Journal of Oleo Science
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doi : 10.5650/jos.ess18134
J. Oleo Sci. **67**, *(12) 1535-1541 (2018)*

The Antioxidation of Different Fractions of Dill (*Anethum graveolens*) and Their Influences on Cytokines in Macrophages RAW264.7

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Abstract: Dill (*Anethum graveolens L.*) has been shown strong antioxidative and immune propertise, but the precise potency and action mechanisms remain largely elusive. This study is to dissect the different fractions' antioxidant power and antiinflammatory function. We extracted 4 fractions from China original dill with ether (DI-E), ethyl acetate (DI-EA), *n*-butanol (DI-B) and water (DI-W), and performed 4 different kinds of antioxidative analysis together with vitamine C (Vc): DPPH, ABTS, reducing power and TPTZ-FRAP. For correlated compounds in antioxidant assays Folin-Ciocalteu's analysis was performed. For antiinflammation, cell proliferation by MTT, NO molecules and interleukin-1 and 6 in supernatant were detected by Griess reaction and Elisa, respectively, and gene expression of inducible nitric oxide synthase (iNOS) was analyzed by RT-PCR. The strength of antioxidant activity was Vc > DI-EA > DI-B > DI-W > DI-E. Folin-Ciocalteu's analysis showed that antioxidant power was correlated to phenolic compounds. However, in antiinflammatory assays DI-E was most active one by cell proliferation, iNOS's gene expression, and secretion of interleukin IL-1 and 6 in macrophage RAW264.7. The antioxidant fraction and anti-inflammatory fraction of the dill were determined. The certain fractions of dill may be strong at antioxidation, but weak at antiinflammation, vice versa. Thus dill has anti-ageing and anticancer potential, a good resource for functional food and ancillary drugs of rehabilitation.

Key words: dill (*Anethum graveolens L.*), antioxidant, antiinflammation, macrophage RAW264.7, iNOS

1 Introduction

Dill (*Anethum graveolens L.*), an important member of the Umbelliferae family native to southwest Asia or southeast Europe, is widely used for flavoring foods and beverages, and for the treatment of many pathological conditions such as diseases of the uterus, cervical ectropion, flatulence, indigestion, stomachache, colic, and gas in the intestinal tract[1–3]. Dill has been also reported to possess antibacterial property[4] which is believed to be the reason widely used in cans for sour cucumber and other food processes. Dill is also known for its antihyperlipidemic, and antihypercholesterolemic properties[5]. As a traditional medicinal vegetable, dill increases milk production and promotes menstruation[2]. However, the significant medicinal activity of dill is mainly related to its antioxidant power[6] and anti-inflammatory properties[7].

Internal and external process of oxidation/antioxidation influences cell senescences and ageing. One of the antioxidant signaling pathways is Nrf2 controlled antioxidative responsive elements (ARE) and these antioxidant systems include enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, macromolecules such as albumin, ceruloplasmin, and ferritin, and certain small molecules. The sum of endogenous and external food-derived antioxidants represents the total antioxidant activity of the system against attack by reactive oxygen species (ROS) or nitrogen species[8, 9]. The antioxidant power of some fraction of dill has been preliminarily studied[5]. To our knowledge and according to a literature survey, both dill's antioxidative and the antinflammatory mechanisms as whole

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Accepted September 21, 2018 (received for review July 8, 2018)
Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online
http://www.jstage.jst.go.jp/browse/jos/ http://mc.manusriptcentral.com/jjocs

remain elusive and dill is becoming popular as spices and for medicinal purposes in China. Since inducible nitric oxide synthase (iNOS) is a very important signal molecule, together with cytokines in the process of phyisological and pathological process, especially in the inflammatory process[10, 11], thus, a further exploration of dill as a dietary and medicinal resource is scientifically, economically and socially demanded.

