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Modulation of Cytokine Secretion by Garlic Oil Derivatives Is Associated with Suppressed Nitric Oxide Production in Stimulated Macrophages

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We previously described that garlic oil derivatives differentially suppress the production of nitric oxide (NO) and prostaglandin E_2 (PGE_2) in activated macrophages. In the present study, we investigated the effects of the garlic derivatives, diallyl sulfide (DAS), diallyl disulfide (DADS), and allyl methyl sulfide (AMS), on cytokine production in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells, and the association between modulation of cytokines and inhibition of NO production was also assessed. The results indicated that these garlic compounds had different effects on the secretion of activated cytokines, including proinflammatory tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6, as well as the antiinflammatory, IL-10. DAS inhibited the production of all stimulated cytokines in a concentration-dependent manner, and the inhibition was closely associated with the suppression of NO and PGE_2 production. DADS repressed the production of stimulated TNF-α and IL-10 and increased the production of activated IL-1β and, to a lesser extent, IL-6; but only the decreased IL-10 production was associated with DADS-induced NO inhibition. Yet, the DAS- and DADS-suppressed NO production was independent of TNF-α. AMS, on the other hand, slightly suppressed the stimulated TNF-α but enhanced IL-10 production, and such modulation was closely associated with the decrease in NO production.

KEYWORDS: garlic; organosulfur compounds; cytokine; nitric oxide; inflammation

INTRODUCTION

Garlic (*Allium sativum* L.) has been widely used as a flavoring agent and as a traditional medicine to treat diseases including microbial infections, hyperlipidemia, and heart diseases (*1*, *2*). Recently, more attention has been paid to its anticarcinogenic and immunomodulatory effects (*3*−*7*). A variety of organosulfur compounds isolated from various preparations have been identified as being responsible for the biological effects of garlic. For example, diallyl sulfide (DAS), diallyl disulfide (DADS), allyl methyl sulfide (AMS), and diallyl trisulfide (DAT) are principal compounds in garlic oil; *S*-allyl cysteine (SAC) is the major compound in aged garlic extract (*8*, *9*); and allicin is mainly present in the aqueous garlic extract (*9*). Many of these organosulfur compounds have been identified as playing protective roles in carcinogenic processes (*4*).

Macrophages play an important role in host defense against infection and cancer. Activation of macrophages by stimuli, such as lipopolysaccharide (LPS), increases the production and secretion of numerous inflammatory mediators, including nitric oxide (NO), prostaglandin E_2 (PGE_2), and a variety of cytokines. Under physiological conditions, these mediators are involved in antipathogenic processes and are well-regulated. However, different pathological conditions, such as chronic inflammation, autoimmune diseases, and cancer, are closely associated with the production of excess amounts of NO and PGE_2 and inappropriate expression of cytokines (*10*−*13*). Hence, agents that regulate the production of various cytokines and suppress the overproduction of NO and PGE_2 may have protective roles in inflammation-related diseases.

There is increasing interest in using natural products to modulate immune responses and neutralize inflammatory processes because of the fewer effects and lower cytotoxicities (*13*). We previously reported that the principal components in garlic oil, DAS, DADS, and AMS, differentially suppress NO and PGE_2 production in LPS-activated macrophages, in which all three compounds suppress stimulated NO production, but only DAS inhibits PGE_2 production (*14*). Because cytokines can be induced by LPS treatment and because NO and PGE_2 produced by inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), respectively, can be regulated by a variety of cytokines, this study was aimed at examining the effects of these individual components in garlic oil on the production of various cytokines, including TNF-α, IL-1β, IL-6, and IL-10, in LPS-activated RAW 264.7 cells. Moreover, the association between the suppression of NO production and modulation of cytokine production by these derivatives was also assessed. Results from

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this study may increase understanding of how compounds from garlic modulate immune function and how these compounds might be used in the treatment of inflammation-related diseases.

MATERIALS AND METHODS

Chemicals and Biochemicals. DAS, DADS, LPS, sulfanilamide, *N*-(1-naphthyl)ethylenediamine dihydrochloride, and sodium nitrite were commercially obtained from Sigma Chemical (St. Louis, MO). AMS was from ACROS (Fairlawn, NJ), and absolute ethanol was purchased from Merck (Darmstadt, Germany). Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from GIBCO (Grand Island, NY). Recombinant murine TNF-α (rTNF-α) was from PeProTech EC (London, U.K.). All other laboratory chemicals were of the highest quality available and were purchased from Sigma and USB (Cleveland, OH).

