium persulfate. When ABTS radical cations react with antioxidants, it is converted back to its colorless neutral form due to the electron donating capacity of antioxidant.

All four extracts showed a strong ABTS radical scavenging capacity (Fig. 2). The results showed that the ABTS radical scavenging capacity of all four extracts was almost similar to that of ascorbic acid. There was no significant difference in the scavenging capacity of the extracts and ascorbic acid in the 0.5 ㎎/㎖ to 4 ㎎/㎖ concentration range, which suggested the EU and REU extracts has a strong ABTS radical scavenging activity at these concentration. The scavenging activities ranged from 67.43% - 112.76% and 53.41% - 99.45% (at 0.25 - 4 ㎎/㎖) with IC_{50} valuesof 0.17 ㎎/㎖ and 0.16 ㎎/㎖ for water and ethanol EU extracts respectively (Table 2).

For the water and ethanol REU extracts, scavenging activity ranged from 89.91% - 99.85% and 98.36% - 98.65% respectively, with IC_{50} values of 0.13 ㎎/㎖ and 0.11 ㎎/㎖ respectively. As it noticed a very low IC_{50} values for all four extracts, this demonstrates that all four extracts were very productive scavengers of ABTS free radicals (Table 2).

4. Nitrite radical-scavenging capacity

Nitrite is considered as a free radical owing to its unpaired electron, and shows important reactivity with certain types of proteins. Nitrite can produce nitrosamine by reacting with amine compounds present in protein-containing food, medicine, and residual pesticides. Leafy and root vegetables, as well as meat, also contain large amounts of nitrite and amine compounds.

In many studies, it has been noted that nitrosamine has a carcinogenic effect, and therefore can be attributed to an elevated risk of cell damage and cancer (Lee *et al*., 2009). Hence, it is crucial to evaluate the nitrite radical scavenging activity of the extracts.

In this study, EU and REU extracts exhibited concentration-dependent nitrite radical scavenging capacity at concentrations ranging from 0.25 ㎎/㎖ to 4 ㎎/㎖. The water and ethanol EU extracts showed scavenging capacity of 19.6% and 18.7% at 2 ㎎/㎖ concentration, respectively (Table 2).

The water extract of REU showed a slightly higher scavenging capacity of 27.7% than the 25.8% nitrite scavenging capacity of the REU ethanol extract at 2 ㎎/㎖ concentration of 25.8% at 2 ㎎/㎖ concentration. While the highest scavenging activity for the ethanol REU extract at the same concentration was 25.8%. ascorbic acid used as a standard, exhibited 54.0% nitrite radical scavenging capacity at the 2 ㎎/㎖ concentration.

According to the observation, the extracts were not high nitrite radical scavenger, but the extracts could scavenge nitrite radical to some extent. The extracts were less potent in nitrite radical scavenging capacity than ascorbic acid.

5. Estimation of reducing power

The ability to reduce a compound usually depends on the presence of reductants (Duh *et al*., 1999). Reductants exhibit

