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## The Effects of Aged Garlic Aqueous Extracts on Toxicity Caused by Hydroxyl Radical Species

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### Abstract

Previous study shows that aged garlic dried for 15 days and beyond exhibited the highest levels of total reducing power, total polyphenol content, and DPPH-scavenging ability. Here, the effects of a more diffusible radical species, hydroxyl radical ( $\cdot\text{OH}$ ), generated via a Fenton-type reaction were evaluated to examine its toxicity in the presence of aged garlic aqueous extract. In ribose degradation assay, the amount of ribose degraded by  $\cdot\text{OH}$  in the presence of aged garlic dried for 0-10 days was shown to decrease from 0- to 10-day drying, but slightly increase with garlic prepared by 20-day drying. In DNA integrity assay, the relaxation of supercoiled plasmid DNA, pGEM-7zf(-), due to DNA single-stranded breakage caused by  $\cdot\text{OH}$  was diminished in the presence of aged garlic aqueous extract with 20-day drying garlic reaching plateau, consistent with the radical scavenging effects observed previously. We also developed an assay to examine the  $\cdot\text{OH}$  toxicity on the integrity of plasmid DNA within *E. coli* since  $\cdot\text{OH}$  is a neutral species that can cross cell membranes without much difficulty. In the integrity assay of DNA transformed into *E. coli*, aged garlic aqueous extract of 20-day drying was shown to exhibit a slightly lower protecting ability against  $\cdot\text{OH}$ , compared to its wild garlic counterpart. Lastly, to resolve these conflicting results, an EPR spectrometer was used to examine the radical scavenging effects of both wild and aged garlic dried for 5 to 20 days. We found that wild garlic exhibited a much stronger effects on scavenging  $\cdot\text{OH}$ , whereas aged garlic lost much of this ability regardless the time of their fermentation.

Keyword: aged garlic; hydroxyl radical; 2'-deoxy-D-ribose; plasmid DNA; EPR; Fenton's reaction

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## Introduction

Fresh garlic extracted over a prolonged period (up to 20 months) produces odourless aged garlic extract containing stable and water soluble organosulphur compounds that prevent oxidative damage by scavenging free radicals, such as DPPH, a relatively stable radical species [1]. The levels of DPPH-scavenging activities, total reducing power, and total polyphenols monitored were all increased in a time-dependent manner for garlic aged during a drying process for different days, compared with their wild garlic counterpart prepared via lyophilization (unpublished data). However, the protective ability of garlic on genetic materials, such as DNA, against a far more reactive radical species, hydroxyl radical,  $\cdot\text{OH}$ , was never addressed.

Hydroxyl radical has been known to cause DNA strand cleavage by attack ribose moieties via abstraction of hydrogen atoms on the ribose sugar ring, depending on the accessibility of hydrogen atoms to the aqueous phase [2, 3]. Additionally, in spin-trapping experiments using spin trap, DMPO, wild-type garlic aqueous extract was shown to diminish the level of  $\cdot\text{OH}$  [4]. The polyphenol content were proportional to the DPPH scavenging ability, suggesting a radical scavenging ability exhibited by aged garlic was likely due an increased amount of polyphenol [1]. Nevertheless, the reactivity of DPPH is far weaker, compared to  $\cdot\text{OH}$ . Whether the increased total polyphenol content in aged garlic has any scavenging effect on  $\cdot\text{OH}$  remains to be elucidated.

Hydroxyl radical, a neutral species, is believed to cross the hydrophobic region of cell membrane phospholipid bilayers without much difficulty. In *E. coli*, transformed plasmid DNA damage as visualized by its relaxation caused by  $\cdot\text{OH}$  can therefore be used to evaluate the protective effects of aged garlic aqueous extracts or any other biochemical compounds on the protection of DNA within *E. coli* against  $\cdot\text{OH}$ .

In this study, we found that aged garlic dried for different days exhibited contradicting results by increasingly protecting supercoiled plasmid DNA and those transformed into *E. coli* against  $\cdot\text{OH}$ , while in ribose degradation assay garlic dried for 20 days was shown to increase the levels of degraded ribose. Finally, EPR spectroscopy was used to examine the  $\cdot\text{OH}$  scavenging ability of garlic aged for various days in experiments using spin trap, DMPO, to resolve these conflicting results.

