In Vitro Virucidal Effects of *Allium sativum* (Garlic) Extract and Compounds

Norbert D. Weber[1], *Douglas O. Andersen*[1], *James A. North*[1], *Byron K. Murray*[1], *Larry D. Lawson*[2], *and Bronwyn G. Hughes*[2,3]

[1] Department of Microbiology, Brigham Young University, Provo, Utah 84602, U.S.A.
[2] Murdock Healthcare Springville, Utah 84663, U.S.A.
[3] Address for correspondence

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Abstract

Garlic (*Allium sativum*) has been shown to have antiviral activity, but the compounds responsible have not been identified. Using direct pre-infection incubation assays, we determined the *in vitro* virucidal effects of fresh garlic extract, its polar fraction, and the following garlic associated compounds: diallyl thiosulfinate (allicin), allyl methyl thiosulfinate, methyl allyl thiosulfinate, ajoene, alliin, deoxyalliin, diallyl disulfide, and diallyl trisulfide. Activity was determined against selected viruses including, herpes simplex virus type 1, herpes simplex virus type 2, parainfluenza virus type 3, vaccinia virus, vesicular stomatitis virus, and human rhinovirus type 2. The order for virucidal activity generally was: ajoene > allicin > allyl methyl thiosulfinate > methyl allyl thiosulfinate. Ajoene was found in oil-macerates of garlic but not in fresh garlic extracts. No activity was found for the garlic polar fraction, alliin, deoxyalliin, diallyl disulfide, or diallyl trisulfide. Fresh garlic extract, in which thiosulfinates appeared to be the active components, was virucidal to each virus tested. The predominant thiosulfinate in fresh garlic extract was allicin. Lack of reduction in yields of infectious virus indicated undetectable levels of intracellular antiviral activity for either allicin or fresh garlic extract. Furthermore, concentrations that were virucidal were also toxic to HeLa and Vero cells. Virucidal assay results were not influenced by cytotoxicity since the compounds were diluted below toxic levels prior to assaying for infectious virus. These results indicate that virucidal activity and cytotoxicity may have depended upon the viral envelope and cell membrane, respectively. However, activity against non-enveloped virus may have been due to inhibition of viral adsorption or penetration. Additionally, the composition of various commercial garlic products, including garlic powder tablets and capsules, oil-macerated garlic, steam-distilled garlic oils, garlic aged in aqueous alcohol, and fermented garlic oil was determined as well as the virucidal activities of the products against herpes simplex virus type 1 and parainfluenza virus type 3. Virucidal activities of commercial products were dependent upon their preparation process. Those products producing the highest levels of allicin and other thiosulfinates had the best virucidal activities.

Key words

Allium sativum, allicin, antiviral, garlic, thiosulfinates, virucidal activity.

Introduction

Garlic, *Allium sativum* L. (Liliaceae), has been used traditionally to treat a number of infectious diseases including those now known to be caused by bacteria, fungi, protozoa, and viruses (1–3). Antibacterial, antifungal, and antiprotozoal effects have been substantiated *in vitro* and found to be due to diallyl thiosulfinate (allicin), methyl allyl thiosulfinate, and allyl methyl thiosulfinate (first named residue linked to the thio, second named group linked to the sulfinate) (3–6). Thiosulfinate compounds are released from garlic cloves after tissue disintegration caused by chewing, cutting, or pressing. Such mechanical action allows alliinase (alliin alkyl sulfenate lyase, EC 4.4.1.4) to convert the (+)-*S*-alk(en)yl-L-cysteine sulfoxides to the corresponding thiosulfinates (shown in Fig. 1) (7). In addition to the endogenous (+)-*S*-alk(en)yl-L-cysteine sulfoxide content of garlic cloves, a reserve for the sulfoxides (and hence the thiosulfinates) also exists in precursor compounds which are γ-glutamyl-*S*-alk(en)yl cysteines (8).