Cell Culture. Murine monocyte-macrophage RAW 264.7 cells were obtained from the Culture Collection and Research Center, Hsinchu, Taiwan (CRCC 60001). Cells were grown as monolayers in DMEM supplemented with 10% FBS at 37 °C in an atmosphere of 95% air and 5% CO_2. DAS, DADS, and AMS were dissolved in absolute ethanol, and the concentration of absolute ethanol added to the media never exceeded 0.2% (v/v). Because DAS (1−10 μM), DADS (0.1−0.2 μM), and AMS (2−20 μM) inhibited LPS-activated NO production and showed no significant cytotoxicity (*14*), these concentrations were used in this study.

Assay for Cytokine Production and Release. The cytokines released into culture medium were determined by commercial cytokine ELISA kits (Amersham Biosciences, Buckinghamshire, England). Basically, cells were treated with LPS and various concentrations of DAS, DADS, and AMS for 24 h, and the cultured medium was then collected for the analysis of cytokines according to the manufacturer's instructions.

Assay for Cytotoxicity. The viability of cells was examined with a CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI). Basically, cells were treated with LPS and various concentrations of DAS, DADS, and AMS for 24 h and then were harvested to test for cytotoxicity. Live cells convert 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)- 2*H*-tetrazolium (MTS) to a formazan dye that can be detected at OD_{492nm} by a microplate reader.

Assay for NO and PGE_2 Production and Release. The nitrite concentration in the culture medium was determined as an index of NO production. Nitrite was quantified spectrophotometrically after its reaction with Griess reagent (1:1 mixture of 1% sulfanilamide/5% H_3PO_4 and 0.1% naphthylethylenediamine dihydrochloride) using sodium nitrite as a standard (*15*). The production and release of PGE_2 in the culture medium were determined using a commercial PGE_2 EIA kit (Amersham Biosciences).

Statistical Analysis. Values are expressed as the mean ± standard deviation (SD), and were analyzed using SAS software version 6.12 (SAS Institute, Cary, NC). One-way ANOVA followed by Fisher's least significant difference test as well as Student's *t*-test were used to determine statistical differences between groups. Pearson's correlation analysis was used to analyze the association between the production of NO and various cytokines. The significance of mean differences was based on a p value of <0.05.

RESULTS

Effects of DAS, DADS, and AMS on Cytokine Production. The effects of DAS, DADS, and AMS on the production and release of cytokines were examined by ELISA assay kits. None of the cytokines was affected by treatment with DAS, DADS, or AMS for 24 h, but production of the proinflammatory cytokines, TNF-α, IL-1β, and IL-6, as well as the antiinflammatory cytokine, IL-10, was enhanced by LPS treatment in RAW 264.7 cells (**Figure 1**). As shown in **Figure 1A**, co-treatment with DAS inhibited the increased production of both pro- and antiinflammatory cytokines in a concentration-dependent manner. On the other hand, DADS suppressed the production of TNF-α and IL-10 but enhanced the production of IL-1β and, to a lesser extent, IL-6 in macrophages stimulated with LPS (**Figure 1B**). Co-treatment with AMS showed a more-diverse response to activated cytokine production. AMS slightly inhibited TNF-α but enhanced IL-10 production in stimulated macrophages. In addition, activated IL-1β production was enhanced by a low concentration (2 μM) but inhibited by a high concentration (20 μM) of AMS, and stimulated IL-6 production was also slightly enhanced by 10 μM of AMS (**Figure 1C**).

