Original Article

Antioxidant and Anti-inflammatory Activity Study of Fulvic Acid

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Abstract

Background/Objectives: This study attempted to confirm the possibility of fulvic acid as a functional material. **Methods/Statistical analysis:** Anti-inflammatory effects due to antioxidant measurement, as well as the cytotoxicity and NO production inhibition in RAW 264.7 cells were measured using fulvic acid. **Findings:** In this study, it was shown that fulvic acid enhances DPPH radical elimination and SOD activity. No cytotoxicity was identified in RAW 264.7 cells. Also, after inducing inflammation of RAW 264.7 cells by LPS, NO production inhibiting ability related to inflammatory reaction was measured to confirm excellent NO production inhibiting effect. **Improvements/Applications:** Based on these results, it is concluded that, fulvic acid has low toxicity to cells, is safe, has excellent antioxidant activity, and has excellent inhibition of NO production related to inflammation, so it can be used as a functional material.

Keywords: Antioxidant, DPPH Radical Scavenging, Fulvic Acid, Nitric oxide, SOD

Introduction

The human body is constantly in need of oxygen throughout the breathing process, and some of the oxygen inhaled (about 2-3%) is converted to toxic substances called Reactive Oxygen Species (ROS), which causes cell damage.[1] Types of active oxygen species include free radical and hydrogen peroxide (H2O2), which are at odds with each other, such as single antioxidant (1O2), superoxide radical (O2•), and hydroxyl radical (OH•). Such active oxygen species are highly unstable in structure and attack normal cells causing oxidative damage.[2] Active oxygen is also a major cause of skin aging and is known to cause inflammatory reactions and carcinogenesis.[3] The internal inflammatory response is the normal protection of the body against external stimuli. RAW 264.7 is a typical cell involved in the inflammatory response. It is activated by a variety of stimuli and cytokines secreted by immune cell to produce pro flammatory cytokines, NO (nitric oxide) and PGE2 (prostaglandinE2).[4] In particular, NO is a highly reactive biogenic molecule produced in L-arginine by NOS (nitric oxide synthase), and in particular, iNOS (inducible NOS) participates in inflammatory reactions.[5] Recently there is growing interest in antioxidants, which can remove active oxygen. Natural substances known to remove active oxygen include vitamin C, vitamin E, polyphenols, carotenoids and superoxide dismutase (SOD), synthetic antioxidants Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT).[6,7]

Humic substances are brown and black organic polymer compounds naturally produced during the decomposition process of animals and plants.[8,9] Humic substances are classified into humin, humic acid, fulvic acid, and ulmic acid according to the solubility in acid and alkali

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solutions. Humic acid, followed by humic acid, followed by a kind of depolymerization. Unlike humin, which is insoluble in all pH conditions, fulvic acid used in this study is soluble in all pH regions of the mixture. Fulvic Acid increases the activity of some enzymes, such as transaminase, invertase, and alkaline phosphatase, which act as electron donors and receptors as needed. Fulvic acid participates in oxidation-reduction, causing transition metals reactions, and removes free radicals with strong natural electrolytes,which are antioxidants. In addition, fulvic acid is involved in redox reactions and removes pre-radical groups with strong natural electrolytes, resulting in transition metal reaction and antioxidant activity.

Fulvic Acid varies in color from yellow to tawny and has a molecular weight from hundreds to tens of thousands of Daltons. Fulvic Acid is a type of hindwing acid in which copper and plant deposits are decomposed and synthesized by microorganisms for a long time. It is known to improve immunity of organisms, treat diseases, decipher toxic substances, and remove them from living organisms.[10,11] Also, when plant cells are exposed to fluvic acid, cell growth is accelerated and life support activities of the cells are accelerated. It also promotes carbohydrate metabolism and leads to the accumulation of soluble sugar. Sugar accumulation increases reverse osmotic pressure inside the cell wall to prevent damage and stimulates the immune system to increase immunity.[12]

In recent years, scientists have become more interested in natural substances due to the potential side effects of synthetic substances. In relation to this, research is being conducted to find substances that have low toxicity and high physiological activity to the human body using naturally derived substances.

