

Comparative antibacterial activity of garlic essential oil extracted by hydro–distillation and diethyl ether extraction methods on four pathogenic bacteria

Abstract

Antibacterial activity of different concentrations of garlic oil crude extracts (50–1000µg/ml) and main isolation fraction from garlic extract utilizing TLC (10–100µg/ml) against four pathogenic bacteria, *Agrobacterium tumefaciens*, *Erwinia amylovora*, *Rhodococcus fascians* and *Pseudomonas solanacearum*, were investigated. Agar dilution method was used for determination of minimum inhibitory concentration (MIC) of the garlic extracts. Diethyl ether crude garlic oil extract exhibited the strongest bacterial action against *R. fascians* (MIC=100µg/ml) followed by *E. amylovora* (MIC=200µg/ml), *A. tumefaciens* (MIC=300µg/ml) and showed low activity against *P. solanacearum* (MIC=400µg/ml). *R. fascians*, *A. tumefaciens* and *E. amylovora* (MIC=250–300µg/ml) were more sensitive to the inhibitory activity of the hydro–distillation garlic oil extract than *P. solanacearum* (MIC=750µg/ml). The main isolating fraction from garlic oil crude that was extracted by hydro–distillation method exhibited the strongest bacterial action against *R. fascians* (MIC=20µg/ml) followed by *E. amylovora* (MIC=60µg/ml) and exhibited low activity antibacterial against *A. tumefaciens* and *P. solanacearum* (MIC=>100µg/ml). Essential oil component isolated from the garlic oil extracted by diethyl solvent method exhibited marked inhibition activity against *R. fascians* (MIC=10µg/ml) that was comparable to ampicillin bactericidal activity (MIC=6µg/ml)

Volume 4 Issue 2 - 2016

Hamdy R Soltan, Soad M Ahmed, Doaa A Emam

Department of Pesticide Chemistry, Faculty of Agriculture, Egypt

Correspondence: Hamdy R. Soltan, Department of Pesticide Chemistry, Faculty of Agriculture, Alexandria University, Alexandria, Egypt, Email hrsoltan@hotmail.com**Received:** March 18, 2016 | **Published:** July 21, 2016

Introduction

The environmental problems caused by overuse of pesticides have been the matter of concern for both scientists and public in recent years. A widespread use of pesticides leads to their accumulation in soil, water basins, groundwater, and fruits with subsequent transmission through the food chain to humans and mammals. Thus, on the one hand, one needs to search the new highly selective and biodegradable pesticides to solve the problem of long term toxicity to mammals and, on the other hand, one must study the environmental friendly pesticides and develop techniques that can be used to reduce pesticide use while maintaining crop yields. More recently, the encouragement of use of products from natural resources and even the extremely biodegradable synthetic and semi synthetic products in pest management, has been considered to constitute the umbrella of green pesticides.^{1–5} *Allium sativum* L., garlic, is one of the most representative species of the genus, used as food, spice and medicinal plant since ancient times. The medicinal value of the plant is consistent with various biological properties such as its antimicrobial, cardiovascular and anticancer effects.^{6–8} Garlic is still being employed in folk medicine all over the world for the treatment of a variety of diseases.⁹ Evidence from several investigations suggests that the biological and medical function of garlic is mainly due to their high organo–sulphur compounds content.¹⁰ The primary sulphur–containing constituents in hole vegetable is the S–alk(en)yl–L–cysteine sulphoxides (ACSOs), such as alliin, and γ–glutamylcysteines, which, besides to serve as important storage peptides, are biosynthetic intermediates for corresponding ACSOs from which, and by different metabolic pathways in each vegetable, volatile, such as allacin, and lipid–soluble sulphur compounds, such

as diallyl sulphide (DAS), diallyl disulphide (DADS) and others, are originated.¹¹ These compounds provide to garlic and onion their characteristic odour and flavour, as well as most of their biological properties.¹² This agent exhibits anti–bacterial, anti–fungal and anti–viral properties.¹³ The actual use of these products in the control of plant diseases is, however, still limited. Literature on the medicinal values of garlic abound. There are, however, very few references on the use of garlic clove extracts to control plant pathogens. The aim of this investigation was to study the efficacy of essential oil extracts by hydro–distillation and diethyl ether method of fresh garlic bulb against four major bacterial pathogens.