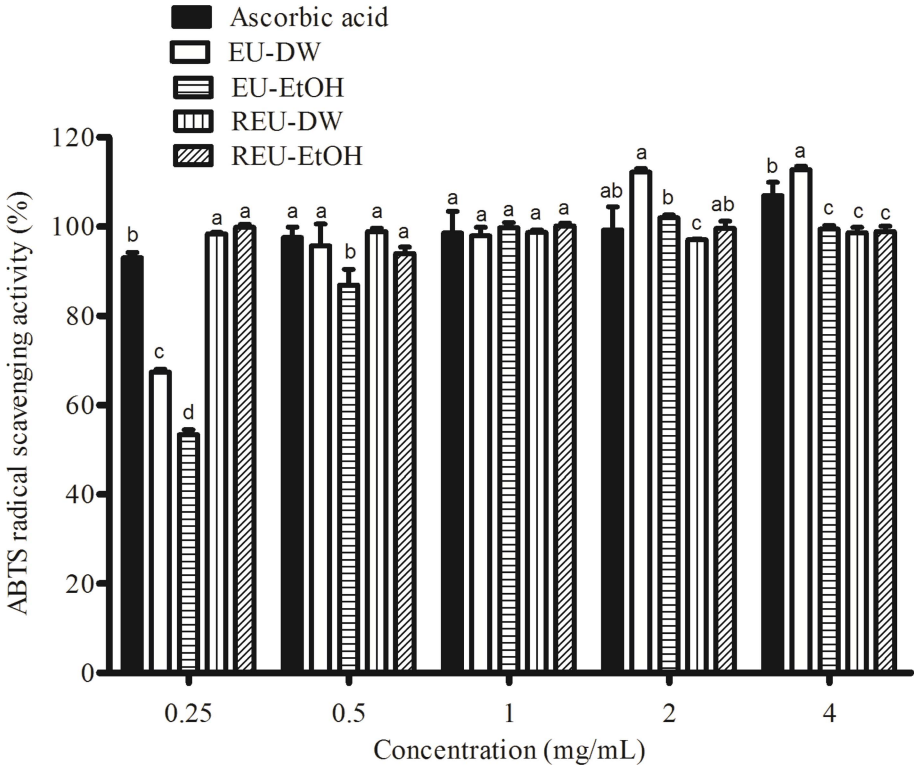

Fig. 2. ABTS radical scavenging activity of the extracts with various concentration in non-roasted *E. ulmoides* (EU) and roasted EU (REU) cortex. Ascorbic acid was used as a positive control. Each value is expressed as the means ± SD (n = 3). EU-DW; *E. ulmoides* water extract, EU-EtOH; *E. ulmoides* ethanol extract, REU-DW; roasted *E. ulmoides* water extract, REU-EtOH; roasted *E. ulmoides* ethanol extract. *Means not sharing the same letter are significantly different. *p*-values were obtained by One-way ANOVA followed by Duncan's Multiple Range Test (DMRT, $p < 0.05$).

antioxidant potential by donating hydrogen ions and breaking the free radical chain (Gordon, 1990). Reducing ability has been determined by evaluating the transformation of Fe (III) to Fe (II) in the presence of sample extracts (Gülçın *et al*., 2003). An increased absorbance indicates a high reducing activity.

In this study, a concentration-dependent Fe (III) reducing capacity were observed for all four extracts. Ascorbic acid was used as reference due to a well-known antioxidant properties. These results represented that confirm that a reducing capacity increased in a dose-dependently manner from 0.12 to 0.55 and 0.13 to 0.57 ㎎/㎖ for water and ethanol EU extracts respectively at a concentration range of 0.25 - 4 ㎎/㎖ (Fig. 3a). Both EU extracts showed low reducing capacity and ascorbic acid had more potent reducing capacity than EU extracts.

On the other hand, the reducing capacity of REU extracts increased from 0.13 to 0.98 and 0.18 to 1.01 ㎎/㎖ for water and ethanol extracts respectively at the concentration of 0.25 ㎎/㎖ to 4 ㎎/㎖. The reducing capacity of REU extracts was similarly strong to that of ascorbic acid at a concentration of 4 ㎎/㎖ though the reducing capacity of the positive control was higher than EU extracts.

The Half maximal effective concentration (EC_{50}) values are shown in Fig. 3(b). The EC_{50} values of both EU extracts was higher than REU. From these results, the REU ethanol extract demonstrated to have a strong reducing capacity at a high concentration with the low EC_{50} values (1.22 ㎎/㎖). Hence, the presence of reductants in the extracts contributes to the reduction of Fe (III) to Fe (II).

6. Oxidative DNA damage prevention activity

In plasmids, DNA damage results in a cleavage of one of the phosphodiester chains of the supercoiled DNA, creating an open circular form. Near the first breakage, further cleavage leads to linear double-stranded DNA molecules. An indicator of single-strand breaks is, the formation of circular DNA,