## Material and Methods

## Chemicals and Solutions

All chemicals were reagent grade or better and used without further purification. Deionized (D.I.) water used to make solutions was redistilled by Waters water distillation system. Fresh garlic was purchased from a local food store in Taoyuan, Taiwan, and preserved immediately upon arrival by freezing at  $-20^{\circ}\text{C}$  until experiments. Ferrous ammonium sulfate 6-hydrate  $[\text{Fe}(\text{NH}_4)_2(\text{SO}_4)\cdot 6\text{H}_2\text{O}]$  was purchased from J.T. Baker (Phillipsburg, NJ). 2'-Deoxy-D-ribose, 2-thiobarbituric acid (TBA), and tris(hydroxymethyl)aminomethane (Trizma base) were purchased from Sigma-Aldrich (St. Louis, MO). Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) was purchased from T.K. Chemical Co. (Tainan, Taiwan). The reagent used to stain DNA was HealthView Nucleic Acid Stain purchased from Genomics Co. (New Taipei City, Taiwan).

All solutions were made in D.I. water or the solvent will be notified, otherwise. Stock solutions of ferrous ammonium sulfate (6 mM) and 2-deoxy-D-ribose (1 mM in 60 mM Tris-HCl at pH 7.0) were prepared, loaded in 1.5 ml Eppendorf tubes, and frozen at  $-20^{\circ}\text{C}$  until experiments. Due to their instability, garlic aqueous extract solution,  $\text{H}_2\text{O}_2$  (60 mM), and TBA (1% in 50 mM NaOH) were all freshly made prior to each experiment.

## Preparation of Garlic Aqueous Extract Solution

Garlic was either lyophilized and ground to powder, *i.e.*, 0-day drying, or heat-dried for 5 to 20 days to become aged garlic, followed by grinding to powder for long-term anaerobic storage. Prior to each experiment, garlic powder was dissolved in water to make a solution (10%, w/v), vigorously vortexed, and centrifuged at 15,000 *g* for 1 min to remove insoluble debris from the aqueous extract. Garlic solution was freshly made in each experiment and discarded afterwards.

## The Assay of 2'-Deoxy-D-ribose Degradation

The generation of  $\cdot\text{OH}$  radicals via a Fenton-type reaction ( $\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \cdot\text{OH}$ ) was quantified using 2'-deoxy-D-ribose oxidative degradation as described previously with minor modifications [5]. The principle of the assay is the quantification of the degraded 2'-deoxy-D-ribose product, malonaldehyde (MDA), by its condensation with TBA. In a typical experiment, 30  $\mu\text{l}$  2'-deoxy-D-ribose (1 mM in 60 mM Tris-HCl at pH 7.0) was first added to the bottom of a 1.5 ml Eppendorf tube. Then, solutions of  $\text{H}_2\text{O}_2$  (60 mM) and D.I. water (control) or garlic aqueous extract of 10  $\mu\text{l}$  each were added as two individual drops to the wall of the tube containing 2'-deoxy-D-ribose solution. Each reaction was started by mixing the two solution drops on the wall first with a pipette tip containing 10  $\mu\text{l}$   $\text{Fe}^{2+}$  (6 mM in 60 mM Tris-HCl at pH 7.0) and bringing the mixed solution all the way to the 2'-deoxy-D-ribose solution, followed by injecting  $\text{Fe}^{2+}$  solution to the reaction solution.

Therefore, the final volume of each reaction solution was 60  $\mu\text{l}$ , and the concentrations of 2'-deoxy-D-ribose,  $\text{H}_2\text{O}_2$ , and  $\text{Fe}^{2+}$  were 0.5, 10, and 1 mM, respectively. After 80 sec (1.33 min), the reaction was stopped by adding 4% phosphoric acid (v/v) of 60  $\mu\text{l}$ , followed by 1% TBA of 60  $\mu\text{l}$ . After boiling for 15 min, 60 mM Tris-HCl at pH 7.0 of 720  $\mu\text{l}$  was then added to each solution to make a final volume of 900  $\mu\text{l}$  prior to measurement of absorbance at 532 nm. Absorbance measurement of each reaction solution was achieved on a Perkin-Elmer spectrophotometer of type Lambda35 equipped with a quartz cuvette of 1 cm light path and conducted at room temperature. In order to remove the absorbance from garlic solution in each experiment, the absorbance from the garlic aqueous extract solution processed under the same conditions in the absence of 2'-deoxy-D-ribose was measured and subtracted from the total absorbance.