Material and methods

Sample preparation

Hydro distillation Technique (Steam distillation): The essential oils were obtained by hydro distillation in a vertical distillation unit under vacuum (0.4)m bar from fresh garlic bulbs (*Allium sativum*) which were purchased from the local market in Alexandria, Egypt. Garlic cloves Samples (1000 g) were chopped in small pieces, homogenized in (1L) distilled water using a domestic blender (model MX–X61–W, National, Japan) during 1 min at medium speed, then homogenate was macerated during 1h. A flask containing the homogenate was heated during 3h and the vapor condensed and separated throughout an auto–oil/water separator. Low pressure pipe water was constantly switched on to cool down the heated garlic oil. Each extraction was running in triplicate.¹⁴ The essential oil obtained was dried with anhydrous sodium sulfate and then stored at 4°C in tubes under protection from light until use.

Extraction by Solvent Extraction: Garlic bulbs (500g) were separated into cloves, cleansed and the skin was peeled off. The peeled cloves (400g) were cut into small pieces and soaked in 500mL of diethyl ether for 48 h after which the supernatant was decanted. Extraction of oil from the garlic residue left behind was repeated thrice with more solvents. The oil was recovered from the combined extracts by distilling off the solvent at 40°C. The oil was shaken with 20 volumes of redistilled petroleum ether (boiling point 40–60°C) and centrifuged. The clear supernatant was removed and the petroleum ether soluble fraction of the oil was prepared by distilling off the solvent at 60°C.¹⁵

Analytical tools

TLC analysis of sulfur compounds: TLC analysis of garlic oil extract samples were carried out according to the method described by Itakura et al.,¹⁶ Garlic samples were spotted onto preparative thin layer chromatography (0.5cm thickness) Silica gel G60 plates (Merck, Darmstadt, Germany) and developed using chloroform/methanol/water (6:4:1) as an eluting solvent. After the solvent front reached the top of the plate, it was taken out from the chromatographic tank and allowed for air drying. Plates were exposed to the iodine vapor reagent for detection of sulfur compounds. The main spot has R_f value equal 0.8 was scraped from plate, extracted by petroleum ether 40–60°C, evaporated to dryness by rotary evaporator and redissolved in appropriate solvent suitable for antibacterial activity test.

Gas chromatographic–mass spectrometry analysis: A gas chromatograph Hewlett–Packard 5890 (series II), which was modified for a glass capillary column coupled to a HP GC–mass selective detector (5971B MSD) was used. Hydrocarbons and the methyl esters of fatty acids were analyzed by gas chromatography on two capillary coupled columns as described previously,¹⁷ and also: HP–5, 10m, ID–0.32mm, film thickness 0.25 mm, coupled with a second capillary column RTX–1701 (Restek, PA, USA) 30m, internal diameter is 0.32mm, 0.25mm film, and coupled with a third capillary column HP–FFAP, 30m, 0.32mm, 0.25 mm film. GC oven was programmed: 40°C 2min, 2°C/min to 300°C, 20min at 300°C. Injector temperature was kept on 180°C (split less mode). Flow rate of the carrier gas (helium) was 25cm/s. MS detector was operated at 194°C, ionization energy was 70eV. Scan range was from 30 to 650m/z at scan rate of 0.9s–1. Solvent delay was 10min. Organ sulfur compounds were identified by NIST (National Institute for Standards and Technology) mass spectral library.

Tested Bacteria

Four bacteria species *Agrobacterium tumefaciens* (crown gall disease), *Erwinia amylovora* (Fire blight on apple, pear, and other rosaceous crops), *Rhodococcus fascians* (Known as *Corynebacterium fascians* until 1984, causes leafy gall disease) and *Ralstonia solanacearum* (*Ralstonia* was recently classified as *Pseudomonas* is soil-borne and motile with polar flagellar tuft, bacterial wilts of tomato, pepper, eggplant and Irish potato), were provided by microbiology laboratory, Department of Plant Pathology, Alexandria University. The bacteria species were maintained on Nutrient Agar (NA: peptone 10g, meat extract 5g, sodium chloride 2.5g and agar 10g in 1000ml distilled water at pH 6.5–6.6) medium.