The traditional antiviral uses for garlic include treatment of chicken pox, measles, the common cold, and influenza (5). Few confirmatory reports have been published regarding these traditional antiviral uses. Tsai et al. (9) found that a commercial garlic extract from Shanghai, China had *in vitro* antiviral activity against herpes simplex virus type 1 (HSV-1) and influenza B virus, but not coxsackie B1 virus. Studies using mice indicated that aqueous ethanolic extracts of garlic administered orally 15 days before experimental infection with influenza virus A0/PR 8 strain were protective, while administration of this extract at the time of viral infection had no effect

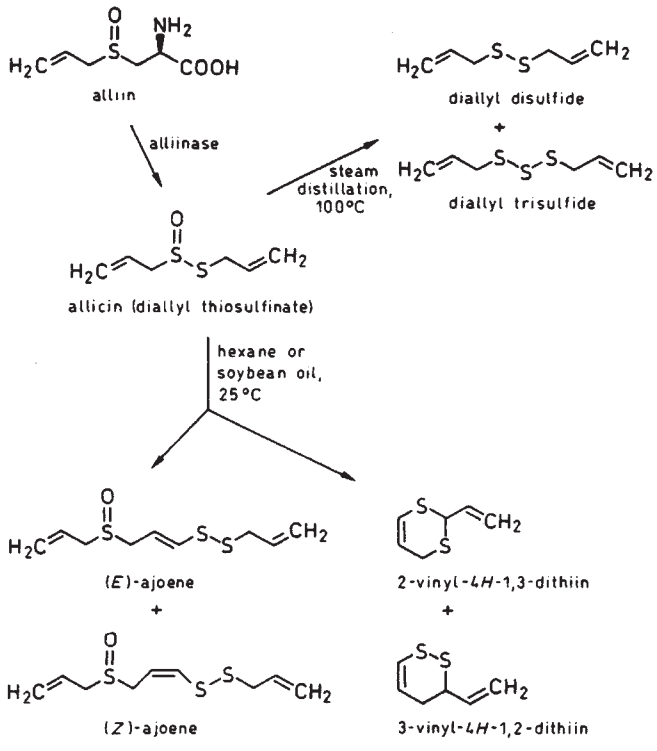

Fig. 1 Reaction scheme showing the formation of allicin from alliin in chewed, cut or pressed garlic and the subsequent allicin transformation compounds found in steam-distilled garlic oils or in hexane or soybean oil incubates.

(10). Garlic extract was not effective against two strains of Japanese encephalitis virus (Nakayama and JaGAr 01 strains). Activity against influenza virus (A/PR 8/34, H1N1 strain) in mice was also demonstrated for an aqueous garlic extract administered by intranasal or intramuscular routes (11). Treatment 3 days before inoculation with virus significantly decreased viral hemagglutination titers in lung homogenates and moderately increased mean survival length. Post-inoculation treatment (after 1 h) had no effect on mortality or mean survival length, but led to significant decreases in hemagglutination titers.

The antiviral properties of garlic may be due to a direct antiviral effect, to immune modulation, or to some combination of these events. Garlic intake in humans has been reported to enhance natural killer cell activity (12), and a number of human immune functions were found to be enhanced *in vitro* by aqueous garlic extract, its polar and thiosulfinate fractions (13). Both the aqueous extract and the polar fraction increased interleukin-1 production while the thiosulfinate fraction stimulated natural killer cell activity. The aqueous extract, polar and thiosulfinate fractions were also found to increase interleukin-2 production.

Active antiviral compounds in garlic have not been identified, so an evaluation of the *in vitro* effectiveness of several compounds now known to be present in aqueous extracts of garlic cloves, garlic powder, oil-macerated garlic, or steam-distilled garlic oils was determined. The antiviral activity of several commercial garlic supplement products was also determined. This paper reports antiviral activity, including direct virucidal action, against both DNA and RNA enveloped and non-enveloped viruses. Experimental results suggested that garlic extract and allicin had direct virucidal activity and significant cytotoxicity but no intracellular antiviral properties.

Materials and Methods

Preparation of fresh garlic extract, garlic powder, and garlic supplement products

Garlic cloves (from local stores) were stripped of their outer skin and finely grated into a pulp. This pulp was placed into a disposable 50 ml syringe with cotton gauze in the tip and the liquid extract was forced from the garlic by squeezing. The liquid extract was collected in microcentrifuge tubes then centrifuged at 14,000 rpm using an Eppendorf 5415 microcentrifuge at 4 °C. The supernatant fluid was collected, filtered through a 0.45 µm Acrodisc (Gelman Sciences, Ann Arbor, MI), and stored on ice prior to use. Garlic extract was prepared fresh daily prior to each series of experiments. High quality garlic powder (commercially manufactured using a minimum amount of slicing before drying) samples were prepared by first adding 0.83 g of powder to 5 ml of Dulbecco's phosphate buffered saline (DPBS) then vortexing for 2 min. These preparations were centrifuged, filtered, and stored in the same manner as fresh garlic extract. Garlic products that were tablets or in hard-gelatin capsules were ground to a fine powder using a mortar and pestle, while the contents of products in soft-gelatin capsules were removed using a sterile needle and syringe. Six milliliters of gelatin lactalbumin hydrolysate buffer (GLB, pH 7.0) (14) were added to 2.0 g of the ground tablets or hard-capsule contents and 3.0 ml of GLB were added to 1.0 g of the products removed from soft-gelatin capsules. These mixtures were vortexed for 2 min and then centrifuged 20 min at 14,000 rpm at 4 °C. The supernatant fractions were collected, filtered, and adjusted to pH 7.0 using either 1N HCl or NaOH.