### **DNA Integrity Assay**

DNA integrity assay was performed using supercoiled plasmid DNA as the target molecule. Plasmid DNA, pGEM-7Zf (-), was transformed into *E. coli*, DH5 $\alpha$  strain, and grown in LB broth at 37°C for overnight. The culture was then harvested and DNA was purified using Plasmid Miniprep kit (BioKit, Miaoli, Taiwan). After purification, 6  $\mu\text{l}$  plasmid DNA dissolved in D.I. water was added as a drop of solution onto a piece of parafilm prior to the addition of other reagents. Then, 2  $\mu\text{l}$  D.I. water (or garlic aqueous solution, 10% w/v) and 2  $\mu\text{l}$  60 mM  $\text{H}_2\text{O}_2$  were added as two spots close to the plasmid DNA solution. Finally, a pipet tip holding 6 mM  $\text{Fe}^{2+}$  of 2  $\mu\text{l}$  in 60 mM Tris-HCl at pH7.0 was used to mix both water (or garlic aqueous extract) and  $\text{H}_2\text{O}_2$  together and move this mixed solution into the DNA solution, followed by injecting  $\text{Fe}^{2+}$  solution to start the reaction. After 80 sec, the reaction was quenched by adding 2  $\mu\text{l}$  loading dye (0.25% bromophenol blue and 40% sucrose), prior to electrophoresis in a 1.5% agarose gel. Plasmid DNA was visualized by internally staining the gel with HealthView Nucleic Acid Stain. Quantification of DNA band intensity was achieved via software, ImageJ, developed by National Institute of Health (Bethesda, MD).

### **Plasmid DNA Cleavage in *E. coli***

*E. coli* transformed with plasmid DNA, pGEM-7Zf (-), was grown overnight in LB broth at 37°C. Thirty  $\mu\text{l}$  culture of the *E. coli* was loaded in a 1.5 ml Eppendorf tube, followed by centrifugation at 6,000  $g$  for 2 min. The LB broth was removed by pipetting as much as possible. Six- $\mu\text{l}$  D.I. water was added to the pellet, followed by gently resuspending the cellular pellet in water. Two  $\mu\text{l}$  D.I. water (or garlic aqueous extract) and 2  $\mu\text{l}$  60 mM  $\text{H}_2\text{O}_2$  were added directly onto the inner wall of the Eppendorf tube as two separate spots. Then, a pipet tip holding 6 mM  $\text{Fe}^{2+}$  of 2  $\mu\text{l}$  was used to mix both water (or garlic aqueous extract) and  $\text{H}_2\text{O}_2$  together and move

this mixed solution into the resuspended *E. coli* cellular solution, followed by injecting  $\text{Fe}^{2+}$  solution to start the reaction. After 80 sec, 28  $\mu\text{l}$  loading dye described above was added to quench the reaction, prior to adding chloroform/phenol mixture (1:1, v/v) of 14  $\mu\text{l}$ . After vortexing, the reaction solution was centrifuged at 15,000  $g$  for 2 min and a layer of cell debris can be seen between the aqueous (upper and blue) phase and organic phase (bottom). Ten  $\mu\text{l}$  aliquot of the aqueous phase was carefully taken out without disturbing the cell debris and loaded into a well of a 1.2% agarose gel for analysis. DNA was visualized by staining the gel with HealthView Nucleic Acid Stain as described above.