Antibacterial activity test: Agar dilution method was used, as recommended by European Society of Clinical Microbiology and Infectious diseases,¹⁸ for determination of minimum inhibitory

concentration (MIC) of the garlic extracts. Garlic extracts were dissolved in acetone. Appropriate volumes of the stock solution were added to molten nutrient agar to obtain a range of concentrations 50, 100, 200, 300 and 400ppm (in the case of crude extracts) before pouring to Petri dishes. Also, another separate experiment was carried out for determination of minimum inhibitory concentration (MIC) of the garlic extracts to Bacteria species, *A. tumefaciens*, *E. amylovora* and *R. fascians* a range of concentrations 225, 250 and 275ppm, *P. solanacearum* a range of concentrations 500, 750 and 1000ppm. After solidifications, 6µl of bacterial cultures grown in nutrient broth for 12 hours (approximately 108 CFU/ml) was spotted (three spots per each plate) using 2µl standard loop on the surface of agar. The inoculum spots were allowed to dry before inverting the plates for incubation at 35°C for 24h. The antibiotic, Ampicillin (1–10), was used for comparison. Concentration of Antibiotic, Ampicillin, was used for comparison to *Pseudomonas solanacearum* 15, 20, 25 and 50ppm. The MIC is the lowest concentration of the agent that completely inhibits visible growth as judged by the naked eye, disregarding a single colony or a thin haze within the area of the inoculated spot.

Another separate experiment was carried out for the main organo sulfure compound (R_f=0.8) that isolated from the garlic oil crude, either isolated from garlic oil crude extracted obtained by hydro distillation or solvent method, using TLC method described previously. This compound was tested for its bactericidal efficacy against the four bacteria. Appropriate volumes of the stock solution were added to molten nutrient agar to obtain a range of concentrations 10, 20, 40, 60, 80 and 100ppm before pouring to Petri dishes and the lowest concentration of the agent that completely inhibits visible growth as judged by the naked eye.

Results and discussion

Yield and chemical composition

The average yield of essential oil of *Allium sativum* is 0.11%. The results of chemical analysis of the essential oil are contained in Table 1. The components determined by GC–MS in the garlic oils obtained by hydro-distillation were identified consisting of sulfides with different percentages with allyl methyl sulfide (13.105%), allyl methyl disulfide (7.231%), diallyl trisulfide (24.253%) and diallyl disulfide (34.276%) are the major components of this essential oil while the other components are identified in a relatively small percentage: 1-oxa-4-6-diazocyclooctane-5thione (8.398%), 3H-1,2-dithiol-3-one,4,5-dimethyl (2.174%), 1-3 Dithiane (1.57%), and Dimethyl trisulfide (2.304%). In our study, chemical analysis performed by GC/MS revealed that essential oil of *Allium sativum* is characterized by the presence of two major compounds, which are: diallyl trisulfide (24.253%) and diallyl disulfide (34.276%) together with other constituents at relatively low levels. This essential oil is substantially not similar in chemical composition to that found by O’Gara et al.,¹⁹ for a plant of the same species from India whose oil essential is mainly composed by diallyl disulfide (53.00%) and diallyl trisulfide (11.5%) while the profile found by Pyun et al.,²⁰ with a plant native to Korea, however, is significantly different with certain compounds that are not detected in our study as: N,N-dimethylthiourea (1.46%), 3-vinyl-4H-1,2-dithiine (1.99%), 3,3-thio bis-1-propene (0.87%). The difference in composition found on the essential oils investigated is likely to be related to the extraction method. Garlic oil is mostly prepared by solvent extraction consists of the diallyl (52%), allyl methyl (29%) and dimethyl (6.6%) di, tri and tetra sulfides. A typical

