Medical Laboratory Journal
Online ISSN: 2538-4449
Golestan University of Medical Sciences

Original Article

Phytochemical Analysis of Garlic Hydro-alcoholic Extract and Evaluation of its Anti-bacterial Effect on Enterohemorrhagic *Escherichia coli* in Vitro and ex Vivo

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Received: 2021/04/06
Revised: 2021/05/20
Accepted: 2021/05/22

DOI: 10.29252/mlj.16.1.25

ABSTRACT

Background and objectives: Garlic is a medicinal plant with various health promoting properties including antimicrobial effects. In this study, we investigated in vitro antibacterial effects of garlic hydro-alcoholic extract against enterohemorrhagic *Escherichia coli* (EHEC).

Methods: Garlic hydro-alcoholic extract was prepared by maceration method. Phytochemical analysis of the extract was carried out using gas chromatography-mass spectrometry. Minimum inhibitory concentration (MIC) of the extract against EHEC was determined by micro-dilution assay. Cytotoxic effect of the garlic extract on human colon adenocarcinoma cell line (SW480) was assessed using MTT assay. Micro-dilution assay was also used to determine the MIC of the extract against EHEC when co-cultured with SW480 cells.

Results: The amount of organosulfur in garlic extract was 70.91% and the most common organosulfur compounds were trisulfide, di-2-propenyl (34.8%) and diallyl disulfide (14.83%). The MIC of garlic hydro-alcoholic extract on EHEC alone and when co-cultured with SW480 was 12.5 mg/ml. Concentrations of 12.5 mg/ml and 25 mg/ml of the extract significantly reduced the viability of SW480 cells compared to control and concentration of 6.25 mg/ml of garlic extract ($p < 0.0001$).

Conclusion: The garlic hydro-alcoholic extract has inhibitory effects on EHEC in vitro. Therefore, it can be considered a suitable candidate for controlling infections caused by EHEC.

Keywords: Cell line, Plant extracts, Garlic.

INTRODUCTION

Enterohemorrhagic *Escherichia coli* (EHEC) O157: H7 is one of the most important *E. coli* pathotypes, which is responsible for the occurrence of bloody diarrhea, hemorrhagic colitis and hemolytic uremic syndrome worldwide (1). The low infectious dose of this bacterium (10-100 CFU) can be one reason for its high prevalence (2, 3). EHEC is colonized in the epithelial cells of the human colon, causing damages known as attaching and effacing lesions (4).

Treatment of EHEC infections with antibiotics is not recommended as they may increase the risk of hemolytic uremic syndrome (5). Hence, there is a need for finding novel or complementary treatments for EHEC infections. One of these approaches is the use of medicinal plants that contain chemical compounds active against a wide variety of disease-causing agents that can help maintain public health (6,7).

Garlic is a member of *Liliaceae* with the scientific name *Allium sativum*, a plant that has been long used to fight bacterial, viral and fungal infections (8, 9). The antibacterial properties of garlic are attributed to the presence of allicin and other sulfur-derived compounds (9). This compound is volatile and rapidly degraded to other sulfur compounds (10, 11). Allicin exerts its antimicrobial effects by various mechanisms, including the thiol-disulfide exchange, reaction with free thiol groups of various enzymes, binding to acetyl coenzyme A synthase and thus inhibiting DNA and RNA synthesis, reaction with enzymes and permeability through membrane phospholipids (12, 13). In this study, we evaluate the in vitro antibacterial effect of garlic hydro-alcoholic extract on EHEC bacteria.

MATERIALS AND METHODS

E. coli ATCC 43894 was cultured in BHI broth (Merck, Germany), and after ensuring purity, single colonies were used in subsequent steps. Fresh garlic bulbs were purchased (Gorgan, Iran), peeled, weighed (500 g) and cleaned. The garlics were sterilized using ethanol and homogenized aseptically. Ethanolic extraction was performed by maceration method. The obtained supernatant was then passed through a 0.45 µm syringe filter. Finally, a thick yellow extract with a pungent garlic odor was obtained (10).