### **EPR experiments**

Previously, our results show that DMPO can be oxidized to generate EPR-active species, DMPO-OH, under acidic conditions (pH 3.0-6.0) without involving  $\cdot\text{OH}$  (data not shown). The EPR experiments in this study were therefore carried out under slightly alkaline conditions (pH8.0) to avoid this situation. Stock DMPO solution of 200 mM was made by dissolving DMPO in Tris-HCl (100 mM at pH8.0), followed by washing with charcoal to remove impurities and filtering through an acrodisc of 0.45  $\mu\text{m}$ . In a typical EPR experiment, 45  $\mu\text{l}$  DMPO solution was first added onto a piece of parafilm. Fifteen  $\mu\text{l}$  D.I. water (or 10% garlic aqueous extract, w/v) and 15  $\mu\text{l}$   $\text{H}_2\text{O}_2$  were added as two drops right next to the DMPO solution. Then, a pipet tip holding 15  $\mu\text{l}$   $\text{Fe}^{2+}$  of 6 mM in 100 mM Tris-HCl at pH8.0 was used to mix water (or garlic aqueous extracts) and  $\text{H}_2\text{O}_2$  together and move the mixed solution to the DMPO solution, followed by adding  $\text{Fe}^{2+}$  solution. Then, the whole reaction solution was drawn into a capillary tube and the tube was sealed with Vaseline grease. The capillary tube was dropped into an EPR quartz tube. The reaction solution was allowed to continue for 8 min prior to EPR data collection. A Bruker EMX-10 X-band EPR spectrometer was used for measurement of DMPO-OH at room temperature. The parameters used to collect EPR spectra are listed as follows. Instrumental conditions were: microwave frequency, 9.765 GHz; microwave power, 19.97 mW; modulation amplitude, 1 G; scan time, 168 sec; time constant, 20.48 msec and scan range, 100 G. The EPR measurements for DMPO alone were performed before and after the whole experiment to ensure that EPR-active species due to oxidation of DMPO was not interfering the experiments and the results showed that no such detectable EPR signals due to these species were observed.

### **Statistical Analysis**

A one-way analysis of variance (ANOVA) was performed when comparing significant differences in more than three groups. When statistically significant differences were indicated, unpaired Student's *t*-test was used. The results were expressed as mean  $\pm$

standard deviation (SD). The level set for statistical significance was  $p < 0.05$ .

## Results

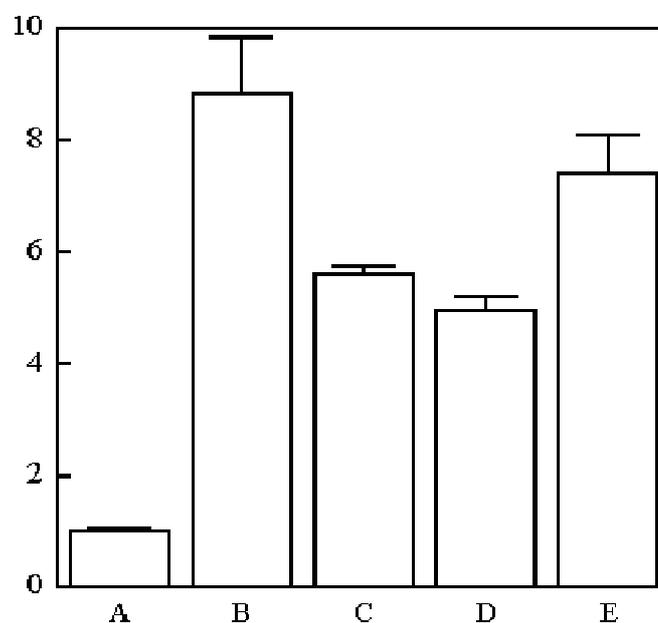
### 2'-Dexoy-D-ribose degradation

In order to examine the effects of aged garlic aqueous extract on 2'-dexoy-D-ribose degradation caused by  $\cdot\text{OH}$  generated via a Fenton-type reaction in the absence or presence of aqueous extract of garlic, the reactive fragmented ribose product, malondialdehyde (MDA), and thiobarbituric acid (TBA) adduct, MDA-TBA (or TBARS) was spectrophotometrically determined at 532 nm. As shown in Figure 1, lyophilized wild garlic (0-day drying) aqueous extract exhibited the highest absorbance at 532 nm, suggesting that the ingredients within wild garlic enhanced the generation of  $\cdot\text{OH}$  in a Fenton's reaction by reducing  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$ . The levels of TBARS absorbance were shown to slightly decrease in the aqueous extracts of garlic dried from 5 to 20 days. For 20-day dried garlic, the TBARS level was the highest among all the aged garlic examined herein, albeit to a less extent when compared with that in the presence of lyophilized wild garlic. The results presented here suggest that garlic aqueous extracts, whether wild or heat-dried, lack the ability to scavenge  $\cdot\text{OH}$  as expected, but instead enhance the generation of  $\cdot\text{OH}$  presumably by reducing  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$ , as shown previously in the presence of ascorbate [6]. Additionally, although the reducing power of garlic was slightly decreased during an early period of drying (5- to 10-day drying), it was significantly increased in a longer period of drying, consistent with previous findings, *i.e.*, the total reducing power of aged garlic was increased during the drying process.