commercial preparation of garlic oil contains diallyl disulfide (DADS, 28.4%), diallyl trisulfide (DATS, 20.4%), allyl methyl trisulfide (18.3%), allyl methyl disulfide (4%), 1-3 Dithiane (5.5%), diallyl tetrasulfide (0.7%), allyl methyl sulfide (9.3%), 3-Vinyl-[4H]-1,2-dithiin (4%), dimethyl tetrasulfide (2.4%), dimethyl trisulfide (2.2%) and diallyl sulfide (2.3%). Diethyl ether extracted garlic oil contains nine times as much of the dithiins (9.5 mg/gm) and 1-oxa-4-6-diazocyclooctane-5-thione (8.398mg/g) and ten times as much of the allyl methyl trisulfide (16.3mg/g) than garlic oil extracted by hydro-distillation method. The results suggest that with extraction methods at high temperatures cyclic compounds are obtained and that processes at room temperature favor the formation of cyclic compounds in greater proportion. Those results obtained were agreed with that found by Lawson.²¹

Isolation and identification methods

Analysis of oil extract on thin layer chromatography (silica gel G) using solvent system chloroform/methanol/water (6:4:1) showed the sulfur compounds detected as pale yellow spots diallyl disulphide (DADS) as the major constituent with an Rf value of 0.8 as reported earlier by Mathew et al., 1996. This spot band have this Rf value was scaped from the silica gel plate, re-dissolved in appropriate organic solvent to evaluate its bioactivity against bacteria.²²

Antibacterial activity of garlic oil

The minimum inhibitory concentrations (MIC) are the lowest concentration of the agent that completely inhibits visible growth as judged by the naked eye, disregarding a single colony or a thin haze within the area of the inoculated spot. The results presented in Table 2 showed that the garlic oil crudes exhibited variable degrees of antibacterial activity against all of tested bacterial strains. In the dose response study, the inhibition zone increased with an increasing concentration of extracts. Low concentrations inhibited weakly the development of bacteria. However, *Rhodococcus fascians* was more sensitive than other tested bacteria. Hydro-distillation of crude garlic oil exhibited the strongest bacterial action against *R. fascians* (MIC=250µg/ml) followed by *Agrobacterium tumefaciens* and *Erwinia amylovolora* (MIC=300µg/ml) which exhibited moderate activity. On the other hand, this crude showed low activity against *Pseudomonas solanacerum* (MIC=750µg/ml).

In general, crude garlic oil extracted by diethyl ether solvent exhibited marked inhibition activity against bacteria, and inhibition was stronger than those of garlic oil extracted by hydro-distillation. Comparatively, *R. fascians* and *E. amylovolora* were more sensitive to the inhibitory activity of the diethyl ether solvent than *P. solanacerum* and *A. tumefaciens*. According to the MIC values, the results cited that the strong antibacterial activity against *R. fascians* and *E. amylovolora* were 100µg/ml and 200µg/ml, respectively. Whereas, it showed low bactericidal activity against *P. solanacerum* (MIC=300µg/ml) and weakly active results against *A. tumefaciens* (MIC=400µg/ml). The bactericidal activity of ampicillin expressed as MIC value was 6µg/ml against *A. tumefaciens*, *E. amylovolora* and *R. fascians* and 15µg/ml against *P. solanacerum* as represented in Table 2.

Bactericidal activity of main organosulfur isolated using TLC technique from garlic oil extracted by the two extraction methods against *A. tumefaciens*, *P. solanacerum*, *E. amylovolora* and *R. fascians* by minimum inhibitory concentration (Table 3). The obtained data revealed that different concentration was required of the main component isolated from garlic extract to kill different types of bacteria and also indicated that each bacterium had its own resistant and susceptibility level against antibacterial substances. The results

presented in Table 3 showed that the isolating fraction from garlic oil crude extracted by hydro-distillation method exhibited the strongest antibacterial action against *R. fascians* (MIC=20µg/ml) followed by *E. amylovolora* (MIC=60µg/ml) and exhibited low activity antibacterial against *A. tumefaciens* and *P. solanacerum* (MIC=>100µg/ml). Whereas, the fraction isolated from garlic oil crude that was extracted by diethyl ether showed the strongest bactericidal activity against *R. fascians* and *E. amylovolora* with MIC=10µg/ml and 20µg/ml, respectively. However, it was posses weakly bactericidal activity against *P. solanacerum* and *A. tumefaciens* (MIC=>100µg/ml). The bactericidal activity of the standard bactericide ampicillin against *A. tumefaciens*, *E. amylovolora* and *R. fascians* was 6µg/ml while for *P. solanacerum* was 15µg/ml.