2 Materials and methods

2.1 Chemicals, Regents and RAW264.7 cell line

Dill was purchased from Huaqin food Co. Qindao, Shangdong, 2,4,6-tris-2,4,6-tripyridyl-2-triazine (TPTZ), 2,2′ -azino-bis- 3-ethylbenzthiazoline-6-sulphonic acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich Chemical Co. The organic reagents were purchased from local companies. RPMI 1640 medium and fetal bovine serum (FBS) were from Gibco. 3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), lipopolysaccharides (LPS) and dimethyl sulfoxide (DMSO), penicillin and streptomycin were purchased from Sigma-Aldrich Chemicals (St Louis, MO, USA). NO assay kit was purchased from Jiancheng Co., Nanjing, China). RNA Mini kit was purchased from Qiagen (Hilden, Germany), RevertAidTM First Strand cDNA Synthesis Kit was from Fermentas (Hanover, Maryland, USA). The RAW264.7 cell line was a gift from Beijing Institute of Biotechnology, Chinese Academy of Military Medical & Sciences.

2.2 Preparation of dill extracts

Fifty grams of dried dill leaves was extracted three times with 0.5 L of 80% ethanol at 40℃ for 4 h. The extracts were filtrated, and enriched following by using a rotary evaporator (Yarong Co., Shanghai, China) at 42℃. The extracts were dispersed with 150 mL distilled water, and further subjected to liquid–liquid partition successively with 450 mL of petroleum ether, as fraction DI-E, then extracted with 700 mL of ethyl acetate, as DI-EA, then 800 mL of *n*-butanol as DI-B, and finally 50mL left in bottom as water fraction (DI-W). The different fractions were concentrated using rotary evaporator, then freeze-dry to obtain DI-E (10.41%), DI-EA (2.87%), DI-B (5.22%), and DI-W (13.5%), respectively. The samples for antioxidant assay were dissolved in methanol at a concentration of 1mg/mL then diluted in series of 6.25, 12.5, 25, 50, 100, 200, 400, 800 (μg/mL) for antioxidant assays.

2.3 Scavenging assay of DPPH free radicals by dill extracts

DPPH is a stable free radical molecule with red color (absorbs at 517 nm). If free radicals have been scavenged by candidate antioxidants, DPPH will change its color to yellow. 100 μL samples were mixed with 100 μL of DPPH (0.2 mM) in methanol. After 30 min incubation at room temperature, read the absorbance at 517 nm[11]. Vc was used as positive control. The inhibitory percentage of DPPH was calculated according to the following equation: DPPH scavenging rate $= \frac{A_0 - A_1 + A_2}{A_0}$. A_0 = absorbance (DPPH solution in methanol), A_1 = absorbance of sample, A_2 = absorbance of sample's background.

2.4 Reducing power

Reducing power of ionic was determined with Fe^{3+} [12]. An aliquot of each sample (100 μL) was mixed with 100 μL of sodium phosphate buffer (0.2 M, pH 6.6) and 100 μL of 1% $K_3Fe(CN)_6$ followed by incubation at 50℃ for 20 min, then let it cool quickly. After adding 100 μL of 10% trichloroacetic acid, the mixture was centrifuged at 3000 rpm/min for 10 min. The supernatant solution (100 μL) was mixed with 100 μL of ddH_2O and 20 μL of 1% ferric chloride to react for 10 min. Subsequently, the absorbance was measured at 700 nm.

2.5 $ABTS^+$ radical scavenging activity

$ABTS^+$ was prepared by mixing an ABTS stock solution (7 mM in H_2O) with 2.45 mM potassium persulfate[13]. This mixture was incubated for 12-16 h in dark before use. The $ABTS^+$ solution was diluted with methanol to an absorbance of 0.700 ± 0.01 at 734 nm. An aliquot of each sample (30 μL) was mixed with 300 μL of the $ABTS^+$ solution for 6 min at 30℃ and the absorbance was determined at 700 nm. The absorbance at each time point was corrected for the absorbance of an ABTS blank.

$$ABTS^+ \text{ scavenging rate} = \frac{A_{control} - A_{sample}}{A_{control}}$$

2.6 FRAP method for antioxidant assay

The total antioxidant capacity of the samples was determined using a modification of the FRAP assay[14]. FRAP reagent was prepared from 300 mM acetate and glacial acetic acid buffer (pH 3.6), 20 mM ferric chloride and 10 mM 4,6-tripryridyl-s-triazine (TPTZ) made up in 40 mM HCl. All three solutions were mixed together in the ratio 10:1:1. The FRAP assay was performed by warming 150 μL of ddH_2O to 37℃ before adding 30 μL of sample and 150 μL of FRAP reagent, and incubating for 6 min at 37℃. Absorbance at 593 nm was determined by subtracting a reagent blank. The total antioxidant capacity of samples was determined against a standard of known FRAP value, ferrous sulphate.