Identification of extract constituents was carried out by gas chromatography–mass spectrometry (GC-MS) analysis using the method described by Pure et al. (14). The minimum inhibitory concentration (MIC) of garlic hydro-alcoholic extract (0.6-100 mg/ml) was determined by micro-dilution assay according to the Clinical and Laboratory Standards Institute (CLSI) (15). Briefly, serial dilutions of the garlic extract were prepared ranging from 0.6 to 100 mg/ml. Then, 100 µl from each dilution were added to each well of a 96-well plate containing 100 µl of BHI broth inoculated with 10 µl of bacterial suspension (10^5 CFU/ml). Well containing BHI broth alone, BHI broth and bacteria (positive control) and extract and medium (negative control) were considered as the controls. The plates were incubated at 37 °C for 24 hours. The lowest concentration of the extract that inhibited bacterial growth was selected as the MIC. To determine MBC, the MIC and two higher concentrations of the extract were inoculated into BHI Agar, and the lowest concentration in which there was no bacterial colony was considered as the MBC. The EHEC growth curve (absorbance at 570 nm) during 24 hours of incubation in presence of different garlic extract concentrations was plotted and compared with growth on free garlic extract. Next, 2×10^4 SW480 cells were seeded onto wells of a 96-well flat bottom microtiter plate containing RPMI 1640 medium with GlutaMax (Gibco Inc., America), 10% heat-inactivated fetal bovine serum (Gibco Inc., America) and 1% penicillin-streptomycin (Bio-idea Inc., Iran). The plate was incubated at 37 °C in a CO_2 incubator to reach a confluency of about 70%. Then, different concentrations (6.25, 12.5 and 25 mg/ml) of garlic hydro-alcoholic extract were added to the wells, and the plate was incubated for 24 and 48 hours at 37 °C and 5% CO_2. The effect of garlic extract on SW480 cells was assessed using MTT assay as described previously (16). The percentage of living cells was calculated using the following formula (17): Cell viability (%) = {(Absorbance of treated - Absorbance of Blank) / (Absorbance of Control - Absorbance of Blank)} ×100. The effect of garlic extract on morphology of SW480 cells was investigated using an invert microscope (*Nikon Eclipse TS100, Japan*) (18).

A confluent monolayer of SW480 cells in 96-well plate was washed with phosphate buffered saline three times. The cells were co-cultured with 10^5 CFU/ml bacterial suspension (MOI=100) treated with serial dilutions of garlic extract (0.02-50mg/ml). The RPMI medium with and without garlic extract were used as negative controls and RPMI with EHEC was used as the positive control. The plate was incubated at 37 °C overnight in a CO_2 incubator (19). Since a rapid decline in pH and the subsequent yellow color (indicating acidity) is a sign of bacterial contamination, bacterial growth was assessed by changing color from pink to yellow (20). All experiments were performed independently three times, using duplicate samples each time.

Statistical analysis was performed using the SPSS 16 software (SPSS, Inc., Chicago, IL), and one-way analysis of variance (ANOVA) was applied at statistical significance of ≤ 0.05.

RESULTS

The results of the GC-MS chromatogram analysis of garlic hydro-alcoholic extract are summarized in table 1. Based on the results, 22 compounds were identified, 70.91% of which belonged to organosulfure compounds. Trisulfide, di-2-propenyl (34.8%) and diallyl disulfide (14.83%) were the predominant components of the extract. Acetic acid, 2-furancarboxaldehyde and hexadecanoic acid were the most common non-sulfur components.

Table 1- Organosulfur and non-sulfur compounds in garlic hydro-alcoholic extract

	Components	Area %	Assurance %
Organosulfure components	Trisulfide, di-2-propenyl	34.8	76
	Diallyl disulfide	14.83	72
	Trisulfide, methyl 2-propenyl	11.49	64
	Dithio(1-Propenyl)Propionate	2.97	49
	3-Vinyl-1,2-dithiocyclohex-5-ene	1.78	92
	Diallyl tetrasulphide	1.6	91
	3-Vinyl-1,2-dithiocyclohex-4-ene	1.42	96
	Thiophene, 2-propyl-	1.3	45
	1,2-Dithiolane	0.72	46
Non-sulfure components	Acetic acid, methyl ester	3.6	47
	2-Furancarboxaldehyde, 5-(hydroxy methyl)	2.95	94
	Hexadecanoic acid	2.33	99
	Trimethylsilyldiazomethane	2.03	46
	Phenol, 2,4-bis(1,1-dimethylethyl)-	1.87	97
	Cyclotrisiloxane, hexamethyl-	1.06	35
	Mannitol	0.96	43
	Leucine	0.88	64
	Hydrazinecarbodithioic acid, 1-methyl ester	0.81	35
	Bis(2-ethylhexyl) phthalate	0.71	86
	N,N-Dimethylhexanamide	0.69	38
	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	0.67	72
	2-Furancarboxaldehyde, 5-(hydroxy methyl)	0.16	94

The susceptibility of EHEC to garlic hydro-alcoholic extract was determined quantitatively by determining MIC and MBC values. The MIC and MBC of garlic extract on EHEC was 12.5 mg/ml and 25 mg/ml, respectively. At all concentrations except 1.56 mg/ml, the extract significantly reduced growth of EHEC, especially before 14:00 hour compared to control ($p \leq 0.05$, $F = 29.86$) (Figure 1).