Preparation and characterization of garlic compounds

Deoxyalliin (*S*-allylcysteine) was synthesized and characterized in our laboratory as previously described (8). Alliin was prepared by oxidation of deoxyalliin according to Stoll and Seebeck (15). Allicin and methyl allyl and allyl methyl thiosulfinates were either isolated from aqueous garlic extracts or synthesized as described previously (16). Since the antiviral effects of synthesized allicin, methyl allyl thiosulfinate, and allyl methyl thiosulfinate had been determined in our laboratory to be similar to those of their isolated counterparts, synthesized compounds were used in this study. *E*- and *Z*-ajoene and diallyl trisulfide were prepared and characterized as before (17). Diallyl disulfide was purchased from Aldrich and purified by distillation.

Cells and viruses

Vero cells (African green monkey kidney, ATCC CCL 81, American Type Culture Collection, Rockville, MD) and HeLa cells (human epithelial cervical carcinoma, Flow Labs, Inglewood, CA) were used to grow virus pools and to monitor viral infectivity. Both cell lines were grown in Eagle minimal essential media (MEM, GIBCO, Grand Island, NY) containing Earle's balanced salts and supplemented with 10 % bovine calf serum (BCS, HyClone Laboratories, Logan, UT) and 20 mM HEPES buffer (pH 7.35, Research Organics, Cleveland, OH). For infectivity assays, overlay medium consisting of MEM with 2 % BCS and 1 % methylcellulose was supplemented with penicillin and streptomycin. Virus stock pools were prepared by twice freezing infected cells at −70 °C and thawing as previously described for our laboratory (18).

Herpes simplex virus type 1 (HSV-1) strain KOS and herpes simplex virus type 2 (HSV-2) strain 333 were grown in Vero cells, while parainfluenza virus type 3 (Para-3) strain C-243 and human rhinovirus type 2 (HRV-2) strain HGP were grown in HeLa cells. Additionally, vesicular stomatitis virus (VSV) strain Indiana and vaccinia virus (VV) strain Elstree were propagated in both cell lines. Approximate titers of the virus pools used for this study, given in plaque forming units per ml, were: HSV-1, 10^8; HSV-2, 10^7; VV grown in Vero cells, 10^5; VSV grown in Vero cells, 10^8; Para-3, 10^7; HRV-2, 10^7; VV grown in HeLa cells, 10^6; and VSV grown in HeLa cells, 10^8.

Direct pre-infection incubation (DPI) assay

This assay was performed as previously described (19, 20). Fresh garlic extract (1,100 mg/ml) and other compounds to be tested for virucidal activity were first diluted to the desired concentrations in GLB or DPBS. Thirty microliters of stock virus was added to 270 µl of these dilutions and the mixtures were incubated at 37 °C for 6 h. As controls, virus stock was added to DPBS or GLB and incubated at the same temperature and time as the test samples. After incubation, the samples were serially diluted (typically to 10^{-6}) in DPBS or GLB and 0.2 ml portions were assayed for remaining viral infectivity by standard plaque assay techniques using confluent monolayers of HeLa or Vero cells (approximately 2.5×10^5 cells per well in 24-well plates). Duplicate wells were infected with each dilution of the samples. Diluting the samples lowered their concentrations well below those levels which were cytotoxic. Viral adsorption was for 30 min at 37 °C. Following adsorption, unadsorbed virus was removed and overlay medium was added. Infected cells were incubated at 37 °C in a humidified 5 % CO_2 environment until viral plaques were readily visible upon microscopic examination. Cells were fixed using 10 % formalin and stained with 1 % crystal violet.

Effect of time, temperature, and pH on virucidal activity of fresh garlic extract

These parameters were determined by appropriate modifications of the DPI assay. Dilutions of extract were incubated with Para-3 or HSV-1 for 0, 1, 2, 4, 6, 8, or 10 h at 37 °C. Also, Para-3 and HSV-1 were incubated with extract samples for 6 h at 4, 25, or 37 °C to determine optimal virucidal temperature. Following incubation, viral infectivity was measured as just described. The effect of pH variation on DPI reduction of infectious HSV-1, HSV-2, Para-3 and VV was determined by adjusting the extract pH from its normal 6.0 to 7.0 prior to the 6 h incubation. Additionally, the effect of pH 6.0 on the DPI was determined by maintaining the extract at its normal pH and adjusting the pH of the virus control samples to 6.0 prior to their 6 h incubation.

