

# Antioxidative and Antigenotoxic Effects of Garlic (*Allium sativum* L.) Prepared by Different Processing Methods

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Published online: 27 August 2009  
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**Abstract** This study describes the antioxidant activities and antigenotoxic effects of garlic extracts prepared by different processing methods. Aged-garlic extract (AGE) showed a significantly higher total phenolic content ( $562.6 \pm 1.92$  mg/100 g garlic acid equivalents) than those of raw garlic extract (RGE) or heated garlic extract (HGE). The  $SC_{50}$  for DPPH RSA in HGE was significantly the highest at 2.1 mg/ml. The  $SC_{50}$  for SOD-like activity in garlic extracts was, in decreasing order, RGE (7.3 mg/ml) > AGE (8.5 mg/ml) > HGE (9.2 mg/ml). The  $ED_{50}$  of AGE was the highest (19.3  $\mu$ g/ml) regarding  $H_2O_2$  induced DNA damage and its inhibition rate was 70.8%. The  $ED_{50}$  of RGE for 4-hydroxynonenal (a lipid peroxidation product) induced DNA damage was 38.6  $\mu$ g/ml, followed by AGE > HGE. Although the heat treatment of garlic tended to decrease the TPC and SOD-like activity and increased DPPH RSA, garlic, in general, has significant antioxidant activity and protective effects against oxidative DNA damage regardless of processing method.

**Keywords** Aged-garlic · Total polyphenol · DPPH · SOD-like activity · Antigenotoxic effect

## Abbreviations

RGE Raw garlic extract  
HGE Heated garlic extract  
AGE Aged garlic extract

$ED_{50}$  The estimated dose for 50% reduction in oxidative DNA damage  
 $SC_{50}$  The concentration required for scavenging 50% of activity  
DPPH 2,2-Diphenyl-1-picrylhydrazyl  
DMSO Dimethyl sulfoxide  
TPC Total phenolic contents  
RSA Radical scavenging activity  
SOD Superoxide dismutase  
FCR Folin–Ciocalteu reagent  
HNE 4-hydroxynonenal

## Introduction

Epidemiological studies have associated the consumption of high amounts of garlic with substantial reductions in cancer [1]. Garlic (*Allium sativum* L.) is one of the world's oldest medicines and has been employed not only for flavouring but also as a medical herb due to its diverse biological activities, including antiatherosclerotic, anticarcinogenic and antioxidant effects [2–4]. Garlic is composed mainly of fructose-containing carbohydrates and sulfur compounds.

Excess production of oxygen radical species such as hydrogen peroxide, superoxide anion radical, and the hydroxyl radical are thought to cause damage in cells [5]. The oxidative damage to cells is one of several factors causing many diseases, including atherosclerosis, diabetes, and cancer [6]. Many researchers have focused on antioxidant defense, in particular the prevention of oxidative stress by dietary antioxidants [7–9]. Dietary foods contain a wide variety of free radical scavenging antioxidants, such as flavonoids, vitamin C, and so on [10].

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Studies show that fruits and vegetables tend to lose certain aspects of their bioactivities when stored at high temperatures [11], or cooked [12]. AGE made by an aging process is commonly used as a functional product in Korea. AGE and its constituents have been shown to prevent oxidative injury in endothelial cells [13] and suppress cancer growth [14].

We investigated the antioxidant and antigenotoxic effects of AGE compared with RGE and HGE made by different processing methods. The antioxidant activities of garlic extracts were evaluated with regard to TPC, DPPH RSA and SOD-like activity. The antigenotoxic effects of AGE were assessed using the comet assay.

## Materials and Methods

### Material

All garlic (*Allium sativum* L.) was collected in 2007 at rural district of Namhae, Korea. Aged garlic was supplied by Doul farm (Namhae, Korea) and was made by drying with soaked raw garlic in water for 330–360 h at 68–72 °C. DPPH, DMSO and Folin–Ciocalteu reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hydrogen peroxide, sodium chloride, sodium hydroxide, potassium chloride, and potassium phosphate were also purchased from Sigma Chemical Co. (St. Louis, MO).

### Preparation of Extracts from Garlic

Raw garlic was freeze-dried after steam blanching for preventing oxidation, then ground. The heated garlic powder was prepared by hot-air dry oven with freeze-dried garlic powder. Each 5 g of garlic was extracted with 0.1 L of methanol for 3 days at room temperature and filtered through a Whatman No. 1 filter paper (Tokyo, Japan). Solvents were then removed by evaporation to obtain the extracts. The extracts were then dissolved in DMSO at a concentration of 50 mg/ml, and diluted with DMSO when needed.