Therefore, in order to confirm the antioxidant activity, fulvic acid is used as a functional material by measuring the total content of DPPH Radical Scavenging Activity, SOD-like activity, total polyphenol, and total flavonoid content, and measuring the cytotoxicity and NO activity inhibiting capacity of RAW 264.7 cells.

Materials and Methods

DPPH Radical Scavenging Measurement

Hydrogen donation effect of Fulvic Acid on DPPH (1,1-diphenyl-2-picryl-hydrazyl) was measured the using DPPH Radical Scavanging method. Dilute fulvic acid by concentration to 0.01, 0.025, 0.05, 0.1 and 0.2%. Mix 180μL of a 10 mM DPPH solution with 20μL of fulvic acid in a 96 well plates for 30 minutes at 37°C and measure absorbance at 517 nm. Ascorbic acid was used as a standard substance.

Total Phenolic Compound Content Measurement

The total polyphenol content of fulvic acid was determined by the Folin-Denis method.[13] Dilute fulvic acid by concentration to 0.01, 0.025, 0.05, 0.1, 0.2%. After mixing 400μL of Fulvic Acid with 400μL of Folin-Denis reagent for 3 minutes, mix 400μL of 10% Na2CO3 in a dark room. After 60 minutes, 200μL of the superior liquid was taken on a 96 well plate and the absorbance at 760 nm was measured.

Total Flavonoid Content Measurement

The total flavonoid content of fulvic acid was measured using the Moreno method.[14] Following dilution to 0.01, 0.025, 0.05, 0.1, 0.2%, 100μL of fulvic acid, 20μL of 10% aluminum nitrate, 20μL of 1M potassium acetate, and 860μL of ethanol are mixed. After leaving it at room temperature for 40 minutes, the suspended matter was sunk by a centrifuge, and 200μL of each 96 well plate was scrambled to measure absorbance at 415 nm.

SOD-Like Activity Measurement

The SOD-like assessment demonstrated the automatic oxidation of pyrogallol, which catalyzes the reaction with hydrogen peroxide (H2O2), as SOD-like activity after deforming the Marklund method.[15] We diluted fulvic acid by concentration to 0.01, 0.025, 0.05, 0.1, 0.2%. Add Tris-H buffer (50 mM Tris-cacodylic acid buffer pH 8.20, 10 mM EDTA, pH 8.5) 3 mL and 7.2 mM pyrogallol l0.2 mL to react at 25°C for 10 minutes and add 1 mL of 1N HCL to complete the reaction. After that, the absorbance was measured at 420 nm using an ELISA reader.

Cell Culture

RAW 264.7 cells used in this experiment were obtained from Korea Cell Line Bank (KCLB) and added to high glucoseDulbecco's Modified Eagle's Medium (DMEM, Sigma, USA) with 10% fetal bovine serum (FBS, Sigma, USA).

Cytotoxicity Measurement

Add Raw 264.7 cells to a 96 well plate at a concentration of 3×104 cells well per well and incubate them in an incubator for 24 hours. After 24 hours, dilute the fulvic acid to concentrations of 0.1, 0.25, 0.5, 1, and 5% and incubate it in an incubator at 37°C and 5% CO2 for 48 hours. Replace the cultured cell culture solution with a serum-free medium containing 1% NR solution, incubate it for 3 hours, and check the crystallization of NR under a microscope. Add a 10% formaldehyde solution to phosphate-buffered saline (PBS) and deposit 100μL each well for 20 minutes. The NR desorb solution (50% distilled water, 49% ethanol, 1% glacial acid) was absorbed at 540 nm by micro plater after extracting 100μL of NR in each well.

Nitric Oxide (NO) Production Inhibitory Capacity Measurement

To measure the inhibition of NO production of fulvic

acid, the amount of NO in the cell culture solution was measured. After adding RAW 264.7 cells to 96 well plates at a concentration of 5×104 cell wells per well, incubate them in a 5% CO2 incubator at 37℃ for 24 hours. After removing the batch after 24 hours , add 0.1, 0.25, 0.5, 1, 5% fulvic acid to the medium treated with 1 microgram LPS (lipopolysaccharide) concentration and incubate it for 48 hours. After adding 100μL of Griess reagent and 100μL of cultured upper layer cell culture solution to a new 96 well plate and reacting it in a shaded state for 10 minutes, the absorbance was measured at 540 nm.