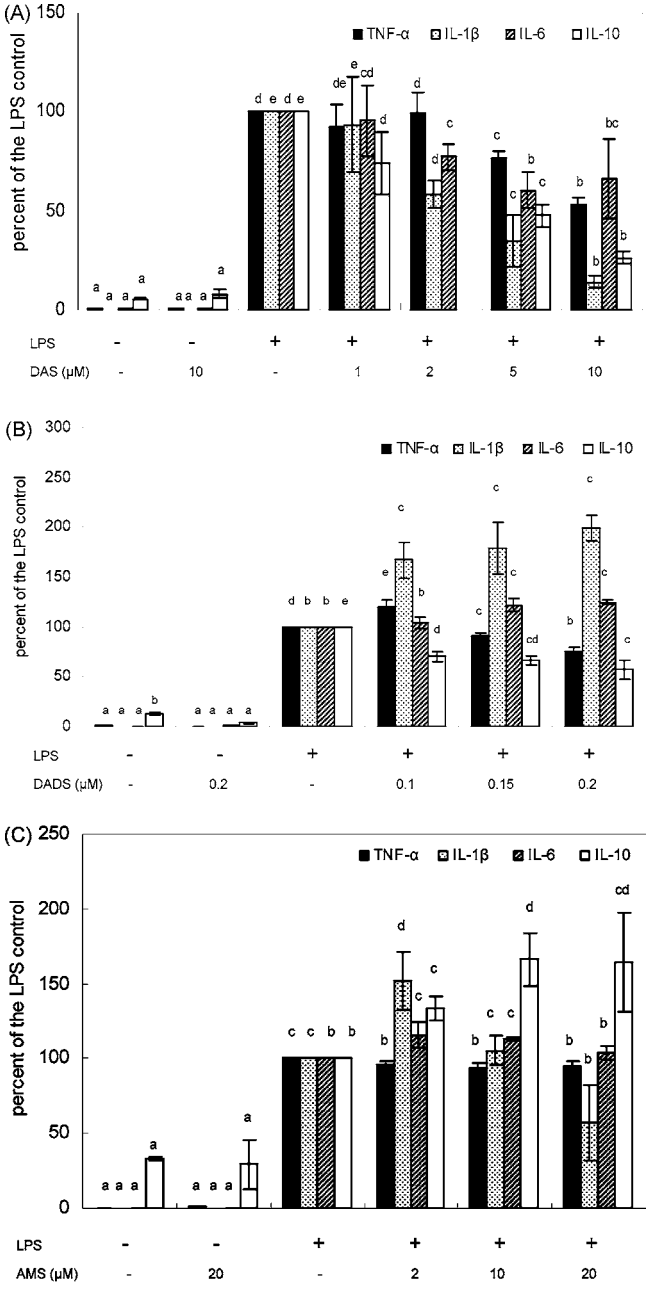

Figure 1. Effects of DAS (**A**), DADS (**B**), and AMS (**C**) on LPS-activated cytokines released into the medium by RAW 264.7 cells. Cells were co-treated with LPS (330 ng/mL) and various concentrations of DAS, DADS, and AMS for 24 h. The levels of cytokines in the medium were then determined using commercial ELISA kits. Values represent the mean ± SD from three measurements (labeled a–e). Data in the same cytokine group with different labels significantly differ ($p < 0.05$).

Effects of DAS, DADS, and AMS on NO and PGE_2 Production. To understand whether the modulation of activated cytokine production by these garlic derivatives is associated with