### Measurement of Total Phenolic Content

Each garlic extract was mixed with 2 ml of 1 N Folin–Ciocalteu reagent and left at room temperature for 3 min. The mixture was kept at room temperature for 1 h after the addition of 2 ml of 10% Na<sub>2</sub>CO<sub>3</sub>. The absorbance was measured by ELISA (Shimadzu UV-1601, Tokyo, Japan) at 700 nm. Total phenolic contents were expressed as garlic acid equivalents.

### Measurement of DPPH Radical Scavenging Activity

Eighty µl of 0.2 mM DPPH ethanol solution was added to 20 µl of sample solution at different concentrations, and allowed to react at room temperature. The control consisted of 20 µl of DMSO and 80 µl of 0.2 mM DPPH. The mixtures were kept at room temperature for 10 min and then the absorbance was measured at 492 nm. The ability to scavenge the DPPH radical was calculated as percent RSA using the following equation:  $RSA(\%) = (1 - A/B \times 100)$ . Where A was the absorbance of sample, and B was the absorbance of the control.

### Measurement of SOD-like Activity

The reaction mixture contained 50 µl Tris-HCl buffer and 7.2 mM pyrogallol, with or without sample. After incubation at 25 °C for 45 min, the absorbance at 405 nm was determined against the blank. Superoxide dismutase (SOD)-like activity was calculated using the following equation:  $SOD - \text{like activity}(\%) = (1 - A/B) \times 100$ . Where A was the absorbance of sample and, B was the absorbance of the control.

### DNA Damage Determination by Alkaline Comet Assay

Leukocytes were isolated in a fraction of mononuclear cells (containing lymphocytes and monocytes) from anonymous buffy coat preserves by gradient centrifugation with HISTOPAQUE®-1077 (Sigma, Deisenhofen, Germany). Leukocytes were incubated with methanol extracts of garlic dissolved in DMSO and diluted to concentrations 1, 5, 10, and 50 µg/ml for 30 min at 37 °C in a dark incubator. For oxidative stimulus they were then resuspended in PBS with 200 µM H<sub>2</sub>O<sub>2</sub> for 5 min on ice and 200 µM HNE for 30 min at 37 °C, respectively. After each treatment, samples were centrifuged at 250×g for 5 min and washed with PBS. 1% DMSO without oxidative stimulus was used as the negative control. The leukocytes were then mixed with 75 µl of 0.7% low melting agarose, and added to the slides precoated with 0.5% agarose. The slide was then immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, and 1% sodium laurylsarcosine; 1% Triton X-100 and 10% DMSO) for 1 h at 4 °C. The slides were next placed into an electrophoresis tank containing 300 mM NaOH and 10 mM Na<sub>2</sub>EDTA (pH 13.0) for 40 min. For electrophoresis of the DNA, an electric current of 25 V/300±3 mA was applied for 20 min at 4 °C. The slides were washed three times with a neutralizing buffer (0.4 M Tris, pH 7.5) for 5 min at 4 °C, and then treated with ethanol for another 5 min before staining with 50 µl of ethidium bromide (20 µg/ml). Measurements were made by image analysis (Kinetic

**Table 1** Total phenolic contents (TPC), DPPH radical scavenging activity (RSA), SOD-like activity of garlic extracts prepared by various processing methods

		RGE	HGE	AGE
TPC	mg/100 g GAE <sup>1)</sup>	173.4±6.92 <sup>b</sup>	75.1±3.98 <sup>c</sup>	562.6±1.92 <sup>a</sup>
DPPH RSA	SC <sub>50</sub> mg/ml	2.6±0.33 <sup>a</sup>	2.1±0.03 <sup>b</sup>	2.7±0.23 <sup>a</sup>
SOD-like activity	SC <sub>50</sub> mg/ml	7.3±1.31 <sup>ns3)</sup>	9.2±1.64	8.5±1.02

Data are mean ± standard deviation values ( $n=3$ )

Significant differences at  $P<0.05$  are indicated by different superscript letters within a row

<sup>1)</sup> GAE garlic acid equivalents

<sup>2)</sup> SC<sub>50</sub> (mg/ml): Concentration in µg/ml required to scavenge 50% of the radical

<sup>3)</sup> NS Not significant

Imaging, Komet 4.0, U.K) and fluorescence microscopy (LEICA DMLB, Germany), determining the percentage of fluorescence in the tail (tail intensity, TI; 50 cells from each of two replicate slides).