2.7 Folin-Ciocalteu method for total polyphenols

The amounts of total phenols were analyzed by a method with Folin–Ciocalteu's phenol reagent using gallic acid as a standard[15]. The sample (1 mL) was added to 0.5 mL Folin–

Ciocalteu Reagent, the mixture was allowed to equilibrate for 3 min and then mixed with 1.5 mL of 60 g/L sodium carbonate solution. After incubation at 70℃ for 10 min, the absorbance of the mixture was read at 725 nm using the corresponding solvent as blank. The results were expressed as μg/mL of ferulic acid equivalents to samples.

2.8 Cytotoxicity assay

The cells were The cells were cultured in RPMI1640 medium supplemented with L-glutamine (1mM), 10% (v/v) heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37℃ in 5% (v/v) CO_2 incubator. Cells were seeded in 96 well plates at a density of 8000 cells/well. After 24 h, the cells were treated with medium or the medium mixed with different concentrations of dill extracts and LPS (1 μg/mL). After incubation for 24, MTT solution (0.5 mg/mL) was added and incubated for another 4 h. Finally, 150 μg/mL of DMSO was added to solubilize the formed formazan crystals after aspiration of MTT solution. The amount of formazan crystal was determined by measuring the absorbance at 570 nm using ELISA reader (Thermon, USA).

2.9 Determination of the Nitrite

Nitrite concentration in the medium was measured as an indicator of nitric oxide (NO) production according to the Griess reaction method. Cells were seeded in 24 well plates at a density of 4×10^4 cells/well were treated with LPS and DI 24 h after 12 h induction. The supernatant was collected by centrifugation at 3000 rpm/min 20 min, the nitrite was measured using NO assay kit (Jiancheng, China) by strictly following the protocol instruction.

2.10 Gene expression of iNOS by RT-PCR

Total RNA was extracted from approximately 2×10^6 of cells for each test following the manual (Qiagen, USA). The fully integrity of total RNA was determined by 1% agarose gel. The total RNA was converted to single cDNA using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, USA). The cDNA was kept in −20℃. The iNOS PCR product with fragment size of 222bp were sense 5'-GCATGGACCAGTATAAGGCAAGCA -3' and antisense 5' -GCTTCTGGTCGATGTCATGAGCAA -3' based on literature[16] and the β-Actin with size of 202 bp : Sense 5'-TGGAGAAGAGCTATGAGCTGCCTG-3' Antisense5'-GTGCCACCAGACAGCACTGTGTTG-3'[17], synthesized by Invitrogen, Shanghai, China. RT-PCR of iNOS was performed as following: 5 μL of 10 × Taq Reaction Buffer, 2 μL of template cDNA, 1.5 μL of primers each, 1 μL of dNTP mix (10mM), 1 μL of Taq DNA polymerase and H_2O up to 50 μL. Reaction started with 94℃ for 30 s, then 94℃, 30 s, 60℃, 45 s, and 72℃, 45 s for 35 cycles, and finally an extension of 10 min at 72℃, then kept at 4℃. The correct fragment of PCR was confirmed by a commercial sequencing service company (BGI, Beijing, China).

2.11 Measurement of IL-1 and 6 of supernatant by ELISA

The supernatant was collected by centrifugation at 3000 rpm/min 20 min after the induction of LPS and DI treatment, the IL-1 and 6 were measured using ELISA kit (R&D system, USA) by following the protocol instruction strictly, and in the sensitive range through a standard curve.