Statistical Processing

These experiments were performed in triplicate or more under the same conditions, and the statistical processing was analyzed using SPSS Window Version 17.0 (SPSS Inc., Illinois, USA).

Results and Discussion

DPPH Radical Scavenging Measurement

Free radicals are known to be a major cause of aging, and DPPH radicals are widely used to verify antioxidants by reducing them to antioxidants.[16] To check the antioxidant effect of fulvic acid, DPPH radical elimination activity is measured and shown in Fig. 1. As a result of this experiment, fulvic acid was used at a concentration of 0.01, 0.025, 0,05, 0.1, 0.2% DPPH radical scavenging activity was confirmed at a concentration-dependent on DPPH radical Scavenging. Ascorbic acid, known for its excellent antioxidant activity, was used as the positive comparator. When comparing the antioxidant activity of fulvic acid and ascorbic acid, the DPPH radical scabbing activity of the ascorbic acid extract was 54% and that of the ascorbic acid extract was 50.25%. Fulvic acid extract has been found to have a similar DPPH radical elimination ability to ascorbic acid. The organic substance fulvic acid is known to have an antioxidant effect as it is known to be removed after adsorption of toxic substances[17], and this study also confirmed the DPPH radiological elimination activity.

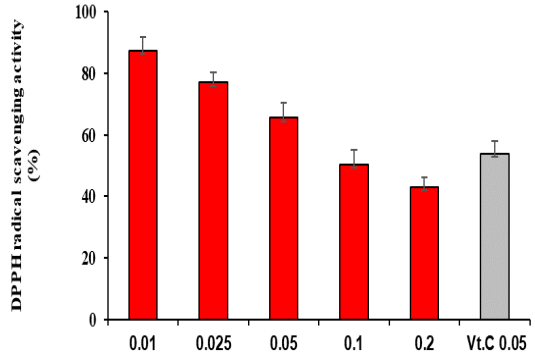

Figure 1: DPPH radical scavenging activity of Fulvic Acid

Concentration of Total Poly Phenol and Total Flavonoid

Total polyphenol and flavonoid content of fulvic acid were determined experimentally, and results are shown in Table 1. As a result of the measurement, the total polyphenol content was 29.24 mg/g and the total flavonoid content 30.39 mg/g from 2% of the concentration used in this experiment.

Table 1: Concentration of Total Poly Phenol and Total Flavonoid (mg/g)

Concentration(%)	Total Poly Phenol	Total Flavonoid
0.01	3.07	1.67
0.025	3.92	2.68
0.05	6.14	6.73
0.1	12.56	13.27
0.2	29.24	30.39

SOD-Like Activity Measurement

SOD is present in organs where life forms consume oxygen and acts to protect cells by catalyzing the reaction of un-equalizing Super - oxidation in the breathing process to O2 and H2O2. In order to check the activity of SOD, the SOD activity of fulvic acid was measured (Fig. 2). Fulvic acid was found to have greater SOD activity than control.. In particular 0.2% SOD activity was found to be 40% higher than control. SOD is an enzyme that participates in the elimination of superoxide in living organisms. It has been reported that active oxygen produced in the body causes oxidative damage to normal cells.[18] This study confirmed that fulvic acid has high antioxidant activity due to its excellent SOD-like activity.