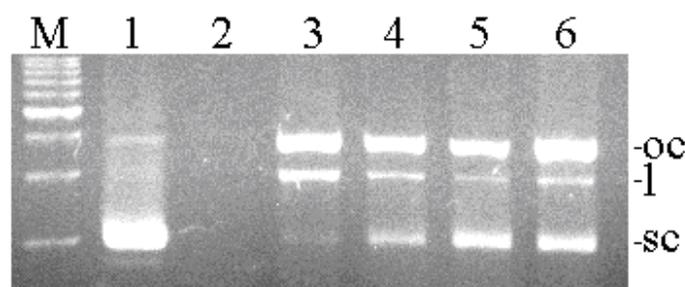
### DNA integrity assay

The mobility of supercoiled plasmid DNA can be retarded and visualized on agarose gel electrophoresis after being relaxed into open circular or linear form by single-stranded breakage caused by  $\cdot\text{OH}$ . Therefore, the DNA damage due to hydrogen abstraction caused by  $\cdot\text{OH}$  can be used to evaluate the effects of ingredients of garlic on genetic materials, such as DNA. As shown in Figure 2 along with DNA band intensity expressed as peak areas and listed in Table 1, in lane 2, plasmid DNA, pGEM-7zf (-), was completely digested by  $\cdot\text{OH}$  generated via a Fenton's reaction. However, in the presence of white garlic aqueous extract (lane 3), open circular form and, to a less extent, linear form of plasmid DNA was noticed, suggesting a radical scavenging ability existing in the wild garlic aqueous extract. Moreover, in the presence of garlic aged for 5-20 days, the levels of supercoiled DNA were significantly increased (lane 4-6), with the 20-day dried black garlic showing

the highest radical scavenging ability in terms of the level of supercoiled plasmid DNA, followed by 5- to 10-day dried garlic aqueous extracts. This is basically in agreement with previous findings that black garlic aged for 15 days and beyond exhibited the highest levels of scavenging power against radical species, DPPH.



**Fig 1. 2'-Dexoy-D-ribose degradation.** Ribose degradation denoted as absorption of MDA-TBA at 532 nm by hydroxyl radical generated via a Fenton-type reaction in the presence of (A) D.I. H<sub>2</sub>O, aqueous extracts of garlic dried for (B) 0-day (via lyophilization), (C) 5 days, (D) 10 days, and (E) 20 days. Data in bars were represented by mean  $\pm$  standard deviations, where  $n = 3$ . Notice that values in the y-axis are  $A_{532}$  of each data set normalized according to that of the experiment conducted using D.I. water as a control.



**Fig 2. 2'-Dexoy-D-ribose degradation.** DNA integrity assay for wild and aged garlic aqueous extracts. Lane M, DNA marker in kb. Lane 1, plasmid DNA, pGEM-7zf(-), alone. Lane 2, plasmid DNA + Fenton's reaction. Lane 3, plasmid DNA + Fenton's reaction in the presence of aqueous extract of lyophilized wild garlic. Lane 4-6, the same as lane 3, except in the presence of aqueous extract of aged garlic dried for 5, 10, and 20 days, respectively. The notions, -oc, -l, and -sc, stand for open-circular, linear, and supercoiled plasmid DNA, respectively.

**Table I.** Band intensity of plasmid DNA in integrity assay for wild and aged garlic aqueous extracts. Lanes 1-6 are referred to those described in Figure 2. Individual band intensity are numerically expressed as peak areas and calculated by software ImageJ.

	1	2	3	4	5	6
-oc	113.82	11.278	580.30	468.99	443.15	639.50
-l	100.47	4.6520	300.65	96.963	66.232	154.89
-sc	1077.4	3.5420	26.073	125.72	423.26	408.20