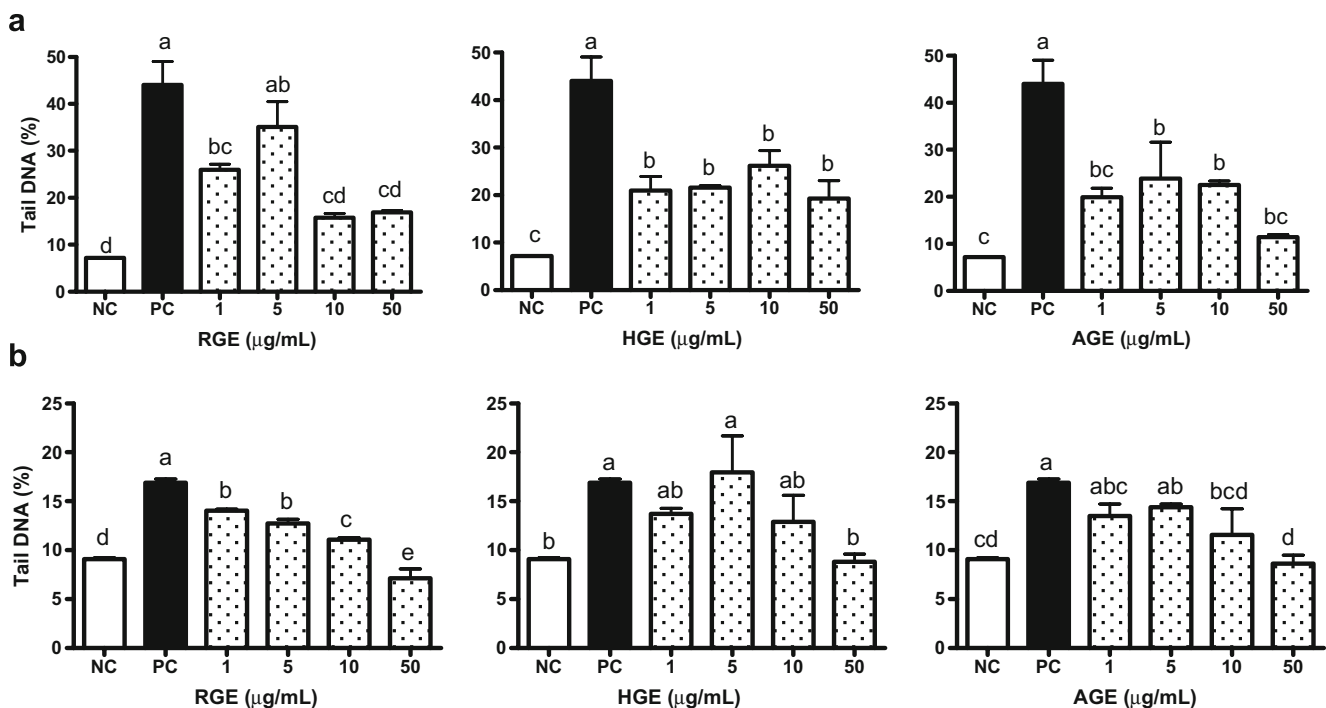
### Statistical Analysis

All measurements were analyzed using the SPSS package for Windows (Ver. 12). The mean values were compared using one-way analysis of variance followed by Duncan's multiple range tests.  $P$ -values of less than 0.05 were considered significant.

## Results and Discussion

### Total Phenolic Contents of Garlic Extracts

Phenolic compounds react with Folin–Ciocalteu reagent (FCR) only under basic conditions (adjusted by aqueous sodium carbonate 5–10%). Dissociation of a phenolic proton in basic medium leads to a phenolate anion, which is capable of reducing FCR in which the molybdate in the testing system is reduced forming blue coloured molybdenum oxide with maximum absorption near 700 nm. The intensity of blue colouration produced is proportional to the