Cytotoxicity of fresh garlic extract and allicin

Toxicity of fresh extract to both HeLa and Vero cells was tested by making serial half-$\log_{10}$ dilutions (10^{-1} through 10^{-4}, corresponding to 110, 35, 11, 3.5, 1.1, 0.4, and 0.1 mg of extract/ml) of extract in MEM and then adding these dilutions, as well as non-diluted extract, to confluent cell monolayers in 24-well plates. After 48 h incubation at 37 °C, monolayers were rinsed with DPBS, trypsinized, and the remaining cells counted using a Coulter counter. Control cells were incubated with MEM only. Allicin toxicity to both HeLa and Vero cells was similarly determined using concentrations of 100, 32, 10, and 3.2 µg/ml (corresponding to 0.62, 0.20, 0.062, and 0.020 mM, respectively).

HPLC analyses of fresh garlic extract, the polar fraction, and allicin

The thiosulfinate content of fresh garlic extract was quantitated by using high performance liquid chromatography (HPLC) according to Lawson et al. (16). The polar fraction was isolated from an aqueous garlic extract that had been prepared according to Lawson et al. (16) by extraction of the nonpolar compounds, including the thiosulfinates, in one volume of chloroform. The remaining aqueous (polar) fraction was analyzed by HPLC methods previously described by Lawson et al. (8). To determine whether virucidal activity was due to allicin or to one of its transformation products, the allicin content in fresh extract was determined using HPLC both before and after the 6 h incubation period of the DPI assay (16, 17).

Virus yield reduction (YR) by fresh garlic extract and allicin

Vero cell monolayers were infected at a multiplicity of infection (MOI) of 0.01 with HSV-1, HSV-2, VSV, or VV. After a 30 min viral adsorption period, the infected cells were rinsed with DPBS and then MEM containing 2 % BCS was added. Serially diluted fresh garlic extract was added to the medium to give final extract dilutions of 10^{-3} (1.1 mg/ml) and 10^{-4} (0.1 mg/ml). Infected cells were incubated 48 h at 37 °C in a humidified 5 % CO_2 atmosphere then were twice frozen at – 70 °C and thawed. Samples were assayed for infectious virus with standard plaque assay methods previously described. Yield reductions were determined similarly for HRV-2, Para-3 and VSV except HeLa cells were used and the fresh garlic extract dilutions tested were 2×10^{-3} (2.2 mg/ml) and 2×10^{-4} (0.2 mg/ml). Yield reductions of Para-3 and HSV-1 by allicin at 100, 32, 10, 3.2, and 1.0 µg/ml were also determined using the same MOI and incubation time. Para-3 and HSV-1 were titered in HeLa cells and Vero cells, respectively.

Results

Fresh garlic extract was determined to have optimal virucidal activity in DPI assays when incubated at 37 °C, with decreased activity at 25 °C and even less activity at 4 °C (data not shown). DPI assays also indicated that maximum activities against Para-3 and HSV-1 were reached in 6 to 8 h at 37 °C. Beyond that time, no further reduction in infectious virus titers was seen. When fresh garlic extract was adjusted from pH 6.0 to pH 7.0 prior to DPI assays, there was no significant difference in its virucidal activity against any of the four viruses tested (not shown). With these reaction parameters established, all subsequent DPI assays were done at 37 °C and pH 7.0 for 6 h. Other data indicated that garlic extract maintained its virucidal activity for over one month when stored at 4 °C. Also, both buffers used (DPBS and GLB) produced similar results in virucidal assays.

Garlic extract was virucidal to each virus against which it was tested (Table 1). At the highest concentration tested (1,000 mg/ml), infectivity of all five viruses was substantially reduced. VSV was most sensitive to garlic extract. HSV-1 and HSV-2 were comparable in their sensitivity followed by, in decreasing order, Para-3 and VV. Virucidal activity of fresh garlic extract against HRV-2, which is non-enveloped, was also tested at an extract concentration of 1,000 mg/ml and the reduction of infectious HRV-2 was determined to be 2.4 $\log_{10}$. It is important to note that following the 6 h incubation period, virucidal compounds were diluted to concentrations significantly below their cytotoxic levels before addition to cells.

Table 1 Inactivation of selected RNA and DNA viruses by fresh garlic extract[a].