From the experiment, we could obviously see that *R. fascians* and *E. amylovolora* were more sensitive to the fraction isolated from garlic oil crude compared to *A. tumefaciens* and *P. solanacerum*. This may be explained by a few factors such as the present of a thick peptidoglycan layer in less sensitive bacteria and thin peptidoglycan layer in more sensitive bacteria. In general garlic oil fraction exhibited different inhibition levels against bacteria strain tested. Main organ sulfur component isolated from the garlic oil crude utilizing TLC which extracted by diethyl solvent method exhibited marked inhibition activity against *R. fascians* (MIC=10µg/ml). This result indicated that there are possibilities to obtain bioactive component gave approximate efficacy nearly to ampicillin bactericidal activity (MIC=6µg/ml) as shown in Table 3.

Finally, it is concluded from the results of this investigation that essential oils extracts of common garlic was found to inhibit bacterial. However, the effectiveness of this inhibition was strongly related to the type of extraction method of garlic oil extracts used. Garlic essential oil extracts exhibited different inhibition levels against tested bacteria. Garlic oil extracts exhibited marked inhibition activity against bacteria at high concentrations. Of course these results obtained *in vitro* are only a first step in finding new antibacterial products offering natural biocide. Additional tests are necessary before the confirmation of the highlighted performances.

Table 1 Results of the GC-MS analysis of garlic extracts

Compound	Percentage	
	Hydro-distillation	Diethyl ether
Diallyl sulfide	2.207	2.3
Diallyl trisulfide	24.253	20.4
Diallyl disulfide	34.276	28.4
Diallyl tetrasulfide	1.019	0.7
Allyl methyl sulfide	13.105	9.3
Allyl methyl disulfide	7.231	0.2
Allyl methyl trisulfide	0.138	16.3
Dimethyl disulfide	0.689	2.2
Dimethyl trisulfide	2.304	2
Dimethyl tetrasulfide	0.893	2.4
1-propenyl methyl disulfide	0.22	0.1
1-oxa-4-6-diazocyclooctane-5thione	8.398	-
3,5 diethyl 1,2,4 trithiolane	0.248	0.2
1-3 Dithiane	1.57	5.5
3H-1,2-dithiol-3-one,4,5 dimethyl	2.174	-
Di 1-propenyl sulfide	0.718	-
5 methyl -1,2,3thiadiazole	0.341	-
1,2-dithiacyclopentane	-	0.4
3-methylthio propanal	-	0.3
3-Vinyl-[4H]-1,2-dithiin	-	4

Table 2 Comparison of minimum inhibitory concentration (ppm) values of garlic oil extraction by steam distillation and solvent extraction by diethyl ether and ampicillin on bacterial strains

Minimal inhibitory concentration (ppm)			
Bacterial strains	Garlic oil extraction by steam distillation	Garlic oil extraction by solvent (Diethyl Ether)	Ampicillin
<i>A. tumefaciens</i>	300	400	6
<i>P. solanacerum</i>	750	300	15
<i>E. amylovolora</i>	300	200	6
<i>R. fascians</i>	250	100	6

Table 3 Comparison of MIC ($\mu\text{g ml}^{-1}$) of garlic oil extraction by fraction (A), fraction (B) and ampicillin on bacterial strains

Minimal inhibitory concentration (Ppm)			
Bacterial strains	Fraction isolated from garlic oil (A)	Fraction isolated from garlic oil (B)	Ampicillin
<i>A. tumefaciens</i>	>100	>100	6
<i>P. solanacerum</i>	>100	>100	15
<i>E. amylovolora</i>	60	20	6
<i>R. fascians</i>	20	10	6

Acknowledgements

None.

Conflict of interest

The author declares no conflict of interest.