2.12 Statistical analysis

Triplicate analyses were conducted for each sample. The experimental data were expressed as mean ± standard deviations of three separate determinations. The significant of changes in the test responses was assessed using independent-samples T test. The level of significance was taken as $p < 0.05$. All statistical procedures were performed using SPSS 17.0 software for Windows (SPSS Inc., Chicago, IL).

3 Results

3.1 Different antioxidant power of the dill fractions

Four different kinds of antioxidant assays gave antioxidant capacity of samples the same rank as Vc > DI-EA > DI-B > DI-W > DI-E (Fig. 1). The scavenging abilities on DPPH radicals at the concentration 50 μg/mL for DI-EA and DI-B were 96.08% and 94.57%, and these were closed to the ability of Vc. Thus this concentration was further applied in all cell based analysis.

3.2 Correlations between the antioxidant capacity and total phenol contents

By Folin-Ciocalteu assay phenolic compounds in the dill extracts were determined, which not only quantitify the type of compounds, but also help to correlate the antioxidant results (Table 1). The equation about the correlations between EC_{50} values of DPPH scavenging and total phenol contents was $y = 2.864x + 3.863$ and $R^2 = 0.9878$. The correlation about $ABTS^+$ was $y = 1.170x - 8.687$ and $R^2 = 0.9871$. The correlation was good enough to indicate phenolic compounds played the important role in antioxidant activity.

3.3 Effects of dill extracts on cell cytotoxicity and cell proliferation

Cytotoxicity of dill different fractions (12.5-100 μg/mL) on LPS-induced macrophages RAW264.7 was measured by MTT assy. There was no cell cytotoxicity for DI extracts under 100 μg/mL, no significant difference on cell proliferation when macrophages were activated by LPS, but a significant difference of inhibition was seen in LPS + DI-E fraction (Fig. 2). Based on cytotoxicity assay the concentration of 50 μg/mL of dill extracts was selected for further studies.

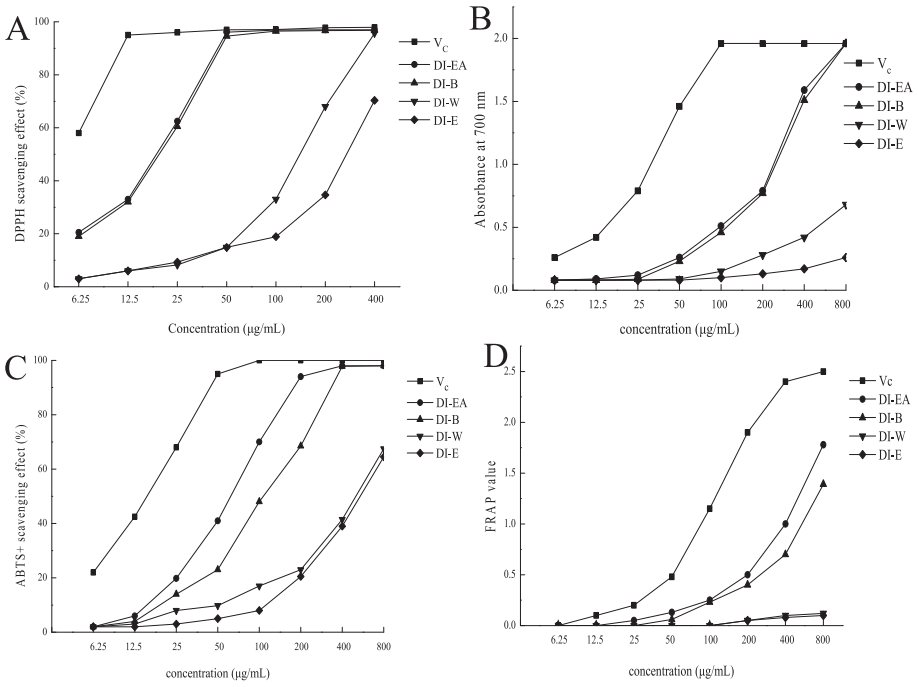

Fig. 1 Antioxidants analysis with different methods for 4 fractions of DI-E, DI-EA, DI-B and BI-W in the corresponding same concentrations. **A**, DPPH scavagening assay. **B**, antioxidant power assay. **C**, ATBS assay. **D**, TPTZ-FRAP assay.