Garlic extract (mg/ml)	Para-3	HSV-1	HSV-2	VV[b]	VSV[c]
1000	$>3.9 \pm 0.4$[d,e]	$>4.1 \pm 0.4$	ND	2.1 ± 0.3	$>3.6 \pm 0.04$
500	2.1 ± 0.2	ND	ND	0.4 ± 0.1	ND
250	ND	ND	2.5 ± 1.0	0.1 ± 0.1	ND
125	ND	ND	1.7 ± 0.2	ND	ND
100	1.5 ± 0.3	ND	ND	ND	ND
63	0.6 ± 0.3	1.5 ± 0.2	1.2 ± 0.5	ND	2.8 ± 0.5
31	ND	1.1 ± 0.6	ND	ND	2.8 ± 0.6
16	ND	ND	ND	ND	2.0 ± 0.2
8	ND	ND	ND	ND	1.7 ± 0.5

[a] Determined by the DPI assay as described in Materials and Methods.
[b] Infectivity assayed using HeLa cells.
[c] Assayed using Vero cells.
[d] Values represent $\log_{10}$ reductions in infectious virus titers ± standard deviation.
[e] > Indicates the reduction in virus titer was beyond the limits detectable by the assay.
[f] ND indicates not determined.

Table 2 Viral inactivation by allicin[a].

Allicin (μg/ml)	Concentration (mM)	Para-3	HSV-1	Allicin (μg/ml)	Concentration (mM)	HSV-2	VSV
500	3.1	1.0 ± 0.3[b]	2.9 ± 0.6	425	2.7	2.0 ± 0.4	ND[c]
250	1.6	0.4 ± 0.4	1.6 ± 0.7	225	1.4	1.0 ± 0.03	ND
150	0.93	0.3 ± 0.1	1.1 ± 0.3	100	0.62	0.7 ± 0.02	ND
70	0.43	0.5 ± 0.5	0.9 ± 0.1	50	0.31	ND	1.5 ± 1.2
25	0.15	0.2 ± 0.1	0.5 ± 0.2	25	0.15	ND	1.3 ± 0.9

[a] Same as in Table 1.
[b] Values represent $\log_{10}$ reductions in infectious virus titers ± standard deviation.
[c] ND indicates not determined.

Table 3 Cytotoxicity of fresh garlic extract and allicin[a].

Fresh extract (mg/ml)	HeLa	Vero	Allicin (μg/ml)	Concentration (mM)	HeLa	Vero
11	99.5 ± 0.05[b]	100 ± 0.1	100	0.62	98.9 ± 0.04	99.9 ± 0.1
3.5	29.8 ± 0.3	99.9 ± 0.2	32	0.20	62.9 ± 0.5	64.6 ± 0.6
1.1	10.0 ± 0.1	70.3 ± 2.2	10	0.062	20.3 ± 0.7	24.1 ± 1.0
0.3	0 ± 0.01	1.1 ± 0.3	3.2	0.020	1.7 ± 1.0	9.2 ± 1.9

[a] Determined as described in Materials and Methods.
[b] Values represent percent reduction in cell numbers with respect to controls.

Allicin concentration in fresh garlic extract (diluted 1/10 in DPBS) remained unchanged throughout the 6 h DPI incubation period at 37 °C, with HPLC data indicating a concentration of 0.25 mg/ml, both before and after incubation.

The thiosulfinate content of fresh garlic extract was found to be (in mg/ml): diallyl thiosulfinate (allicin), 2.5; methyl allyl and allyl methyl thiosulfinates, 0.63; and *trans*-1-propenyl allyl thiosulfinate, 0.23. HPLC analysis of the aqueous (polar) fraction revealed that it contained (in mg/g of garlic): γ-glutamyl-*S*-*trans*-1-propenylcysteine,3.2; γ-glutamyl-*S*-allylcysteine,2.6; and γ-glutamyl-*S*-methylcysteine, 0.5.

Data presented in Table 2 indicated that each of the four viruses tested, all of which were enveloped, were suspectible to inactivation by allicin (structure shown in Fig. 1) at levels as low as 25 μg/ml (0.15 mM) which caused a 1.3 $\log_{10}$ reduction of infectious VSV titers. HSV-1 ranked second to VSV in its sensitivity to allicin, followed by HSV-2 and Para-3. Para-3 infectious titers were reduced only slightly at the highest allicin concentration assayed (500 μg/ml, 3.1 mM). Additional data indicated that allicin at concentrations of 1,100 and 550 μg/ml (6.8 and 3.4 mM) reduced infectious HRV-2 titers by 3.2 and 2.2 $\log_{10}$, respectively, while concentrations of 400 and 200 μg/ml (2.5 and 1.2 mM) reduced VV titers by 0.5 and 0.2 $\log_{10}$, respectively.

