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Full Length Research Paper

Susceptibility of some multiple resistant bacteria to garlic extract

Muhsin A. Jabar[1]* and Amina Al-Mossawi[2]

[1]Microbilogy Department, College of Medicine, University of Kerbala, Iraq.
[2]School of Pharmacy, The Robert Gordon University, Aberdeen, Scotland, U.K.

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Historically, garlic (*Allium sativum*) has been found to possess many therapeutic properties including antimicrobial, antineoplastic, anticardiovascular, immuno-stimulatory and hypoglycaemic activities. Its antimicrobial activity is attributed to its key component allicin, which is rapidly synthesised from its precursor when garlic is crushed. This study assesses the antibacterial potentiality of garlic using modern microplate-based antibacterial assays. To evaluate the potency of raw garlic juice, various extracts (n-hexane, dichloromethane, methanol and water) and commercial preparations of garlic were screened. Three types of assay were performed using different garlic extracts: disc diffusion, checkerboard and resazurin. The results from fresh garlic were promising. However, none of the three commercial preparations tested had any significant activity.

Key words: Garlic, Allicin, Antimicrobial Agents, Resazurin assay.

INTRODUCTION

The use of plants in medicine goes as far back as thousands of years and still continues today (Arora and Kaur, 1999; Cavallito and Bailey, 1994). Many plants are used for the treatment of different diseases and many possess antimicrobial activities ((Arora and Kaur, 1999). Garlic (*Allium sativum*) has been consumed as a spice and medicine for thousands of years. Ancient Egyptians were known to use it for the treatment of diarrhoea; in ancient Greece it was used for intestinal and lung disorders and in Japan it has been used to treat common cold with headache, fever and sore throat. Garlic extracts are also known to be effective against *Helicobacter pylori*, the cause of gastric ulcers (Ankri and Mirelman, 1999). Even today, garlic is popular in use as an alternative remedy in infectious diseases such as otitis media (Klein, 1999).

Garlic has been found to possess many therapeutic properties including antimicrobial, anti-neoplastic, anticardiovascular, immuno-stimulatory and hypoglycaemic activities (Sato and Miyata, 1999). Its antimicrobial properties were first demonstrated in 1858 by Pasteur, and since then, the micro-organisms that have been known to be sensitive to garlic are bacteria, protozoa, fungi and viruses. A striking aspect of the activity of garlic is the apparent inability of most bacteria to develop resistance to it because its mode of action is completely different from that of other antibiotics (Cavallito and Bailey, 1994). It has been proposed that the development of resistance to betalactam antibiotics is 1000 fold easier than the development of resistance to allicin (Ankri and Mirelman, 1999), making garlic a prime candidate for therapeutic use.

The antibacterial property of garlic has been tested in many studies and *in vitro* experiments have shown inhibition of 14 species of bacteria (including *Staphylococcus aureus*, *Escherichia coli, Klebsiella pneumoniae*) by fresh garlic extracts (Farbman et al., 1993; Ankri and mirelman, 1999). The antimicrobial activity is, however, diminished upon boiling, which is attributed to its key component allicin, which is denatured at high temperature (Sato and Miyata, 2000)

Allicin is a volatile molecule, which gives garlic its characteristic odour. It has been shown that it is synthesised by the oxygenation of alliin, its stable precursor, in the presence of an enzyme termed allinase, which is also present in garlic cloves. The transformation of alliin to allicin is extremely rapid, being completed in seconds. Garlic is odour free until it is crushed and studies have shown that alliin and alliinase are stored in different compartments. This suggests that it is designed as a defence

*Corresponding authors E-mail: drmuhsin_jabar@yahoo.co.uk.

mechanism against invading pathogens. The active allicin molecule also has a very short half-life, keeping the defence mechanism rapid and much loc-alised, preserving the rest of the alliin in the clove for future attacks (Ankri and Mirelman, 1999). The main anti-microbial effect of allicin is due to its oxidative interaction with important thiol containing enzymes (Cavallito and Bailey, 1994; Perez-Giralo et al., 2003).

There are many garlic preparations now available in the market, claiming to have "all the benefits of garlic" in capsules, paste, etc. Some have even claimed to have 100% allicin yield. However, since no clinical trials of these products have been performed, the true beneficial potential of these preparations is not really known.