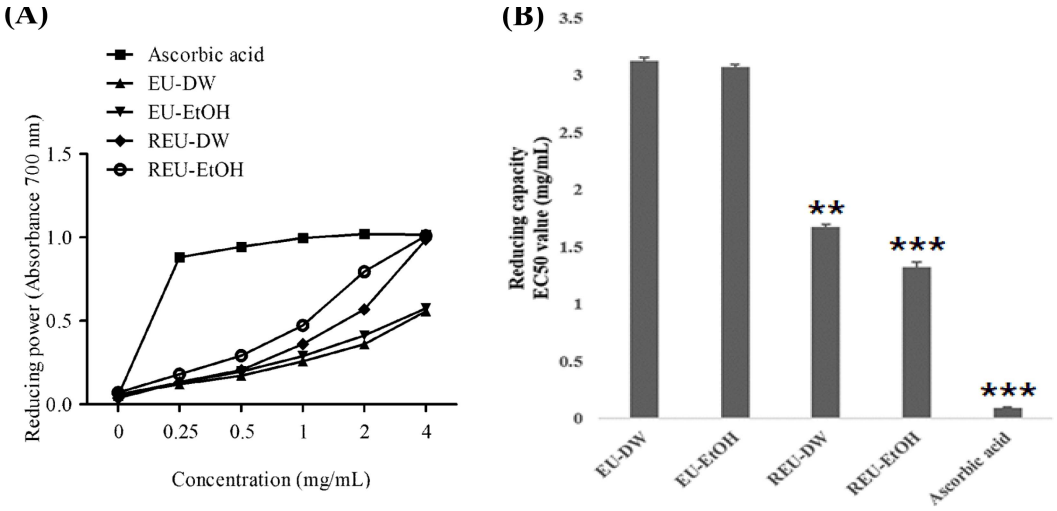

Fig. 3. Reducing power (A) and EC_{50} values (B) of reducing power of extracts in non-roasted *E. ulmoides* (EU) and roasted EU (REU) cortex. EU-DW; *E. ulmoides* water extract, EU-EtOH; *E. ulmoides* ethanol extract, REU-DW; roasted *E. ulmoides* water extract, REU-EtOH; roasted *E. ulmoides* ethanol extract. Ascorbic acid was used as control. Each value is expressed as the means ± SD (n = 3). The EC_{50} value is defined as the effective concentration of the sample that causes a 50% reduction. Statistically significant difference between four extracts was obtained by One-way ANOVA followed by Duncan's Multiple Range Test (DMRT, DW vs EtOH, $^{**}p < 0.01$, $^{***}p < 0.001$).

whereas the formation of linear DNA indicates double strand breaks (Burrows and Muller, 1998).

H_2O_2 and $O_2^{-\bullet}$ are potentially cytotoxic; in biological systems, most of the oxidative damage is caused by OH and $OH^{\bullet}$ is produced by the reaction between H_2O_2 and $O_2^{-\bullet}$ in the presence of redox-active metals. Free radical-mediated oxidation of DNA can be influenced by natural polyphenols through simple mechanisms, including the quenching of ROS by donating hydrogen ions or electrons (Prakash *et al*., 2007).

In this study, the determination of the oxidative DNA damage protective capacity of EU and REU extracts was done by using pBR322 plasmid DNA (Fig. 5). Plasmid DNA was used as a control (Lane 1 of Fig. 5). Due to hydroxyl-, radical-mediated DNA damage, the DNA has been entirely converted from the supercoiled form to the open form. When the DNA was treated with the extracts, supercoiled DNA was restored in a concentration-dependent manner (0.01 to 1 ㎎/㎖). These results showed that both of EU and REU extracts possessed DNA damage protection activity and REU water and ethanol extract had especially higher DNA protecting activity.

In all results of these antioxidant assays, the REU water and ethanol extracts show a stronger antioxidant activity than those from EU water and ethanol extracts. The reason is probably due to the phenolic and flavonoid contents contained in REU water and ethanol extracts. It is well known that phenols and flavonoid compounds of natural substances have excellent antioxidant capacity and the total phenolic and flavonoid compounds have been shown to be positively correlated with antioxidant ability (Babu *et al*., 2013; Hasnat *et al*., 2014; Samad *et al*., 2014).

Previous research (Yen and Hsieh, 1998; Xu *et al*., 2010) reported that the antioxidant activity of *E. ulmoides* leaf and cortex extracts was mainly due to their polyphenol compounds. Yen and Hsueh, (1998) reported that in quantitative analysis results of individual phenolic constituents by HPLC, ethanol extract of roasted Du-zhong cortex contained various phenolic compounds such as chlorogenic acid, caffeic acid, protocatechuic acid, and rutin.