Cytotoxicity assays indicated that fresh garlic extract concentrations of 11 and 3.5 mg/ml were nearly 100% toxic to HeLa and Vero cells, respectively (Table 3). There was negligible toxicity at extract dilutions 10-fold greater (1.1 and 0.3 mg/ml, respectively). Allicin was toxic to HeLa cells at 10 μg/ml (0.062 mM) and higher, while 3.2 μg/ml (0.020 mM) and above was toxic to Vero cells.

Table 4 Viral inactivation by allyl methyl thiosulfinate and methyl allyl thiosulfinate[a].

Allyl methyl thiosulfinate (μg/ml)	Concentration (mM)	Para-3	HSV-1	HSV-2	VV	VSV
940	5.8	2.3[b]	ND[c]	ND	1.0	ND
630	3.9	1.0	ND	ND	0.6	ND
470	2.9	0.7	1.6	0.9	0.5	2.5
235	1.5	0	0.9	0.4	0	1.2
120	0.74	ND	0.8	0.2	ND	0.5
60	0.37	ND	0.7	0	ND	0.4
Methyl allyl thiosulfinate (μg/ml)						
940	5.8	1.3	ND	ND	0.9	ND
630	3.9	0.8	ND	ND	0.6	ND
470	2.9	0.6	1.2	0.4	0.4	2.5
235	1.5	0	0.7	0	0	1.2
120	0.74	ND	0.2	0	ND	0.5
60	0.37	ND	ND	ND	ND	0.4

[a,b,c] Same as in Table **2** except limited availability of the two compounds restricted this experiment to one replication. Residual viral infectivity was assayed using duplicate wells per sample dilution as described in Materials and Methods.

Fresh garlic extract was more toxic to Vero cells than to HeLa cells and was also more virucidal to VSV propagated in the former cell line than in the latter. Activity in 6 h DPI assays against VSV propagated in HeLa cells resulted in a 1.9 $\log_{10}$ reduction, compared to a 3.6 $\log_{10}$ reduction of infectious titer measured for VSV grown in Vero cells.

The YR assay was used as an indicator to determine whether fresh garlic extract or allicin had intracellular antiviral activity. Fresh extract had no antiviral activity against any of the six viruses tested when assayed in YR experiments. Allicin was similarly determined to have no antiviral activity at the concentrations used in YR assays against Para-3 and HSV-1.

Results summarized in Table **4** show the extent of inactivation of Para-3, HSV-1, HSV-2, VV, and VSV by allyl methyl thiosulfinate and methyl allyl thiosulfinate. Allyl methyl thiosulfinate had slightly better virucidal activity against three of the five viruses tested than methyl allyl thiosulfinate. Both compounds were equally effective against VV and VSV. Allyl methyl thiosulfinate was most effective against VSV, followed by HSV-1, HSV-2, Para-3, and VV. Methyl allyl thiosulfinate was also most effective against VSV, followed by HSV-1. However, the effectiveness of this compound against Para-3, HSV-2 and VV was similar in magnitude for each of these viruses.

Tests of viral inactivation by ajoene (structures shown in Fig. **1**), one of the transformation products of allicin, indicated that the *E*- and *Z*-ajoene isomers were equal in their virucidal activities. *E*-ajoene concentrations of 500, 250, and 125 μg/ml (2.1, 1.1 and 0.53 mM) reduced HSV-1 infectivity by 2.6, 1.6, and 1.2 $\log_{10}$, respectively, while 500 and 250 μg/ml reduced infectious Para-3 titers by 2.9 and 1.1 $\log_{10}$, respectively. Additionally, 500 and 250 μg/ml, respectively, reduced titers of HRV-2 by 1.1 and 0.7 $\log_{10}$ and titers of VV by 1.7 and 1.0 $\log_{10}$. Infectious titers of VSV were reduced by 2.6 $\log_{10}$ and HSV-2 titers were reduced by 1.0 $\log_{10}$ by *E*-ajoene at 125 μg/ml.