Many studies have shown the bacterial sensitivity to garlic using classical methods such as agar diffusion methods (Araro and Kaur, 1999). Very few studies, if any, have been performed to assess antibacterial potential of garlic using modern microplate-based antibacterial ass--ays, such as checkerboard assay, resazurin assay etc. To evaluate the potency of raw garlic juice, various extracts (n-hexane, dichloromethane, methanol and water), and commercial preparations of garlic were used in this study.

MATERIALS AND METHODS

Source of samples

1 kg of fresh garlic and 1 tube of Safeway Garlic paste were purchased from Safeway Supermarket, Chricklewood, London NW2, UK. 1 bottle of odourless garlic capsules and a packet of allimax (100% allicin powder) capsules were purchased from Holland and Barrets (UK) health food store.

Preparation of garlic extracts

The fresh garlic was primarily extracted by five solvents (water, ethanol, dichloromethane, n-hexane, and raw juice). Once the most potent extract was determined (water), the garlic preparations were extracted using the same method. The water extract and raw juice were also treated with acid and alkali, and more antibacterial tests were performed.

Water and ethanol extracts: 200 g of fresh chopped garlic were allowed to soak in 1 L of water or ethanol for 20 h, and the extracts were then filtered out. The capsules were emptied out and soaked in water for 20 h. The garlic paste was weighed and soaked in water for 20 h. The water extracts were freeze-dried and the ethanol extract was dried using a rotary evaporator at 30◦C. Water extracts were re-suspended in 40 ml water and ethanol extract in 30 ml ethanol and 10 ml water to achieve stock solutions of 5 g/ml of fresh garlic. For the odourless garlic capsules and garlic paste, the concentration of the stock solution was made up according to their equivalent in fresh garlic. Each capsule contains 50 mg of odourless garlic (equivalent to 500 mg of fresh garlic) where the weight of each capsule is about 0.22 g and the weight of empty capsule is 0.7 g; therefore the weight of contents of each capsule is taken to be 0.15 g. That is for every 0.15 g of capsule contents extracted there is 0.05 g of odourless garlic. The capsule contents of 3 g were weighed, which were taken to be the equivalent of 10 g of fresh garlic. This was extracted and suspended in 2 ml of water to produce 5 g/ml fresh garlic equivalent. The allicin powder was simply suspended in water (8 mg/ml).

Dichloromethane and n-hexane extracts: 200 g of fresh garlic were freeze-dried and coarsely ground using a coffee grinder. The grounds were then soxhlet-extracted (adapted from Prados-Rosales et al., 2003) with 1 L of n-hexane or dichloromethane. The extracts were then dried using a rotary evaporator at 30◦C. N-hexane extract was re-suspended in 40 ml DMSO and dichloromethane extract in 40 ml ethanol to achieve 5 g/ml of fresh garlic.

Raw garlic juice: Weighed, crushed garlic was pressed though filter paper and made up in volume with water to achieve a solution of 5 g/ml of fresh garlic.

Storage of stock solutions

After preparations, all stock solutions except raw garlic juice, which was freshly prepared with each experiment, were kept in the refrigerator to be used for all assays.

Treatment of water extract

5 ml of water extract or raw juice was added to 5 ml of a range of HCl and NaOH concentrations (0.1, 0.01 and 0.001 M) and left for 24 h. The mixtures were then freeze-dried and re-suspended in 5 ml of water.

Antibacterial Tests

Antibacterial activity of the extracts was tested against 8 strains of bacteria. These are four strains of *S. aureus*, with different multiple resistance patterns, two strains of *E. coli,* one strain of *Klebsiella aerogenes*, and one strain of *Salmonella goldcoast*. The bacterial cultures were from the properly identified and appropriately maintained stock cultures from the Microbiological Research Lab, School of Pharmacy, The Robert Gordon University, Aberdeen, UK.

The antibacterial tests performed were: Agar disc diffusion, 96-well micro-plate-based broth dilution method, also known as checkerboard assay (Kumarasamy, 2002; Lorian, 1991), and resazurin assay (adopted from Kuda and Yano, 2002). The calibration curves of absorbance versus colony counts of inoculum suspensions for each of the organisms were previously plotted according to the method described in literature (Pérez-Giraldo, 2003). This was to ensure the final cfu values were valid. Bacterial concentration of 5 x 10^6 cfu/ml was used. All tests were performed in duplicate. Ampicillin was used as a positive control. DMSO, ethanol, and water were used as negative controls

Preparation of bacterial solution

A single colony of bacteria was incubated at 37◦C. The bacterial suspension was centrifuged at 4000 rpm for 7 min, the supernatant was poured off and bacteria were re-suspended in normal saline (0.9 tration of 5 x 10^6 cfu/ml was calculated and the bacterial suspension %, w/v NaCl). This was repeated twice more. After the last run, the strain, the degree of dilution necessary to obtain a fina concen-bacteria were re-suspended in 5 ml normal saline. Using UV spectrophotometer (500 nm) and graphs of viability for the particular was prepared accordingly.