Table 1 EC_{50} against ABTS+ and DPPH free radicals and total phenol contents of different fractions of *Anethum graveolens* L.

Different fractions of dill leaf	EC_{50}(μg/mL)		Total phenol contents (μg/mg)
	DPPH	ABTS	
DI-E	198.187 ± 1.340	460.471 ± 7.493	15.293 ± 0.526
DI-EA	15.933 ± 0.323	51.324 ± 1.066	168.113 ± 1.136
DI-B	20.453 ± 0.898	75.823 ± 2.252	137.927 ± 0.956
DI-W	140.621 ± 0.478	430.991 ± 6.586	28.967 ± 0.683
Vc	5.85 ± 0.038	11.30 ± 0.525	–

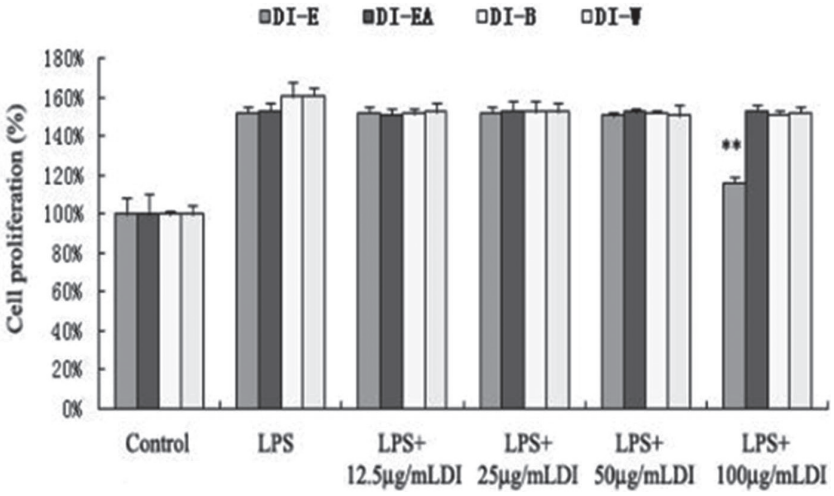

Fig. 2 Cytotoxicity assay based on cell proliferation with 4 different fractions at presence of LPS(1 μg/mL). $^{**}p<0.01$, compared with LPS alone.

3.4 Effects of dill extracts on the production of NO cells in RAW264.7

Nitrite was measured for NO with 1 μg/mL of LPS and 50 μg/mL of dill extracts treated macrophages. A significant difference comparing to control and the group with LPS induction in DI-E fraction was seen (Fig. 3) even though there was no big change for other fractions.

3.5 Dill extracts on iNOS gene expression

Gene expression of iNOS in macrophage RAW264.7 cell by different extracts at 50 μg/mL was demonstrated by RT-PCR with the subtraction of β-actin, a house keeping gene. The gene expression of iNOS was sharply increased by LPS, but all fractions of dill extracts also showed the inhibitory effects, particularly significant inhibition by DI-E was seen (Fig. 4).

3.6 Interleukins -1 and -6 on macrophage RAW264.7 influenced by dill extracts

In the cascade of inflammation, certain types of interleukins secreted by activated macrophages, in turn selectively activate certain kinds of lymphocytes when inflammation or injury took place. ELISA analysis for IL-1 and 6 secretions of macrophage RAW264.7 treated with 1 μg/mL of