These *in vitro* assays demonstrated that EU and REU extracts are a significant source of natural antioxidants, which might be useful in preventing the progression of different diseases caused by free radicals. It could be said that the free radical scavenging activity and the prevention of oxidative DNA damage by EU and REU extracts were due to the presence of the higher phenolic and flavonoid compounds. These results show that the extract of Du-zhong has excellent antioxidant effect. In the study of good faith, the fact that polyphenols are extracted from Du-zhong and that the content

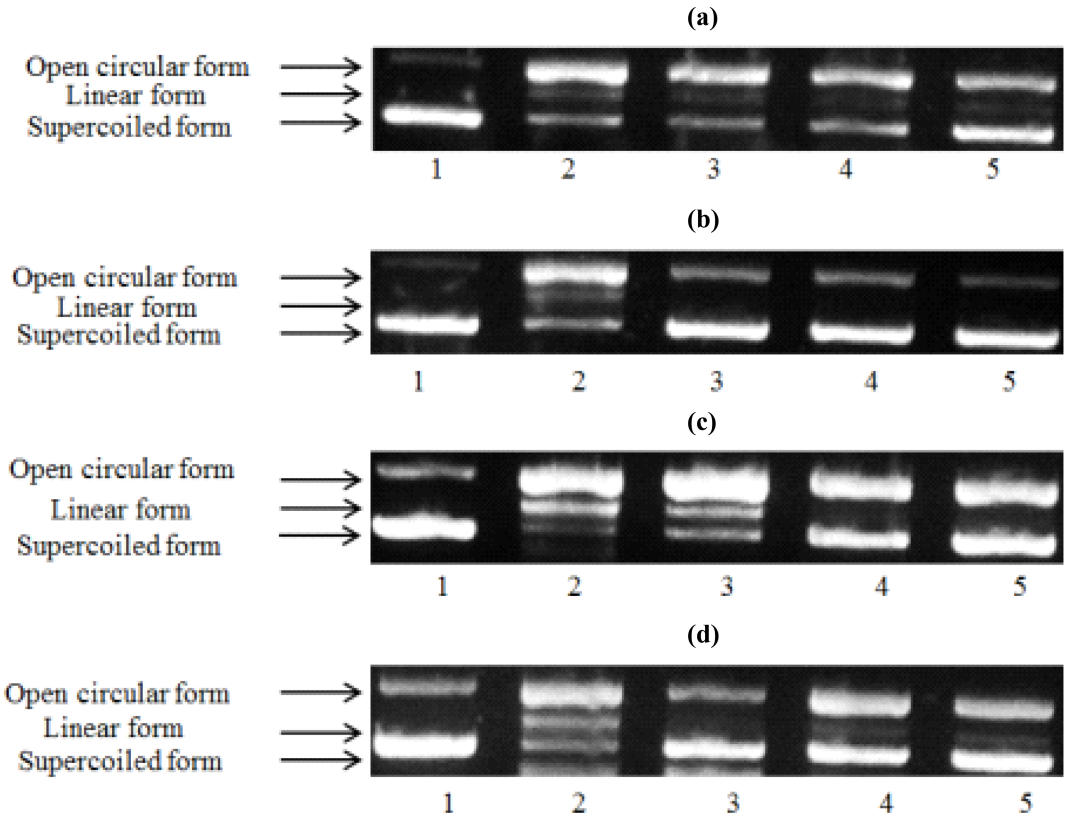

Fig. 5. Electrophoresis patterns for prevention of PBR322 DNA breaks by OH (hydroxyl radical) by water and ethanol cortex extracts of EU and REU. Lane 1; ueated control DNA, lane 2; $FeSO_4$ and H_2O_2 (DNA damage control), lane 3 - 5; $FeSO_4$, DNA and different concentration (0.1, 0.5 and 1 ㎎/㎖, respectively) of extracts in non-roasted *E. ulmoides* (EU) and roasted EU (REU) cortex. (a) EU-DW; *E. ulmoides* water extract, (b) EU-EtOH; *E. ulmoides* ethanol extract, (c) REU-DW; roasted *E. ulmoides* water extract, (d) REU-EtOH; roasted *E. ulmoides* ethanol extract.

of polyphenols is correlated with the ability of antioxidants is inferred from the study of good faith (Kawasaki *et al*., 2000).