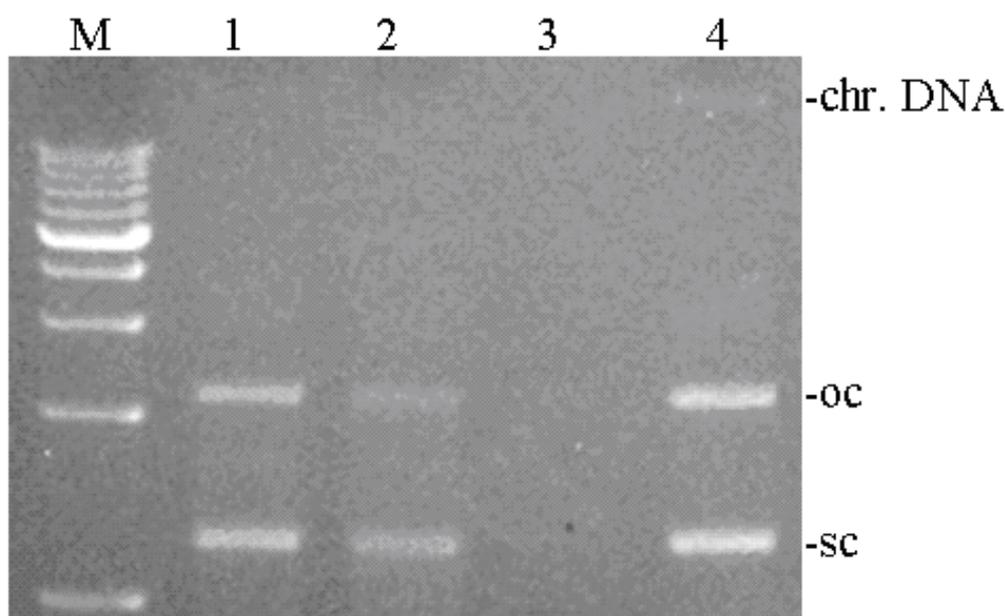
### Integrity assay of transformed DNA in *E. coli*

The DNA integrity assay carried out in this study was merely on the in vitro measurement of how natural ingredients, *e.g.*, garlic aqueous extract, protect genetic materials, DNA, without regard to the fact that cellular organelles are highly compartmentalized. Therefore, a new protocol concerning the integrity of DNA transformed into *E. coli*, similar to the enclosed genetic materials, DNA, within cell nuclei, was developed in this study. As shown in Figure 3, the levels of supercoiled plasmid DNA in *E. coli* was completely destroyed in a Fenton's reaction (lane 3), suggesting that  $\cdot\text{OH}$ , a neutral species, was able to cross the hydrophobic region of cell membranes of *E. coli* and cleave plasmid DNA transformed inside. In lane 1 of Figure 3, the level of supercoiled plasmid DNA was significantly increased in the presence of wild garlic aqueous extract, while the level of supercoiled plasmid DNA in *E. coli* was relatively lower in the presence of aged garlic dried for 20 days (lane 2), suggesting that the radical scavenging ability of aged garlic was decreased during the drying process, compared to that of wild garlic. The levels of supercoiled plasmid DNA transformed into *E. coli* and subjected to a Fenton-type reaction were all similar in the reaction in the presence of aged garlic dried for 5-20 days (data not shown).

### EPR experiments

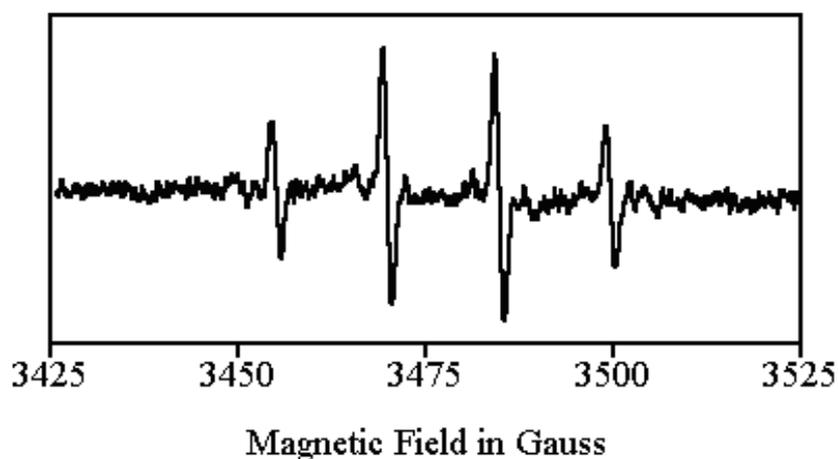
In this study, two contradictory results were obtained. That is, in plasmid DNA integrity assay, aqueous extract of aged garlic dried for different days were shown to increase the levels of supercoiled plasmid DNA, whereas in integrity assay of DNA transformed into *E. coli*, aged garlic showed a slightly lower protecting ability against  $\cdot\text{OH}$ , compared to wild garlic aqueous extract. In order to resolve the conflict in the observations between these two assays, a more direct measurement of  $\cdot\text{OH}$  using EPR and spin trap, DMPO, was used to examine the radical scavenging ability of the aqueous extracts of these two types of garlic, *i.e.*, wild and aged garlic. In the absence of any ingredients,  $\cdot\text{OH}$  generated in a Fenton-type reaction, reacted with