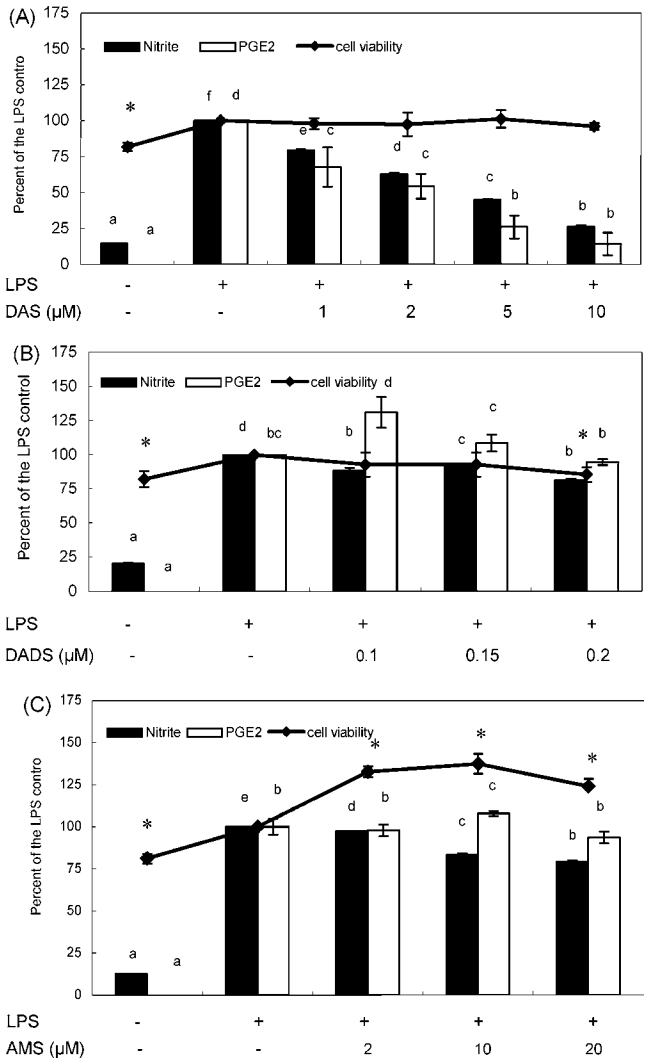

Figure 2. Effects of DAS (**A**), DADS (**B**), and AMS (**C**) on LPS-induced cell viability and release of NO and PGE_2 into the medium by RAW 264.7 cells. Cells were treated with LPS (330 ng/mL) and various concentrations of DAS, DADS, and AMS for 24 h. The nitrite content in the medium was then determined using the Griess reagent; the PGE_2 concentration and cell viability were measured using commercial ELISA kits. Values represent the mean ± SD from three measurements (labeled a–e). Data in the same parameter with different superscripts significantly differ ($p < 0.05$). An asterisk (*) denotes a significant difference from the LPS control ($p < 0.05$).

other inflammatory mediators, the secreted NO and PGE_2 were measured under the same conditions. Consistent with the results we reported previously (*14*), treatment of RAW 264.7 cells with LPS for 24 h significantly enhanced NO and PGE_2 productions, but co-treatment with DAS suppressed such enhancement, which demonstrated the same pattern as that observed in the cytokine measurement (**Figure 2A**). Similarly, DADS and AMS also inhibited LPS-activated NO production. However, neither DADS nor AMS affected the LPS-activated PGE_2 production, which was slightly enhanced at lower concentrations (**Figure 2B,C**). Additionally, most of the concentrations of DAS, DADS, or AMS showed no cytotoxicity to LPS-treated cells, except that 0.2 μM DADS slightly inhibited LPS-stimulated cell growth, suggesting that the inhibition of cytokines by these garlic derivatives was not due to cell death. Moreover, AMS slightly enhanced the proliferation of activated macrophages.

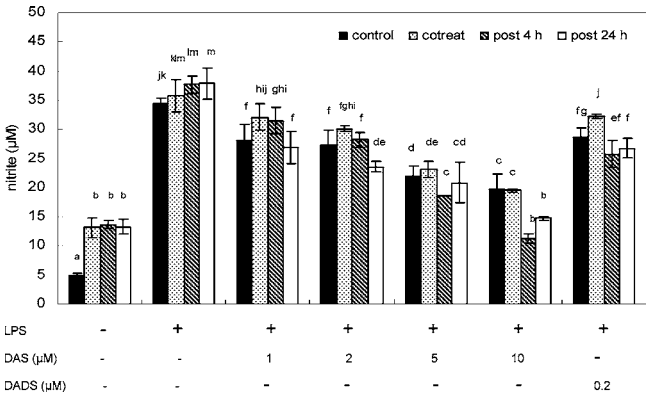

Figure 3. Effects of rTNF-α on LPS-activated NO released into the medium by RAW 264.7 cells. Cells were treated with LPS (100 ng/mL) and various concentrations of DAS or DADS for 24 h. Recombinant TNF-α (10 ng/mL) was either added together with LPS (cotreat) or added in 24 h LPS-treated cells for another 4 h (post 4 h) or 8 h (post 8 h). The nitrite content in the medium was then determined using the Griess reagent. Values represent the mean ± SD from three measurements (labeled a–m). Data with different labels significantly differ ($p < 0.05$).