Table 1. The sensitivity of different bacterial stains to the raw garlic juice and garlic extract dissolved in water.

Bacterial species	Extract used in assay	Assay method		
		Disc Diffusion	Checkerboard	Resazurin
		Zone of inhibition	MIC (mg/ml)	MIC (mg/ml)
Staphylococcus aureus	Water	26	19.53	19.53
	Raw juice	25	19.53	19.53
Staphylococcus aureus	Water	19	9.77	9.77
	Raw juice	26	19.53	19.53
Staphylococcus aureus	Water	18	19.53	19.53
	Raw juice	21	31.06	31.06
Staphylococcus aureus	Water	18	19.53	19.53
	Raw juice	21	31.06	31.06
Escherichia coli	Water	14	19.53	19.53
	Raw juice	17	31.06	31.06
Escherichia coli	Water	15	19.53	19.53
	Raw juice	17	31.06	31.06
Salmonella goldcoast	Water	10	78.12	78.12
	Raw juice	14	39.06	39.06
Klebsiella aerogenes	Water	7	156.24	156.24
	Raw juice	11	78.12	78.12

Preparation of disc diffusion plates

6 mm sterile cellulose discs were loaded, using an eppendorf pipette, with 20 µl of an extract solution or a blank solvent (negative controls) and allowed to dry. Antibacterial discs were used for positive controls. 2 ml of bacterial solution was added to 18 ml of 10/9 strength nutrient agar at 50°C, and poured into a sterile Petri dish. Once the agar has solidified, the previously prepared extract, blank and antibacterial discs were placed on top of the agar (5 discs per plate) and incubated at 37°C overnight.

Preparation of 96-well-microplate

Two wells from each column in row 1 were marked and 100 µl of extract, blank or Ampicillin solution was added. 50 µl of sterile normal saline was added to rows 2 - 11. Two-fold serial dilutions were performed by transferring 50 µl of solution from row 1 to row 2, using a multichannel pipette. This was repeated down the rows to row 12. 40 µl of double strength nutrient broth and 10 µl of bacterial solution was added to all wells, so the final concentration of inoculum in all the wells was 5 x 10^5 cfu/ml. To prevent dehydration, the plates were covered with a plastic cover and then incubated at 37°C overnight. For the resazurin assay, the resazurin solution was prepared by dissolving a single tablet in 40 ml of sterile water. The above procedure was repeated except 10 µl of the resazurin solution was added to each well in the microtitre plate, and 30 µl of 3.3 times strength nutrient broth instead of 40 µl double strength was used in each well.

Minimum inhibitory concentration (MIC)

The plates were examined from below with a reflective viewer. The MIC was taken as the lowest duplicate concentration at which the test organism did not show visible growth. With the resazurin assay, the colour change in the indicator was taken as a sign of bacterial growth.

RESULTS AND DISCUSSION

There were three stages in the study: (i) Assessing the potencies of garlic extracted from different solvents; (ii) comparison of the most potent extract with available garlic products and (iii) assessing the potency of garlic under acid and alkali conditions. Throughout these stages, the three described antimicrobial methods were compared. The water extract of garlic, along with raw garlic juice, were found to have he highest potencies out of all extracts while the rest of the extracts had extremely low, if no potency at all. So that all assays were carried on with water extracts and juice only as presented in Table 1. The water extract and raw juice were also treated with different concentrations of HCl and NaOH andtested against 4 strains of bacteria. The results are shown in Table 2.

The checkerboard assay is probably of the most convenient way of assessing the antibacterial activity of garlic extracts. In this method, the test components can diffuse more easily into the media, producing more quantitative results than the agar diffusion method where only semi-quantitative can be achieved. The resazurin in the assay can be very useful at detecting growth which may not be seen as easily with the naked eye, thus producing slightly more accurate results. The agar diffusion method, however, is very convenient and time saving when it comes to preliminary and qualitative assays (Richards and Xing,

Table 2. The water extract and raw juice treated with different concentrations of HCl and NaOH and tested against strains of bacteria.