References

- Koul O, Dhaliwal GS, Marwaha SS, et al. Future perspectives in biopesticides. In: Koul O et al. editors. *Biopesticides and Pest Management*. 1, New Delhi, India: Campus Books International; 2003. p. 386–388.
- Koul O. *Insect Antifeedants*. Boca Raton, USA: CRC Press; 2005. 1024 p.
- Dhaliwal GS, Koul O. *Biopesticides and Pest Management: Conventional and Biotechnological Approaches*. India: Kalyani Publishers; 2007. 455 p.
- Koul O. Phytochemicals and insect control: An Antifeedant approach. *Crit Rev Plant Sci*. 2008;27(1):1–24.
- Koul O, Singh G, Singh, R, et al. Mortality and reproductive performance of *Tribolium castaneum* exposed to anethole vapours at high temperature. *Biopestic Int*. 2007;3(2):126–137.
- Block E. The organosulfur chemistry of the genus *Allium* – Implications for the organic chemistry of sulfur. *Angew Chem*. 1992;31(9):1135–1178.
- Agarwal KC. Therapeutic actions of garlic constituents. *Med Res Rev*. 1996;16(1):111–124.
- Nagourney RA. Garlic: medicinal food or nutritious medicine? *Med Food*. 2009;1(1):13–28.
- Ali M, Thomson M, Afzal M. Garlic and onions: their effect on eicosanoid metabolism and its clinical relevance. *Prostaglandins Leukot Essent Fatty Acids*. 2000;62(2):55–73.
- Augusti KT, Mathew PT. Lipid lowering effect of allicin (diallyl disulfide oxide) on long-term feeding in normal rats. *Experientia*. 1974;30(5):468–470.
- Lancaster JE, Shaw ML. G–Glutaryl peptides in the biosynthesis of S–alk(en)yl–L–cysteine sulfoxides (flavor precursors) in *Allium*. *Phytochem*. 1989;28(2):455–460.
- Lanzotti V. The analysis of onion and garlic. *J Chromatogr A*. 2006;1112(1–2):3–22.
- Pszczola DE. Antimicrobials: setting up additional hurdles to ensure food safety. *Food Technol*. 2002;56(6):99–107.
- Benkeblia N. Antimicrobial activity of essential oil extracts of various onions (*Allium cepa*) and garlic (*Allium sativum*). *Lebensm–Wissu–Technol*. 2004;37(2):263–268.
- Mukherjee M, Das AS, Das D, et al. Role of peritoneal macrophages and lymphocytes in the development of hypogonadal osteoporosis in an ovariectomized rat model: possible phytoestrogenic efficacy of oil extract of garlic to preserve skeletal health. *Phytother Res*. 2007;21(11):1045–1054.
- Itakura Y, Ichikawa M, Mori Y, et al. How to distinguish garlic from the other *Allium* vegetables. *J Nutr*. 2001;131(3):9635–9675.
- Dembitsky VM, Shkrob I, Dor I. Separation and identification of hydrocarbons and other volatile compounds from cultured blue–green alga *Nostoc sp.* by gas chromatography–mass spectrometry using serially coupled capillary columns with consecutive nonpolar and semipolar stationary phases. *J Chromatogr A*. 1999;862(2):221–229.
- ESCMID. Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by agar dilution. *Clinical Microbiology and Infection*. 2000;6(9):509–515.
- O’Gara EA, Hill DJ, Maslin DJ. Activities of garlic oil, garlic powder, and their diallyl constituents against *Helicobacter pylori*. *Appl Environ Microbiol*. 2000;66(5):2269–2273.
- Pyun MS, Shin S. Antifungal effects of the volatile oils from *Allium* plants against *Trichophyton* species and synergism of the oils with ketoconazole. *Phytomedicine*. 2006;13(6):394–400.
- Lawson LD. Garlic: a review of its medicinal effects and indicated active compounds. In: Lawson LD, et al editors. *Phytomedicines of Europe Series 691*, Washington, USA,; 1998. p. 176–209.
- Hughes BG, Lawson LD. Antimicrobial effects of *Allium sativum* L. (garlic), *Allium ampeloprasum* L. (elephant garlic), and *Allium cepa* L. (onion), garlic compounds and commercial garlic supplements products. *Phytotherapy Res*. 1991;5(4):154–158.