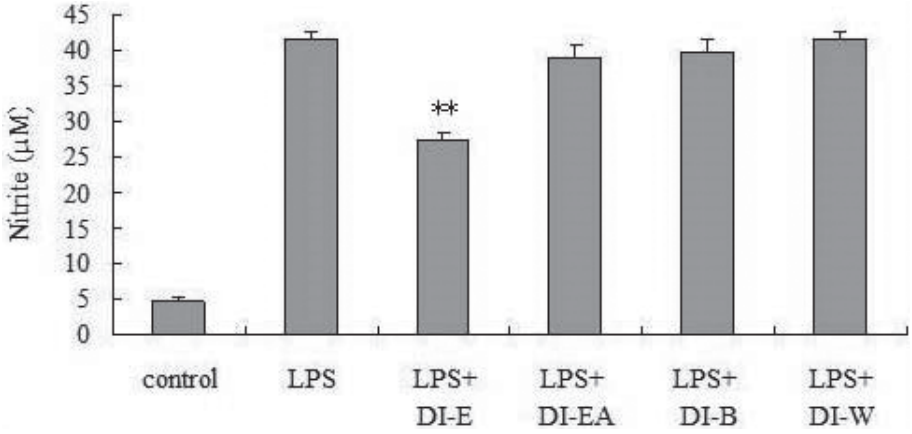

Fig. 3 NO assay in macrophage RAW264.7 cells with 4 different fractions at presence of LPS (1 μg/mL). $^{**}p<0.01$, inhibition of NO, compared with LPS alone.

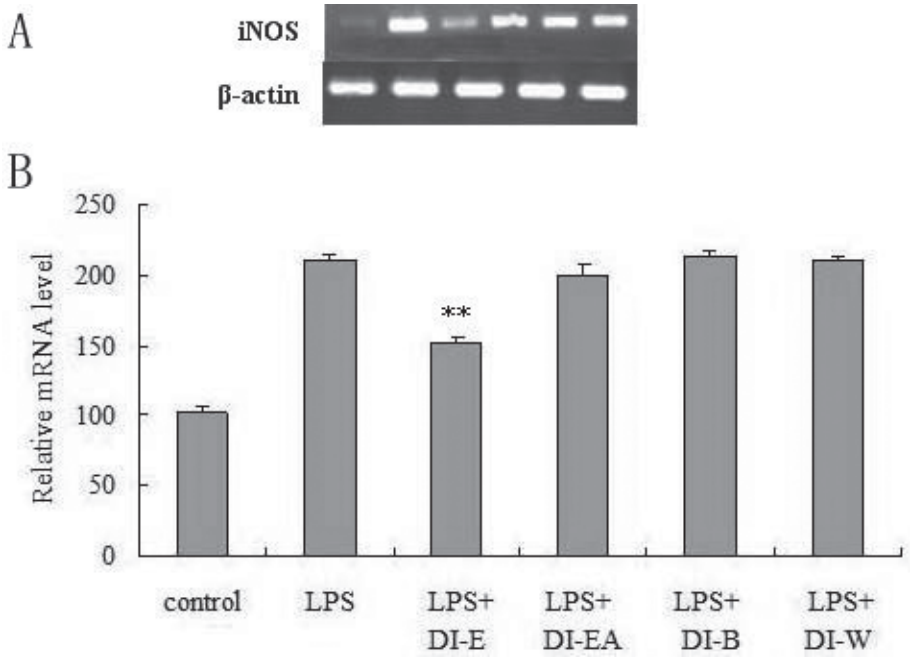

Fig. 4 Gene expression of iNOS in macrophage RAW264.7 cells with 4 different fractions at presence of LPS (1 μg/mL) and absence by RT-PCR. **A**, iNOS DNA by PCR on agarose electrophoresis. **B**, the value of gene expression of iNOS DNA in relative optical density (ROD). $^{**}p<0.01$, compared with LPS alone.