**Fig. 1** The effect of supplementation *in vitro* with different concentrations of garlic extracts prepared by various processing methods on 200 µM H<sub>2</sub>O<sub>2</sub> (a) and HNE (b) induced DNA damage in human

leukocytes. NC, 1% DMSO (without oxidative stimulus) treated negative control; PC, 200 µM H<sub>2</sub>O<sub>2</sub> or HNE treated positive control

total quantity of phenolic compounds present in the sample [15]. AGE showed the significantly highest total phenolic contents ( $562.6 \pm 1.93$  mg/100 g GAE) among the samples tested (RGE:  $173.4 \pm 6.92/100$  g GAE; HE:  $75.1 \pm 3.98/100$  g GAE,  $P < 0.05$ ). According to Choi et al. [16], the total phenolic content of aged-garlic was higher than in fresh and steamed garlic. Aged-garlic is made by aging for 330–360 h at 68–72 °C. The mild-process aging gently modifies harsh and irritating compounds from the raw garlic and naturally generates unique and beneficial compounds through both enzymatic and natural chemical reactions [17]. Kwon et al. [18] reported that various compounds of garlic prepared at high temperature and pressure might be changed to polyphenol compounds. However, HGE made by heat treatment showed a significant decrease of total phenolic contents. The major phenolic loss that typically occurs during processing is brought about by the action of oxidative enzymes such as polyphenoloxidases and peroxidases [19]. These results indicate that grinding the heated garlic may reduce the amount of phenolics by enzymatic oxidation.

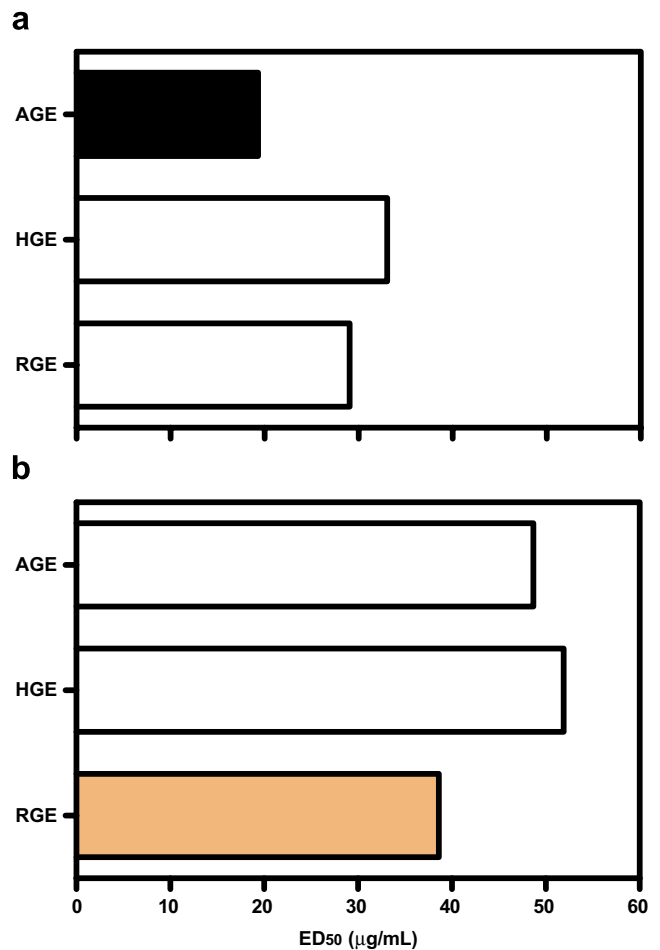
#### Antioxidant Activity of Garlic Extracts

The measurement of radical scavenging activity of any antioxidant is commonly determined by the DPPH method since it is a quick, reliable and reproducible method to assess the *in vitro* antioxidant activity of pure compounds as well as plant extracts [20]. The effect of antioxidants on DPPH is based on their ability to donate a hydrogen atom to DPPH, thus converting the radical into a stable molecule [21]. Scavenging of superoxide anion radicals by antioxidants is of importance for protection against early events in oxidative damage [22]. Garlic extracts showed a highly significant scavenging activities by reducing the stable radical DPPH<sup>•</sup> to yellow-colored diphenyl picrylhydrazine (Table 1). It could be explained by their scavenging ability to donate a hydrogen atom from their phenolic hydroxyl groups [23]. The significant correlation between the phenolic content and the antioxidant activity of various vegetable extracts has been previously reported [24]. However, we could not find correlation between radical scavenge activity and TPC. Although AGE might be expected to have more effective DPPH RSA and SOD-like activity than other garlic extracts due to its high TPC, HGE (2.1 mg/ml) actually had the highest DPPH RSA. RGE (7.3 mg/ml) had the highest SOD-like activity followed by AGE > HGE. This can explain that the processing method of garlic may influence its antioxidant activities. Other researchers have found significant increases in the antioxidant activity of grape seeds [25] and citrus peel [26] after heating, but showing that extremely high temperatures can cause an inverse effect,