Table 1. Relationship between the Production of Cytokines and NO (**A**) or PGE_2 (**B**) in RAW 264.7 Cells Co-treated with LPS and DAS, DADS, or AMS for 24 h

(**A**) NO

	DAS		DADS		AMS	
	r^2	*p* value	r^2	*p* value	r^2	*p* value
TNF-α	0.793 25	0.0012[a]	0.373 25	0.2321	0.804 84	0.0160[b]
IL-1	0.672 57	0.0084[a]	–0.558 86	0.0739	0.193 15	0.6782
IL-6	0.848 78	0.0005[a]	–0.567 9	0.0684	–0.542 29	0.2085
IL-10	0.956 25	<0.0001[a]	0.881 01	0.0002[a]	–0.859 77	0.0062[a]
PGE_2	0.853 71	0.0001[a]	0.227 15	0.4777	0.150 33	0.7233

(**B**) PGE_2

	DAS			DAS	
	r^2	*p* value		r^2	*p* value
TNF-α	0.869 18	0.0001[a]	IL-10	0.861 28	<0.0001[a]
IL-1	0.558 15	0.0381[b]	NO	0.853 71	0.0001[a]
IL-6	0.767 42	0.0036[a]			

[a] $p < 0.01$. [b] $p < 0.05$.

To determine whether there was an association between the modulated cytokines and the decreased NO production, Pearson's correlation analysis was performed. As shown in **Table 1**, the DAS-induced suppression of NO and PGE_2 was strongly associated with the decreased production of cytokines, whereas the DADS-induced inhibition of NO was only correlated with decreased IL-10. Additionally, inhibition of NO by AMS was positively correlated with TNF-α production and showed an inverse relationship with IL-10 production. The results suggest that these inflammatory mediators may be co-regulated by garlic derivatives, especially DAS, and that modulated cytokine production may be involved in the NO-suppressive effects of these garlic derivatives. However, addition of rTNF-α did not alter the DAS- or DADS-suppressed NO production. As shown in **Figure 3**, rTNF-α (10 ng/mL) stimulated NO production in untreated RAW264.7 cells and slightly enhanced NO production in 4 h and 24 h post-LPS-treated cells. On the other hand, rTNF-α, no matter treated together or posttreated with LPS, did not reverse the decreased NO production induced by DAS or DADS in activated macrophages, indicating that the DAS- and DADS-suppressed NO production were independent of TNF-α.

DISCUSSION

In the present study, we demonstrated that DAS, DADS, and AMS, individual bioactive components in garlic oil, had different effects on the production of cytokines in LPS-activated macrophages. DAS inhibited both pro- and antiinflammatory cytokines, including TNF-α, IL-β, IL-6, and IL-10, in stimulated macrophages. DADS enhanced proinflammatory cytokines IL-β and IL-6 but suppressed antiinflammatory cytokine IL-10, indicating the effect of DADS may be more toward proinflammation. On the other hand, AMS, to a lesser extent, decreased production of NO and TNF-α in activated macrophages but significantly enhanced IL-10 production, suggesting that AMS may be a potential antiinflammation compound. Although the effects of garlic extracts on cytokine production in vivo are limited, a number of studies have indicated that its extracts are able to modulate cytokine secretion in different cell systems. Aged garlic extract has been shown to increase the release of IL-2, IL-12, TNF-α, and IFN-γ from mouse splenic cells (*6*). Fresh garlic extract suppresses monocyte production of TNF-α, IL-1α, IL-6, and IL-8 but enhances IL-10 production in LPS-stimulated human whole-blood cultures (*16*). Furthermore, Keiss et al. (*17*) indicated that garlic powder extract and 1 μM DADS, a higher concentration than we used, reduced the LPS-induced production of TNF-α and IL-1β in human whole blood, yet garlic powder extract did not affect IL-10 secretion. Therefore, although the effect of DADS, at concentration ranges of 0.1−0.2 μM, on cytokine regulation, is more toward proinflammation, the net effect in garlic seems to be more antiinflammation. On the other hand, these results also suggest that the immunomodulatory effects of garlic (*6*, *18*) may, at least partially, act through alteration of the production of various cytokines by the individual components of garlic (DAS, DADS, and AMS). However, these possibilities need to be further investigated.