Bacterial species		Extract used in assay	Assay method		
			Disc Diffusion	Checkerboard	Resazurin
			Zone of inhibition	MIC (mg/ml)	MIC (mg/ml)
Staph.aureus	Water	+0.1M HCl	12	78.12	78.12
		+0.01M HCl	12	78.12	78.12
		+0.001M HCl	12	78.12	78.12
		+0.1M NaOH	9	625	625
		+0.01M NaOH	10	78.12	78.12
		+0.001M NaOH	12	39.06	39.06
	Raw juice				
		+0.1M HCl	-	78.12	78.12
		+0.01M HCl	17	78.12	78.12
		+0.001M HCl	25	39.06	39.06
		+0.1M NaOH	-	78.12	78.12
		+0.01M NaOH	16	78.12	78.12
		+0.001M NaOH	17	78.12	78.12
Staph.aureus	Water				
		+0.1M HCl	10	312.5	312.5
		+0.01M HCl	10	312.5	312.5
		+0.001M HCl	20	312.5	625
		+0.1M NaOH	-	-	-
		+0.01M NaOH	-	625	625
		+0.001M NaOH	8	625	625
	Raw juice				
		+0.1M HCl	-	-	-
		+0.01M HCl	18	156.25	78.12
		+0.001M HCl	24	78.12	78.12
		+0.1M NaOH	-	-	-
		+0.01M NaOH	25	312.5	156.25
		+0.001M NaOH	30	78.12	78.12
Escherichia coli	Water	+0.1M HCl	8	312.5	312.5
		+0.01M HCl	8	312.5	312.5
		+0.001M HCl	8	312.5	156.25
		+0.1M NaOH	-	-	-
		+0.01M NaOH	-	625	625
		+0.001M NaOH	-	625	625
	Raw juice				
		+0.1M HCl	-	-	-
		+0.01M HCl	14	78.12	78.12
		+0.001M HCl	14	78.12	78.12
		+0.1M NaOH	-	-	-
		+0.01M NaOH	10	156.25	156.25
		+0.001M NaOH	11	156.25	156.25

Table 2.contd.

Escherichia coli	Water				
		+0.1M HCl	-	156.25	156.25
		+0.01M HCl	-	156.25	156.25
		+0.001M HCl	-	156.25	156.25
		+0.1M NaOH	-	-	-
		+0.01M NaOH	-	625	625
		+0.001M NaOH	-	625	625
	Raw juice				
		+0.1M HCl	-	-	-
		+0.01M HCl	13	78.12	78.12
		+0.001M HCl	13	78.12	78.12
		+0.1M NaOH	-	-	-
		+0.01M NaOH	10	312.5	312.5
		+0.001M NaOH	11	156.25	156.25

1993).

All bacterial strains showed promising sensitivity to both water extract and fresh juice of garlic. The sensitivity of *E. coli* and *S. aureus* to a water extract of garlic agrees with earlier observations (Ankri and Mirelman, 1999). The sensitivity of *Salmonella* and *Klebsiella*, however, was lower than expected, but that is possibly due to the experiments being performed at a later time, with the activity of the water extract gradually decreasing.

Upon treatment with acid and alkali, the antibacterial activity of garlic still continued, but dramatically decreased, with the MIC increasing by 4 - 16 fold. The idea behind the treatment of the garlic extracts with acid and alkali was to slightly imitate stomach and duodenal conditions. However, once treated with the acid or the alkali, little could be done to control the activity. As the acid and alkali conditions were grossly exaggerated compared to the physiological ones, the activity would be expected to be higher in milder conditions.

Although there was no significant activity with extracts of organic solvents, it may be worthwhile to investigate organic solvent extract of the aerial part of garlic plants as it has been found that volatile metabolites also have an effect on bacterial growth (Tirranen et al., 2001).

Conclusions

The aqueous extract of garlic was found to be potent against all bacterial strains tested. Even when treated with acid or alkali, promising results were demonstrated, indicating activity even when taken orally, though more *in vivo* studies are needed to confirm this. The antibacterial activity of garlic has been recognised centuries ago and new methods to exploit this potential are still being developed. Unfortunately, there is a lack of interest of pharmaceutical companies in investing and developing garlic into a drug and performing clinical trials. This is due to the fact that no patents can be submitted on allicin because of its long-standing presence in the public domain (Ankri and Mirelman, 1999). As a result, many unlicensed garlic products have been released in the market, many of which have dubious or no activity at all, as was demonstrated during this study.

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