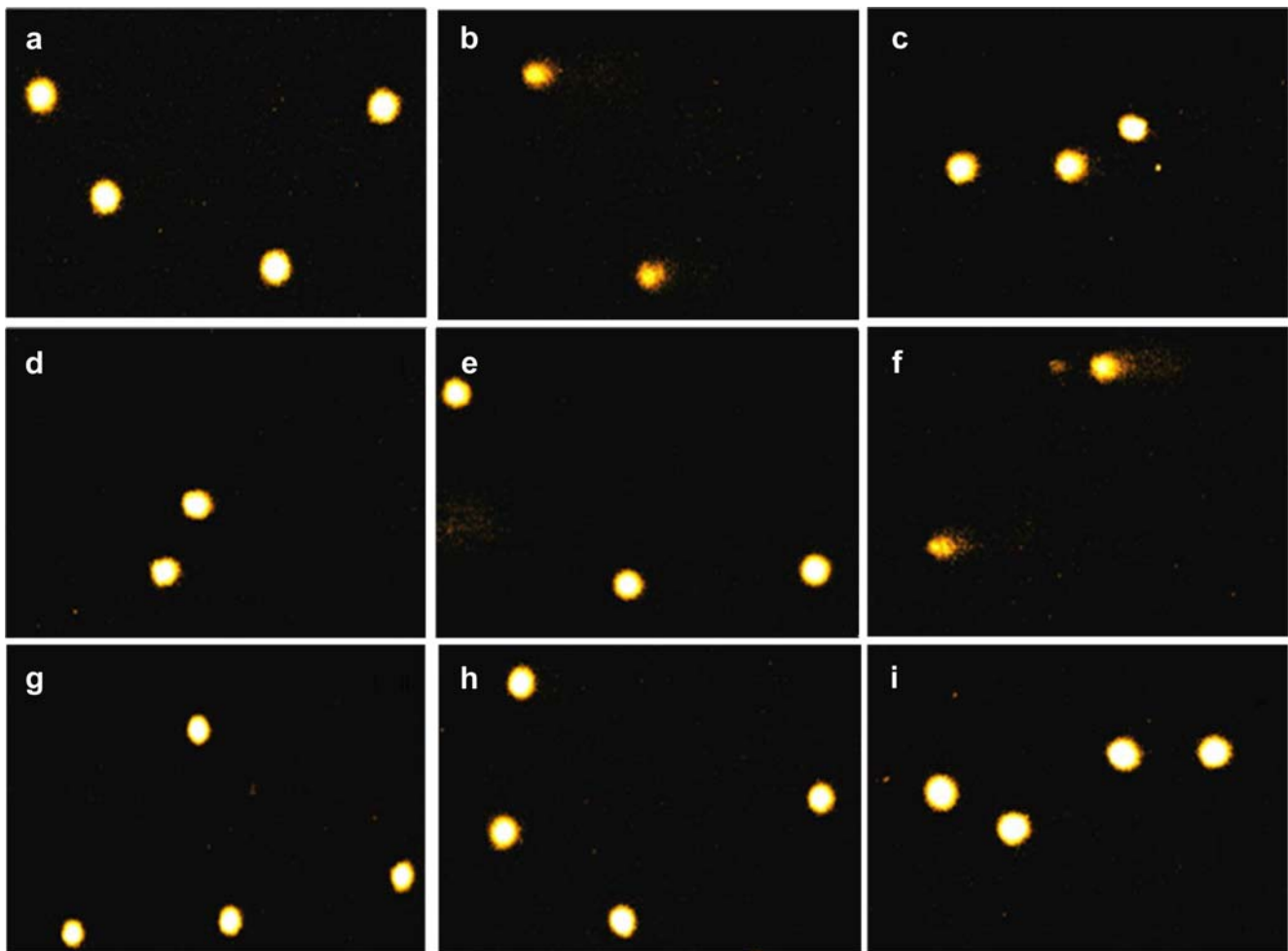
that is to say, a decrease in the antioxidant activity [25]. Gorinstein et al. [27] reported that the change of the bioactive compounds in vegetables after various heat treatments can be explained by their physical properties (texture, color, matrix, softening). We can recognize from these results that in the functional evaluation of food were examined one or more bioactive compounds and biological activities. Therefore, this suggests that the high antioxidant activities of RGE and HGE might be due to antioxidant compounds other than the total phenolic contents.