Previously, we have shown that all three garlic derivatives suppressed activated NO production (*14*), but they had a more-distinct effect on the production of cytokines, indicating the complexity of the system. NO is a small radical molecule that is synthesized from L-arginine by nitric oxide synthase (NOS) in various cells and tissues (*19*). Among the isoforms identified, the inducible form (iNOS) is primarily expressed in macrophages and lymphocytes and is regulated by various modulators, including different cytokines. Proinflammatory cytokines, such as TNF-α and IL-1β (*10*, *20*), act as stimulators, whereas antiinflammatory cytokines, including IL-10, have been reported to be negative regulators of NO (*21*). Similarly, PGE_2 is also synthesized by cyclooxygenase-2 (COX-2) during infection and is inducible after exposure to LPS and cytokines (*10*). Because LPS and proinflammatory cytokines are able to stimulate the transcription of iNOS to enhance NO production and because antiinflammatory cytokines may inhibit NO production, it is conceivable that the NO-inhibitory effect of these compounds may occur through suppressed production of the proinflammatory cytokines, TNF-α, IL-β, and IL-6, or through increased production of antiinflammatory cytokine, IL-10. However, although the decreased production of LPS-stimulated NO, cytokines, and PGE_2 by DAS was highly associated, addition of TNF-α did not reverse the suppression, suggesting that the NO-inhibitory effects of DAS and DADS were not dependent upon TNF-α. Additionally, DAS and DADS significantly inhibited the stimulated production of IL-10, which has been shown to inhibit interferon-γ-stimulated NO production (*21*), so IL-10 may not play a critical role in DAS- and DADS-induced NO inhibition in LPS-activated macrophages. Moreover, pro- and antiinflammatory cytokines are co-regulated; they may act additively or synergistically to affect the NO production. Finally, not only can cytokines modulate NO production but also may NO modulate the secretion of cytokines. NO has been shown to modulate the production of IL-2 and IFN-γ by T cells (*20*) as well as TNF-α and IL-1β in activated alveolar macrophages (*22*), and this may partially explain the differential effects of garlic derivatives on production of cytokines. Because the NO-inhibitory effects of DADS and AMS are less potent than that of DAS, the residual NO in the culture medium may thus affect the secretion of various cytokines.

A 1 g amount of garlic bulb would provide a dose of 30−100 μg of DAS, 530−630 μg of DADS, and 3.8−4.6 μg of AMS (*23*). Ideally, without considering digestion and absorption, this dose of garlic would produce approximately 0.05−0.18, 0.74−0.85, and 0.009−0.01 μM of plasma DAS, DADS, and AMS concentrations, respectively, in a 70 kg person based on a blood volume in the adult human that comprises 7% of body weight. These concentrations of DAS and AMS are much lower than the concentrations we used in this study, but higher plasma concentrations may be achieved by taking garlic supplements which contain higher amounts of these derivatives. On the other hand, although DADS (0.1−0.2 μM) seems to be more toward proinflammation, a higher concentration (1 μM) has been reported to inhibit LPS-induced production of TNF-α and IL-1β. Therefore, usual dietary consumption of garlic or its supplements would be beneficial in terms of its inhibitory effects on excess of NO and PGE_2 production as well as its regulatory effects on cytokine production.

In conclusion, we have demonstrated that DAS inhibits the production of TNF-α, IL-1β, IL-6, and IL-10 in LPS-stimulated macrophages, and the inhibition is closely associated with the suppressed NO and PGE_2. DADS repressed the production of stimulated TNF-α and IL-10, increased the production of activated IL-1β and, to a lesser extent, IL-6, but only the decreased IL-10 production was associated with DADS-induced NO inhibition. AMS, on the other hand, slightly suppressed the stimulated TNF-α but enhanced IL-10 production, and such modulation was closely associated with the decreased NO production. Various pathways have been suggested for the anticarcinogenic activity of garlic oil, including modulation of xenobiotic-metabolizing enzyme activities (*24*−26), inhibition of DNA adduct formation (*27*), induction of apoptosis (*28*), modulation of immune functions (*5*), and antioxidation (*9*). Because immunomodulation plays an important role in cancer development (*13*), the results obtained from this study provide an alternative protective mechanism of garlic oil.

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