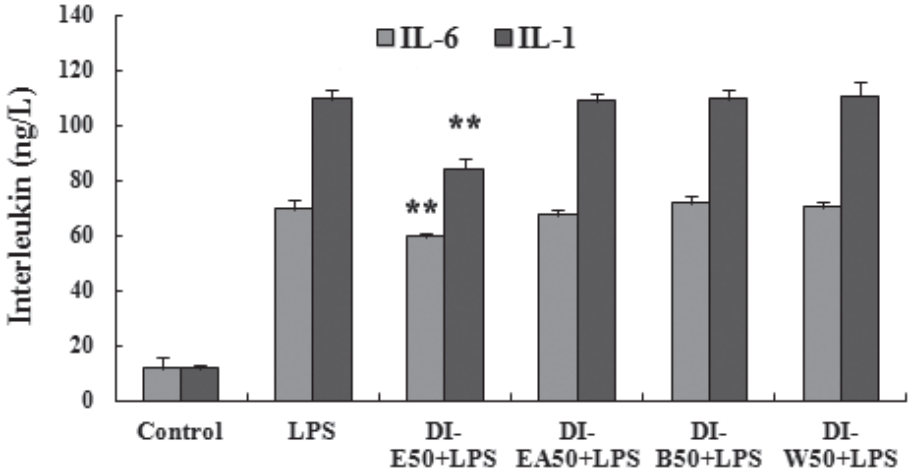

Fig. 5 ELISA for secreted IL-1 and 6 of macrophage RAW264.7 treated with 1 μg/mL of LPS and 50 μg/mL of each dill extract. All the tests were performed with 3-6 duplicates. $^{**}p<0.01$ compared to corresponding LPS plus interleukins.

LPS and 50 μg/mL of each dill extracts was to directly see DI extract capacity of antiinflammation. For in vitro assay LPS has to be added in order to see interleukin secretion. A significant difference was seen with DI-E comparing with LPS induced macrophage RAW264.7 cells, however the influence of other dill extracts on IL-1 and 6 was mild (Fig. 5).

4 Discussion

By analyzing antioxidant capacity of different polar extracts of dill the fraction of DI-EA was found to be the strongest antioxidant comparing to DI-E, DI-B and DI-W fractions, and all showed a dose-dependent during the observation period. By Folin-Ciocalteu analysis DI-EA had highest phenolic value among these fractions, thus it is quite likely that antioxidant power mainly come from these polyphenolic compounds, which also indicates the separative order of released fraction based on the chemistry property, and the accuracy of DPPH, FRAP, ABTS and power reducing methods for the determination of antioxidant activity of the extracts from dill leaves.

RAW264.7 macrophage cells, derived from BALB/c mice ascites, are commonly accepted as a tool to investigate the molecular mechanisms of macrophage involved in regulating inflammation and immunity[18]. However the cells in vitro stay in a dormant status, most genes keep silent, that is why LPS was used in all macrophage RAW264.7 based assays. It is not surprised that DI-E was the only fraction significantly inhibit the cell proliferation, consistently DI-E inhibit the gene expression of iNOS by RT-PCR and also inhibited the IL-1 and 6 by ELISA, so DI-E is the only anti-inflammatory fraction by all assays. At present dill extracts has been reported to be used to lower blood lipid in rat model[3], it is possible that the compounds like in DI-E fraction down regulate the cytokines, and reduce the excessive activation of macrophages, further reduce the blood coagulation because macrophages can form large amount of foam cells in endothelial wall of blood vessels[19, 20]. The inhibition of IL-6 on leukocytes by DI-E fraction is quite possible to be in agreement with the cell proliferation (100 μg/mL). It is common that IL-1 stimulates lymphocyte, and IL-6 normally play inhibitive role. The activation or inhibition of iNOS and IL-1 and 6 in macrophage RAW264.7 cells by dill extracts indicates that dill is important for non-specific human immunocompetence as macrophages are efficient phagocytes that can engulf a considerable number of pathogens or abnormal cells, such as cancer cells.

Interestingly, DI-E had a single main peak in 411 nM, while DI-EA had a main peak in 392 nM in 200-700 nM in full wavelength scanning (data not shown), which indicated that the anti-inflammatory and antioxidant molecules are different. These data collectively show that dill is a good resource of natural antioxidants and antiinflammatory compounds, DI-E fraction with potent antiinflammation, DI-EA with strong antioxidation could be good sources for intestinal digestion and immune integrity. Thus these findings are useful for the process of functional food or ancillary drugs.

Conclusion

The methods of the extraction of active fractions from dill, and their assays of antioxidation and antiinflammation for 4 fractions are of novelty, and can be used for guidance of the natural medicinal process and functional food. The finding of the antioxidant fraction (DI-EA) and antiinflammation (DI-E) could be also used for further studies to pin-

point the precise molecular mechanism.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgements

This project was supported by an initial fund from Tianjin city government for "1000 talents plan" program to C. L

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