The garlic polar fraction had no DPI virucidal activity; however, the highest concentration assayed was 200 μg/ml. Alliin (a compound present in the polar fraction) caused a 1.9 $\log_{10}$ DPI reduction of infectious VSV titers at 320 μg/ml (2.0 mM) when assayed using HeLa cells. However, this concentration was cytotoxic and no virucidal activity was found at 100 μg/ml. Deoxyalliin had no activity at 2,000 μg/ml (12 mM). Diallyl disulfide and diallyl trisulfide were tested at the maximum aqueous dissolution concentrations of 25 and 2.5 μg/ml (0.17 and 0.014 mM), respectively, and neither had virucidal activity.

In comparing virucidal activity with the composition of commercial garlic supplement products (Table **5**) containing or releasing allicin and other thiosulfinates, vinyldithiins (structures shown in Fig. **1**), ajoenes and dialk(en)yl sulfides, those products producing the highest yield of allicin and other thiosulfinates had the greatest virucidal activity against both Para-3 and HSV-1. Fresh garlic extract, garlic powder, and certain garlic powder tablets, dragees, or capsules released high concentrations of allicin and other thiosulfinates. Virucidal activity corresponded directly to the thiosulfinate yield of garlic powder products. Those garlic products producing no detectable allicin or other thiosulfinates (< 5 μg/g product) had no virucidal activity against either virus tested. Additionally, those products containing detectable levels of vinyl dithiins, ajoenes or dialk(en)yl sulfides had no virucidal action at the concentrations used.

Discussion

Results using fresh garlic extract indicated that peak DPI virucidal activity was reached in a 6 to 8 h period at 37 °C and pH 7.0. Of the compounds from fresh extract which were tested, allicin was the most active virucidal component as determined by three lines of evidence. First, stability experiments over the course of the DPI demonstrated that allicin was not converted to its transformation products in any significant amount. Secondly, allicin was generally more active than allyl methyl or methyl allyl thiosulfinates. Thirdly, decreases in the virucidal activities of various commercial garlic products were detected in parallel with decreases in allicin content.

Of the other thiosulfinates tested in this study, allyl methyl thiosulfinate was slightly more virucidal than the methyl allyl form. Allyl methyl thiosulfinate, methyl allyl thiosulfinate and small amounts of *trans*-1-propenyl allyl thiosulfinate were found in those commercial products with virucidal activity. The contribution of these compounds to the overall virucidal activity of such products was difficult to assess, however, since these products also had levels of allicin consistently higher than those of the other thiosulfinates and the *trans*-1-propenyl allyl

Table 5 Viral inactivation by[a] and composition of aqueous extracts of various types and brands of commercial garlic supplement products.

Product	Antiviral activity		Composition (μg/g product)				
	Para-3	HSV-1	Allicin	Other thiosulfinates[b]	Vinyl-dithiins[c]	Ajoenes[d]	Dialk(en)yl sulfides[e]
Garlic powder tablets, dragees, or capsules							
A[f] (U.S.)	>4.1[g,h]	>3.5	3100	1180	n[i]	n	55
B (Germany)	>3.9	>4.8	2240	450	n	n	65
C (Germany)	3.4	4.5	1820	380	n	n	120
D (Germany)	2.0	>4.8	1520	270	n	n	60
E (Germany)	1.6	3.6	260	40	n	n	35
F (Germany)	0.3	1.1	160	30	n	n	50
G (U.S.)	0	0.3	10	n	n	n	n
H (U.S)	0	0	n	n	n	n	n
I, J (Japan)	0	0	n	n	n	n	n
Garlic powder suspended in oil[j]							
A[k] (U.S.)	3.4	>4.4	3050	1020	n	n	60
B[k] (U.S.)	0.7	0.3	110	20	125	80	1210
Oil-macerated garlic[j]							
A (Germany)	0	0	n	n	580	115	170
B (Switzerland)	0	0	n	n	620	110	170
C, D, E (Germany)	0	0	ND[l]	ND	ND	ND	ND
Steam-distilled garlic oils[j]							
A (U.S.)	0	0	n	n	n	n	7340
B (Germany)	0	0	n	n	n	n	4570
C (Germany)	0	0	n	n	n	n	3950
D (U.K.)	0	0	n	n	n	n	4660
Other							
A Aged in aqueous alcohol[m] (Japan)	0	0	n	n	n	n	n
B Fermented garlic oil[j]	0	0	n	n	n	n	n
Garlic extract	>4.1	>4.5	2270	780	n	n	n
Garlic powder	>4.1	>4.8	6900	2170	n	n	50