#### Protective Effects of Garlic on Oxidative DNA Damage in Human Leukocytes

H<sub>2</sub>O<sub>2</sub> is one of the principle reactive products of oxygen metabolism. Although H<sub>2</sub>O<sub>2</sub> produced in the normal metabolism of aerobic organisms is a relatively unreactive species, it is the primary precursor for the generation of



**Fig. 2** Comparison of the antigenotoxic activities of garlic extracts in human leukocytes by comet assay assessed by the estimated dose that would result in a 50% reduction in oxidative DNA damage (ED<sub>50</sub>) from 200 µM H<sub>2</sub>O<sub>2</sub> (a) and HNE (b)



**Fig. 3** Micrographs representing images obtained from the comet assay. **a**, Negative control; **b**, 200  $\mu\text{M}$   $\text{H}_2\text{O}_2$  treated positive control; **c**, RGE + 200  $\mu\text{M}$   $\text{H}_2\text{O}_2$ ; **d**, HGE + 200  $\mu\text{M}$   $\text{H}_2\text{O}_2$ ; **e**, AGE + 200  $\mu\text{M}$

$\text{H}_2\text{O}_2$ ; **f**, 200  $\mu\text{M}$  HNE treated positive control; **g**, RGE + 200  $\mu\text{M}$  HNE; **h**, HGE + 200  $\mu\text{M}$  HNE; **i**, AGE + 200  $\mu\text{M}$  HNE

hydroxyl radicals. Accumulation of  $\text{H}_2\text{O}_2$  can have profoundly deleterious effects on cells through base modifications and strand breakage in genomic DNA [28]. Four-hydroxynonenal (HNE) is formed by radical-initiated degradation of polyunsaturated fatty acids and is a sensitive marker of lipid peroxidation and oxidative stress [29]. HNE has been implicated in a number of pathologies including atherosclerosis [30]. We assessed the susceptibility of normal human leukocytes to treatment with  $\text{H}_2\text{O}_2$  and HNE. All garlic extracts were found to exert an antigentotoxic effect on human leukocytes and the effect was demonstrated remarkably at 50  $\mu\text{g}/\text{ml}$  for all garlic extracts with both  $\text{H}_2\text{O}_2$  and HNE induced DNA damage (Figs. 1 and 3). The  $\text{ED}_{50}$  of AGE containing high total phenolic content (19.3  $\mu\text{g}/\text{ml}$ ) exhibited the greatest protective effect on  $\text{H}_2\text{O}_2$  induced DNA damage (Fig. 2). The  $\text{ED}_{50}$  of RGE in HNE induced DNA damage was the lowest at 38.6  $\mu\text{g}/\text{ml}$ , followed by AGE (48.6  $\mu\text{g}/\text{ml}$ ) > HGE (51.9  $\mu\text{g}/\text{ml}$ ). This result showed a trend similar to the SOD-like

activity. Plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators [31]. The antioxidant activities of the phenolic compounds are attributed to their redox properties, which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers, and they also have metal chelating properties [32]. However, it might be inferred from these data that total phenolics are more potent scavengers of superoxide anion radicals than other molecules. Fabiani et al. [33] reported that phenolic compounds, when used both as purified compounds and in complex crude extracts in olive oil, prevented  $\text{H}_2\text{O}_2$ -induced DNA damage. These results indicated that garlic extracts may efficiently enhance the ability of normal human leukocytes to resist  $\text{H}_2\text{O}_2$  and HNE induced oxidative damage under *ex vivo* conditions (Fig. 3).

In conclusion, heating after grounding produces a reduction in the amount of total phenolic in the garlic, probably owing to its biodegradation at high temperature.



Raw and aged garlic contains high comparable quantities of total phenolic and possess high antigenotoxic effect. It is suggested that a proper heat treatment and aged-processing could be used to enhance the amount of bioactive compounds and antioxidant capacity of garlic. Garlic extracts exhibit significant protective effects against DNA damage induced by H<sub>2</sub>O<sub>2</sub> and HNE, which might be related to antioxidant activity.

**Acknowledgements** This study was supported by Kyungnam University Research Fund, 2009.

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