spin trap, DMPO, forming an EPR-active species, DMPO-OH, that showed typical signal of peak area ratios of 1:2:2:1 [7], whereas in the presence of wild garlic aqueous extract, the DMPO-OH EPR signal was significantly and almost completely diminished, implying that the ingredients in wild garlic aqueous extract possessed the ability to scavenge  $\cdot\text{OH}$  and accordingly decrease the concentration of DMPO-OH, as reported previously [4]. Nevertheless, as shown in Figure 4, in the presence of aqueous extract of aged garlic dried for 20 days, the concentration of DMPO-OH remained basically the same level as that in a traditional Fenton's reaction, suggesting aged garlic in this study lost the  $\cdot\text{OH}$  scavenging ability during the drying process. In addition, the DMPO-OH EPR signals were shown to be of approximately the same intensity in the reaction solutions in the presence of aqueous extracts of garlic dried for 5- and 10-day drying processes (data not shown). Therefore, the EPR experiments conducted in this study explicitly showed that during the fermentation and drying processes aged garlic lost its ability to scavenge hydroxyl radical,  $\cdot\text{OH}$ .



**Fig 3. The effect of 20-day aged garlic on the integrity of plasmid DNA transformed into *E. coli*.**

Lane M, DNA marker in kb. Lane 1, plasmid DNA, pGEM-7zf (-), + Fenton's reaction in the presence of aqueous extract of lyophilized wild garlic. Lane 2, the same as lane 1, except in the presence of aqueous extract of garlic aged for 20 days. Lane 3, plasmid DNA + Fenton's reaction only. Lane 4, plasmid DNA, pGEM-7zf(-), alone. The notions, -chr, -oc, and -sc, stand for chromosomal DNA, open-circular DNA, and supercoiled plasmid DNA, respectively.



**Fig 4. Fenton's reaction** DMPO-OH EPR spectra of Fenton's reaction in the presence of aqueous extract of black garlic dried for 20 days.

## Discussion

In this study, our results show that in ribose degradation both wild and aged garlic enhanced the levels of degraded ribose, while in DNA integrity assay *in vitro* aged garlic extracts exhibited stronger  $\cdot\text{OH}$ -scavenging ability, compared to their wild-type garlic counterpart. In DNA integrity assay within *E. coli*, the aqueous extract of wild-type garlic protects transformed plasmid DNA against  $\cdot\text{OH}$  slightly better than that of the 20-day incubated garlic. Finally, EPR experiments explicitly show that the EPR-active adduct, DMPO-OH, was diminished in the presence of lyophilized wild garlic aqueous extract, whereas 20-day aged garlic one essentially has no effect concerning the generation of  $\cdot\text{OH}$  via a Fenton's reaction.

In ribose degradation experiments, all types of garlic aqueous extracts were shown to increase the levels of degraded ribose with the wild one being the most powerful one. The increased levels of ribose degradation caused by garlic aqueous extracts examined herein are most likely due to their reducing power in garlic by reducing  $\text{Fe}^{3+}$  back to  $\text{Fe}^{2+}$  in a Fenton's reaction and therefore enhancing the generation of  $\cdot\text{OH}$ . Ribose degradation was shown to increase in the aqueous extracts of both types of garlic, while, in gel electrophoreses using supercoiled plasmid DNA, DNA relaxation due to  $\cdot\text{OH}$  was clearly diminished in wild and, particularly, aged garlic fermented in a time-dependent manner. The contradictory findings between ribose degradation and DNA relaxation assays can be explained by the fact that the hydrogen atoms on the five carbon atoms (C5' ~ C1'), H-5' ~ H-1', respectively, of the 2'-deoxy-D-ribose sugar ring are accessible to abstraction by  $\cdot\text{OH}$ , a neutral,