[a] Determined by the DPI assay described in Materials and Methods.
[b] Includes allyl methyl, methyl allyl and minor amounts of *trans*-1-propenyl allyl thiosulfinates.
[c] Includes 2-vinyl-4*H*-1,3-dithiin and 3-vinyl-4*H*-1,2-dithiin.
[d] Includes *E*- and *Z*-ajoene.
[e] Includes diallyl, methyl allyl, and dimethyl sulfides containing one to six sulfur atoms.
[f] Letters designate different brands of garlic products purchased in the countries shown in parentheses.
[g] > Indicates the reduction of infectious virus titer was beyond the limits detectable by the assay.
[h] Values indicate $\log_{10}$ reductions in infectious virus titers and represent the maximum reductions obtained.
[i] n = Not detected. Limit of detection is 5 μg/g product.
[j] Soft-gelatin capsules.
[k] Enteric coated gelatin capsule.
[l] ND indicates not determined.
[m] Liquid in a bottle.

thiosulfinate was not tested for virucidal activity. *E*-and *Z*-ajoene, allicin transformation compounds not found in fresh garlic extract, each had better virucidal activity than allyl methyl thiosulfinate or methyl allyl thiosulfinate and better activity than allicin against Para-3, HSV-1, HSV-2, and VV. Generally, the relative order of virucidal activity was: ajoene > allicin > allyl methyl thiosulfinate > methyl allyl thiosulfinate. Additionally, the garlic polar fraction, alliin, deoxyalliin, diallyl disulfide and diallyl trisulfide had no activity at the concentrations assayed.

In the commercial products tested, ajoene concentrations were well below those having virucidal activity in DPI assays; therefore, the products containing ajoenes had no activity as would have been predicted. Additionally, dialk(en)yl sulfides had no direct virucidal effect based upon their high concentrations in steam-distilled products which had no virucidal activity. Diallyl di- and trisulfide are the most abundant dialk(en)yl sulfides found in steam-distilled garlic oil (17) (Refer to Fig. **1** for reactions and structures). The vinyldithiins, which are formed in garlic vegetable (usually soybean) oil macerates (Fig. **1**), appeared to have no virucidal effect based upon their presence in oil-macerated products which lacked virucidal activity. However, this did not preclude the possibility of activity by vinyldithiins at concentrations higher than those found in the products tested.

Results indicated that the commercial preparation process was directly related to the virucidal activity of the resulting product. Commercial garlic supplement products prepared by oil-maceration, steam-distillation,

aging in aqueous alcohol, or fermentation had no virucidal activity when measured by DPI. The commercial garlic powder products tested differed in virucidal activities. Some powders worked well, while others had no effect.

Cytotoxicity data indicated that both fresh extract and one of its active compounds, allicin, were more toxic to Vero cells than to HeLa cells. Additional evidence indicated that VSV grown in Vero cells was more susceptible to the virucidal effects of extract and allicin than VSV propagated in HeLa cells. Allicin, which had the best virucidal activity of those compounds found in fresh garlic extract and tested by DPI, had no antiviral effect when used in YR assays which indicated a lack of intracellular antiviral activity. Taken together, these data indicate a possible mode of action for allicin in which activity may have been directed against both viral and cellular membranes. Effectiveness against HRV-2, a non-enveloped virus, may have been due to binding of allicin to the viral protein capsid and subsequent inhibition of viral adsorption or penetration. Toxicity data for HeLa cells in our study correlated well with that of Tsai et al. (9). In that study, however, the exact concentration of allicin in the commercial product tested was not known and cytotoxicity was determined solely by visual observation. Our results for garlic extract virucidal activity showed significantly less effectiveness against HSV-1 than that determined by Tsai et al. (9). Possible reasons for the greatly reduced sensitivity of HSV-1 used in our study might have been variation due to virus strain (this study used KOS) or the fact that our study used a different cell line to propagate the virus.

In comparing the virucidal activity of allicin against DNA and RNA viruses, there was little difference in the degree of inactivation based upon nucleic acid type alone. Although VSV was most susceptible to inactivation of those viruses tested, there was no general pattern of RNA viruses being more sensitive than DNA viruses. Susceptibility may have depended more on the nature of the viral envelope than on nucleic acid type. The DNA viruses HSV-1 and HSV-2 were both highly sensitive to inactivation by allicin. However, VV, which is also a DNA virus, but which has a more complex envelope than either HSV-1 or -2, was only slightly susceptible to inactivation by allicin. Results also indicate that fresh garlic extract and allicin were highly toxic to HeLa and Vero cells. The results of DPI assays were not influenced by toxicity since compounds were diluted below their toxic levels prior to addition to cell monolayers for assay of infectious virus. Finally, results of this study suggest that the activity of both fresh garlic extract and allicin against viruses rested exclusively in their direct virucidal properties and not in any intracellular antiviral mechanism.

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