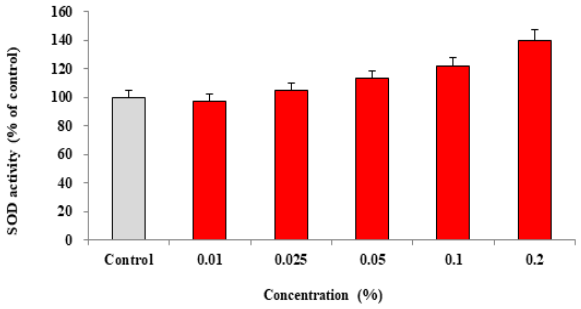

Figure 2: SOD-like activity of Fulvic Acid

Cytotoxicity Measurement

The fulvic acid was treated at a concentration of 0-5% and the cell survival rate due to cytotoxicity in RAW 264.7 cells was confirmed, and the experimentalresults are shown in Fig. 3. As a result of the experiment, no significant cytotoxicity was found in the extract of fulvic acid at 5% and a high cell survival rate of 95% or higher at all concentrations. Based on the results, it was confirmed that fulvic acid is a safe substance to use in the future because it does not show any toxicity to cells.

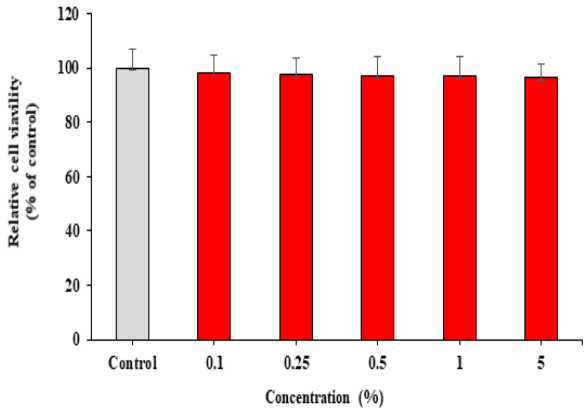

Figure 3: Cytotoxicity of Fulvic Acid

NO (Nitric oxide) Production Inhibitory Measurement

In order to check if fulvic acid affects NO production inhibition, which causes autoimmune and inflammatory diseases, LPS, an inflammatory mediator, was treated at concentrations of 0.1, 0.25, 0.5, 1.5%, and measured in the form of nitrite (NO2-) and nitrate (NO3-) in culture solution. After treating Raw 264.7 cells with LPS to significantly increase NO production by 40% or more, fulvic acid was added by concentration and measured, and the NO production inhibiting effect was found to be superior in all concentrations compared to LPS treatment groups. Especially, it was found that the NO production suppression effect was excellent at 51.3% at 5% concentration (Fig. 4).

According to the previous paper, NO production inhibitory activity in RAW 264.7 cells was reported to help regulate inflammatory response and immune function[19,20], and this study also confirmed that NO production inhibition by fulvic acid helps inflammatory response.

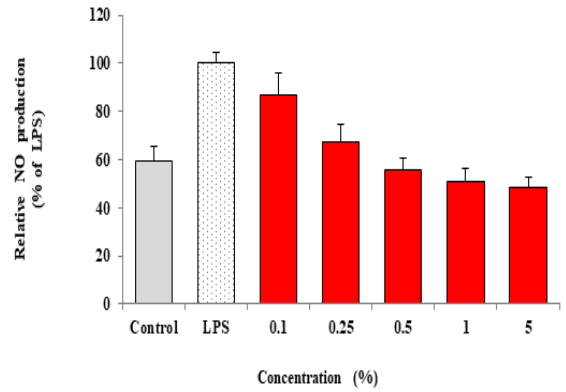

Figure 4: NO production inhibitory of Fulvic Acid

Conclusion

In order to confirm the feasibility of developing fulvic acid as a functional material, the cytotoxicity and anti-inflammatory effects on RAW264.7 cells were studied. Fulvic Acid has been found to have excellent concentration-dependent DPPH radical elimination action and thus significantly increased SOD activity associated with active oxygen removal. However, since the total polyphenol and flavonoid age content of fulvic acid is somewhat low, antioxidant activity is expected to occur by other mechanisms.

Safety against skin cells was confirmed by over 96% cell survival by RAW264.7 cells. In order to confirm the anti-inflammatory effect, inflammation is induced from RAW 264.7 cells by LPS and then the NO production inhibiting effect related to inflammation is confirmed, and excellent NO production from RAW 264.7 cells is suppressed from 5% to 51.3%.

Based on these results, fulvic acid does not exhibit toxicity to skin cells, so it is possible to use it as a functional material with excellent antioxidant and anti-inflammatory effect in the future.

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