non-discriminating and diffusible radical species leading to DNA degradation in ribose degradation. In DNA relaxation experiments, however, duplex DNA, such as the plasmid DNA used in the DNA integrity assay, the preference for individual hydrogen atoms of ribose moieties accessible to abstraction by  $\cdot\text{OH}$  is seen to be in the order: H-5' > H-4' > H-2'  $\approx$  H-3' > H-1', and this order of reactivity correlates well with the solvent accessibility of the individual hydrogen atoms in the sequence studied [3,8]. Therefore, in DNA integrity assay, the number of hydrogen atoms on each DNA sugar ring accessible to  $\cdot\text{OH}$  is indeed very limited and  $\cdot\text{OH}$  is instead presumably scavenged by the ingredients of the aqueous extracts of garlic powders examined here. In addition, the fact that  $\cdot\text{OH}$  is mainly scavenged by aqueous extracts of aged garlic in DNA integrity assay is conceivably due to the higher reducing power of aged garlic compared to that of its wild counterpart.

As for DNA integrity assay within *E. coli*, the wild garlic aqueous extract was slightly more efficient compared to the aged one. This suggests that some ingredients in the wild garlic are preserved that can cross cell membranes with less difficulty and they are most likely lost during the aging process. Likewise, EPR results clearly suggest that in terms of  $\cdot\text{OH}$ -trapping efficiency, wild garlic aqueous extract is much better compared to its aged counterpart. Ajoene (*E,Z*-4,5,9-trithiadodeca-1,6,11-triene-9-oxide), is an oil-soluble product of the rearrangement of allicin, a major component of raw garlic [9]. EPR studies of ajoene on the physico-chemical properties of the platelet plasma and artificial lipid membranes show that ajoene increase mobility of the fatty acid spin label 16-doxyyl stearate, suggesting the existence of a decreased microviscosity of the most internal region within the lipid bilayer membrane without affecting the outer hydrophilic moieties of the bilayer. Besides its lipophilic character, ajoene contains three electron-rich double bonds in addition to a sulfinyl group, S=O. Therefore, ajoene conceivably reacts with  $\cdot\text{OH}$  within the interior of *E. coli* membrane bilayers, and in turn minimizes the intracellular toxicity caused by  $\cdot\text{OH}$ . Whether or not ajoene is degraded during the aging process of aged garlic remains to be explored.

In conclusions, our results presented here show that both wild and aged garlic aqueous extracts exhibit distinct reducing activity against  $\cdot\text{OH}$ . It appears that the Maillard reaction during an aging process degrades some ingredients that exist in wild garlic albeit the anti-oxidative activity was shown to increase in aged garlic. This study can be useful for future studies when studying the effects of species derived from *allium* by taking into account of both wild and aged products.

## Acknowledgements

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# 黑大蒜水萃出物對羥基自由基引起毒害的效應影響

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## 中文摘要

先前研究顯示，野生白大蒜烘培處理 15 天或更久之後，其總還原力、總多酚含量、DPPH 清除能力較白大蒜為高。於本研究中，我們針對不同製備天數(0-20 天)黑大蒜水溶萃取液對清除更為活躍之氫氧自由基( $\cdot\text{OH}$ )之活性作檢測。在核糖降解實驗中，所有大蒜水溶液皆造成核糖降解，而非清除 $\cdot\text{OH}$ 。降解程度以白大蒜最高，5-10 天烘培黑大蒜逐漸遞減。之後，20 天烘培黑大蒜使核糖降解程度略為回升。於 DNA 完整測試， $\cdot\text{OH}$  會造成超螺旋 DNA，pGEM-7zf(-)，之瓦解，白大蒜使完整 DNA 比例略為回升，黑大蒜則有更強 $\cdot\text{OH}$  清除能力，且其隨烘培天數增加而上升。於細胞內 DNA 完整測試，20 天烘培黑大蒜清除能力則較白大蒜略低。電子順磁共振光譜儀實驗顯示，黑大蒜萃取液對於 $\cdot\text{OH}$  自由基產物，DMPO-OH，降低效果遠不及白大蒜萃取液。本研究顯示，大蒜烘培後，抗氧化活性雖有所增加，然而於此梅納反應過程中，若干活性成分亦有所轉變。

關鍵字：黑大蒜、羥基自由基、去氧核糖、質體 DNA、電子順磁共振光譜儀、芬騰反應

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