The results of the MTT assay were analyzed to determine the percentage of living cells after 24 and 48 hours of exposure to different concentrations of the garlic hydro-alcoholic extract. The results showed that cell growth was stopped at concentrations of 12.5 and 25

mg/ml. At concentration of 6.25 mg/ml, about 38% and 62% of the cells remained alive after 24 and 48 hours, respectively.

The cell Viability changed significantly following treatment with different concentrations the extract for 24 and 48 hours ($p \leq 0.0001$) (Figure 3).

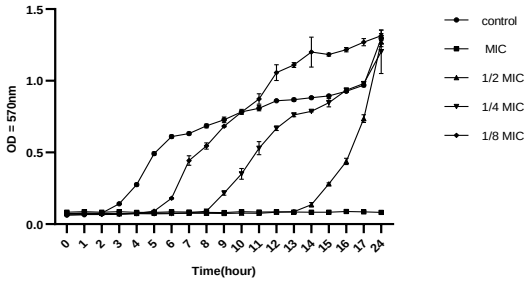

Figure 1- EHEC growth curve in the presence of MIC, ½ MIC, 1/4 MIC and 1/8 MIC of the garlic hydro-alcoholic extract

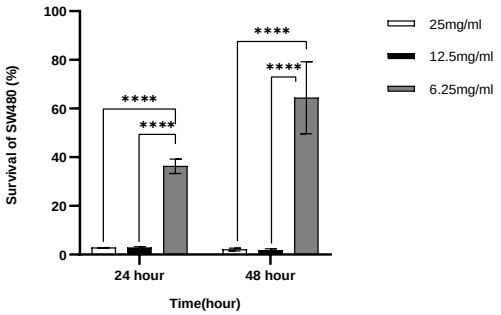

Figure 2- Survival of SW480 cells after exposure to different concentrations of garlic hydro-alcoholic extract for 24 and 48 hours. **** p<0.0001

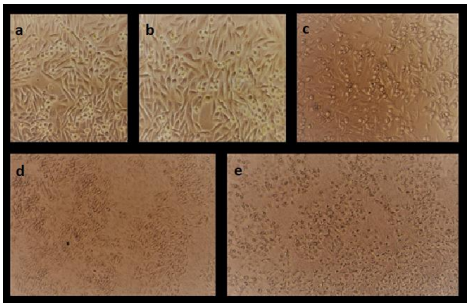

Figure 3- Effect of different concentrations of garlic hydro-alcoholic extract on SW480 cell line. a: Untreated SW480 cell (control), b: 3.12 mg/ml of extract, c: 6.25 mg/ml of extract, d: 12.5 mg/ml of extract, e: 25 mg/ml of extract

At concentrations of 12.5 mg/ml and 25 mg/ml, the extract altered the SW480 cell morphology mostly to round dead cells. At concentration of 6.25 mg/ml, the extract partially affected cell morphology. No morphologic change was noted at concentration of 3.12 mg/ml compared with the control. The MIC of garlic hydro-alcoholic extract against EHEC was 12.5 mg/ml, but it was not clear whether this inhibitory effect was due to the cytotoxic effect of the extract on SW480 cells or bacteria.

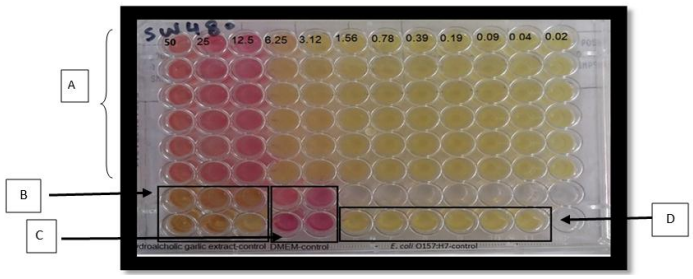

Figure 4- The MIC of garlic hydro-alcoholic extract against EHEC bacteria in SW480 cell line. A: Effect of different concentrations (0.02-50 mg/ml) of the garlic extract on EHEC; B: Negative control (garlic extract without bacteria); C: Negative control (only RPMI medium); D: Positive control (bacteria with no extract). The yellow and pink colors indicate bacterial growth and bacterial inhibition, respectively.

DISCUSSION

The medicinal properties of plants depend on their phytochemical constituents, which can vary according to temperature, pH, method of extracting the active ingredients, solvent, soil where grown, time of harvesting, growth site, etc. (11, 21). In this study, the effect of garlic hydro-alcoholic extract on *E. coli* O157: H7 was investigated under two different in vitro conditions. The total amount of organosulfur compounds was 70.91%, while trisulfide di-2-propenyl and diallyl disulfide were the main components of the extract, which is similar to results of a study by Yu et al. (22). However, in a study by Pure et al., organosulfur compounds constituted 54.92% of fresh garlics from Iran (14). Daniel reported presence of 43 volatile compounds in ethanol extract of raw garlic obtained by maceration method, 86% of which were sulfur compounds and their derivatives (10). In a study by Park et al., the amount of sulfur compound and allyl trisulfide constituted only 5.09% of garlic extract, which is much less than our finding (23). The difference in the results can be due to differences in the preparation method, duration of extraction, type of plant and growth conditions.