7. Inhibition of NO production in LPS-stimulated RAW264.7 cells

NO plays an important role in various kinds of inflammatory responses. There are three isoforms of the enzyme nitric oxide synthase (NOS) by which NO is synthesized: endothelial NOS (eNOS), neural NOS (nNOS), and inducible NOS (iNOS). In living organisms, tissues are damaged due to the overproduction of NO (Beckman and Koppenol, 1996). Immune cells during the process of inflammation generate superoxide radicals, leading to vascular complications (Dröge, 2002). Many diseases, such as diabetes, septic shock, atherosclerosis, and Alzheimer's disease, are related to NO-induced oxidative stress. Overexpression of iNOS can be promoted by various stimuli such as LPS, which causes macrophages to release a large amount of pro-inflammatory cytokines, such as TNF-α, which plays a crucial role in sustaining the inflammatory response. Therefore, the down-regulation of NO is very important to remove these complications.

LPS-stimulated RAW264.7 cells were treated with EU and REU extracts. In conditioned media, LPS-induced cells produced a large amount of NO. EU extracts (10 - 50 ㎍/㎖) did not show any significant ability to reduce NO production (Fig. 6a). However, REU extract treatment (10 - 100 ㎍/㎖) decreased NO production in a concentration-dependent manner (Fig. 6b).

It was found that the REU water and ethanol extract was highly effective in reducing NO production. Kim *et al*. (2012) was reported that water extract of *E. ulmoides* OLIVER has anti-inflammatory activity such as NO production, prostaglandin E2, IL-6 and TNF-α inhibition. In this study, REU extracts were also found to more potent to inhibit NO production than EU extracts.

Therefore, this observation suggests that REU extracts have

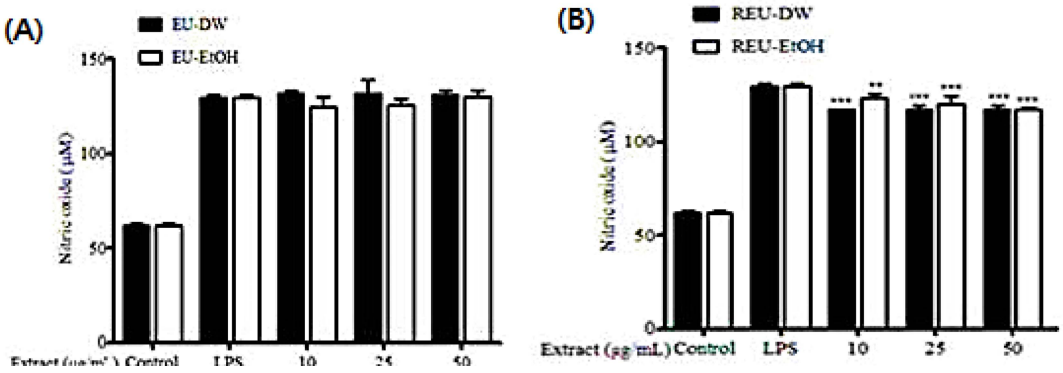

Fig. 6. Inhibition of NO production in RAW264.7 cells treated with extracts of non-roasted *E. ulmoides* (EU) (A) and roasted EU (REU) cortex (B). Inflammatory responses were induced by treating the cells with 1 ㎍/㎖ of lipopolysaccharide. Data are means ± SD of the three independent experiments. Statistically significant difference between the each experimental and the LPS group was performed by *t*-test ($^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$)

the potential to inhibit NO production. In our results, a greater amount of phenolic content has been found compared to a previous study (Wong *et al*., 2006) although the reducing power capacity is almost same.

DPPH and ABTS radical scavenging capacity showed a greater result than some previous report (Wong *et al*., 2006). Moreover, the inhibition power of NO production is poorer than reported in previous study (Kim *et al*., 2012). This comparative study might be helpful for antioxidant study.

In conclusion, these results suggested that REU extracts has a more potential antioxidant capacity compared to EU extracts in DPPH radical, ABTS radical and nitrite scavenging activity and reducing power. Furthermore, the results indicated that REU extracts played an important role in inhibiting NO production, however the cell viability results were not remarkable for all four extracts.

It has been considered that the potent antioxidant and anti-inflammatory activities observed are reasonable given the presence of phenolic constituents, and how hydrogen ion donation from phenolic groups assists the antioxidant capacities of the extracts. However, while both EU and REU extracts showed high antioxidant effects, but the REU extracts were more potent. It is suggested that higher antioxidant potency of REU extracts was due to the roasting process. Nevertheless, further in vivo research is needed to validate these results.

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