In the present study, the amount of non-sulfur compounds (18.72%) was less than sulfur-containing compounds. Numerous studies reported that non-organosulfur compounds from garlic could exert antimicrobial activities. In line with our study, Njue et al. reported presence of low amount of hexadecanoic acid 2,3-dihydroxy propyl ester in garlic extract, which has antimicrobial properties (11). Inconsistent with our findings, in another study, a phytoalexin called allixin (3-hydroxy-5-methoxy -6-methyl-2-penthy l-4H-pyran-4-one) was found in garlic. This non-sulfur compound has been shown to have antioxidant, anti-microbial, anti-tumor and neurotrophic effects (24).

Due to the presence of significant amount of sulfur compounds in the garlic extract, we expected to observe significant antimicrobial effect against EHEC. In a previous study, the MIC and MBC of ethanol extract of garlic on *E. coli* O157 was 200 mg/ml and 300 mg/ml, respectively (25), which are higher than the values obtained in the present study. The MIC of ethanol extract of garlic prepared by percolation method was estimated to be 0.5-1 mg/ml on *E. coli* O157, which is much lower than the value obtained in our study (6). In line with our findings, a study by Lee et al. showed that the aqueous extract of garlic had significant antimicrobial effect on *E. coli* with MIC and MBC values of 24 mg/ml and 96 mg/ml, respectively (26). Overall, evidence suggests that garlic extract has favorable inhibitory effects against *E. coli*.

To understand the potency of garlic ethanolic extract on EHEC for clinical use, the efficacy of this compound was examined against the SW480 cell line. The results of MTT assay showed that the extract significantly reduced the viability of SW480 cells at concentrations higher than 12.5 mg/ml. This finding was also confirmed by morphological observations. Petrovic et al. demonstrated that garlic extract can inhibit various cancer cell lines in vitro, such as multiple myeloma cells (27).

Li et al. showed that injecting raw garlic extract could damage cancer cells in a mouse model, and Gruhlke later attributed this effect to allicin derivatives (28, 29). In 2012, Ilić et al. demonstrated the cytotoxic activity of

allicin against human melanoma FemX and human embryonic lung fibroblast MRC-5 (21). Our results suggest that sulfur compounds other than allicin and its derivative may also contribute to the cytotoxic and antibacterial properties of garlic. Arunkumar et al. reported that the effect of diallyl disulfide on PC3 cancer cells is dose- and time-dependent (30). Our findings confirm the potency of the garlic extract in reducing the viability of cancer cells. In order to evaluate the effect of garlic extract on the growth of EHEC, it was necessary to make a comparison between MIC of garlic extract on EHEC grown in liquid medium and when co-cultured with SW480 cells. The MIC of garlic hydro-alcoholic extract on *E. coli* O157:H7 was 12.5 mg/ml in both situations. Therefore, the inhibitory effect of garlic extract on EHEC did not differ between the planktonic state (in vitro) and when co-cultured with SW480 cells.

CONCLUSION

The garlic hydro-alcoholic extract can inhibit the growth of EHEC in both in the planktonic state and when co-cultured with SW480 cells. The antibacterial effect of the extract might be related to the presence of allicin derivative such as trisulfide, di-2-propenyl, diallyl disulfide and trisulfide, methyl 2-propenyl. Based on the results, it is recommended to investigate efficacy of garlic extract for treatment of hemorrhagic colitis in animal models.

ACKNOWLEDGMENTS

This research was a part of Zynab Badeli's PhD project. We are sincerely grateful to Professor Seyed Shahram Shekarforoush, Naemeh Javid, Hanieh Bagheri, Masoud Bazouri and other members of the Microbiology Department of Golestan University of Medical Sciences for their kind collaboration.

DECLARATIONS

Funding

The study was financially supported by School of Veterinary Medicine, Shiraz University, Shiraz, Iran and the Laboratory Sciences Research Center, Golestan University of Medical Sciences, Gorgan, Iran.

Ethics approvals and consent to participate

This study was approved by the Ethics Committee of the Golestan University of Medical Sciences, Iran (ethical code: IR.GOUMS.REC.1398.010).

Conflicts of interest

The authors declare that there is no conflict of interest.

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How to Cite:
Badeli Z, Haghkhah M, Ghaemi EA [Phytochemical Analysis of Garlic Hydro-alcoholic Extract and Evaluation of its Anti-bacterial Effect on Enterohemorrhagic *Escherichia coli* in Vitro and ex Vivo]. mljgoums. 2021; 16(1): 25-31 DOI: 10.29252/mlj.16.1.25