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Biochemistry & Analytical Biochemistry

Research Article

Synergic Interaction between Aqueous Garlic Extract (Allium Sativum) and Some Antibiotics against Tunisian Isolated Multi-Resistant Salmonella Serovars

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ABSTRACT

The present work aims to study the in vitro antibacterial activities of aqueous garlic extract (A.G.E.) and its effects on the antibiotic susceptibility of some Tunisian isolated Salmonella serovars. The disc diffusion assay revealed an A.G.E. antibacterial activity, characterized by inhibition zones ranging from 6 ± 1.7 to 16 ± 1.2 mm. Their sizes were clearly proportional to the amount of A.G.E. applied. MIC values differ from a serovars to another, and they ranged from 10-12.5 mg/ml, whereas the MBC values ranged from 13-15 mg/ml.The A.G.E. exhibited a synergistic, additive or antagonistic effect against the tested Salmonella serovars when combined with the different antibiotics. These effects were variable from a serovar to another and from an antibiotic to another. The mode of action of the A.G.E. on the Salmonella serovars was visible using TEM. These results support the use of A.G.E. for food bioconservation or microbial infection.

Keywords: Antibacterial activity; Antibiotic resistance; Garlic; Salmonella; Synergic effects; Morphologyeffects.

INTRODUCTION

Antimicrobial resistance (AMR) is a growing public health concern. The increase in the number of antimicrobial resistant pathogens in human medicine has raised both public and scientific interest, and some of this concern has focused on antimicrobial use in livestock production. The World Health Organization (WHO) has recognized the spread of AMR and the appearance of multiple antimicrobial resistant pathogenic bacteria as serious problems that can complicate medical treatments of bacterial infections (WHO, 2001; Yang et al., 2000). Therefore, for the safety of drug and food systems, there is a strong need to continually identify novel antimicrobial agents. Resistance to multiple substances is a problem of public health coming to world-wide level observation since the appearance of antibiotics (Gupta et al., 2001). Indiscriminate and abuse of antibiotics use and the environmental selective pressures induced by disinfectants and antiseptics have generated a microorganism survival answer. It enables the multi-resistant bacteria to efficiently evade the bactericidal action of some of these agents.Plants are known to produce a variety of compounds to protect themselves against a range of microorganisms, plant pathogens, soil and environmental organisms. These are successful defense mechanisms developed by plants. Therefore, plants and their secondary metabolites are promising sources to provide structurally diverse bioactive compounds as potentially therapeutic agents particularly antimicrobials. However, plant-derived antimicrobials are less potent. Hence, it becomes apparent that plants adopt different paradigm-synergy to combat infections (Hemaiswarya et al., 2008).

Garlic (Allium sativum) is a common food spice widely distributed and used worldwide as a spice and herbal medicine for the prevention and treatment of a variety of diseases, ranging from infections to heart diseases. The main antimicrobial constituent of garlic has been identified as the oxygenated sulfur compound, thio-2-propene-1-sulfinic acid S-allyl ester, which is usually referred to as allicin. This molecule is produced catalytically when garlic cloves are crushed and the enzyme allinase (alliinlyase E.C. 4.4.1.4) of the bundle sheath cells mixes

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Received date: April 21, 2021; **Accepted date:** September 14,2021; **Published date**: September 24, 2021

Citation: Hatem B (2021) Unité de Protéomie Fonctionnelle et Potentiel Nutraceutique de la Biodiversité de Tunisie. Institut supérieur des Sciences Biologiques Appliquées de Tunis. Université de Tunis El-Manar, Tunisie. Biochem Anal Biochem 10:10 p150

with its substrate, alliin, which is released from mesophyll cells (Miron et al., 2000; Curtis et al., 2004).Various garlic preparations have been shown to exhibit a wide spectrum of antibacterial activity against Gram-negative and Gram-positive bacteria including such species as Esherichia, Salmonella, Streptococcus, Staphylococcus, Klebsiella, Proteus, and Helicobacter pylori (Ankri and Mirelman, 1999; Reuter et al., 1996; Cutler and Wilson, 2004; Cellini et al., 1996; Jonkers et al., 1999). Even acid-fast bacteria such us Mycobacterium tuberculosis are sensitive to garlic (Uchida et al., 1975).

Salmonella species are one of the common pathogenic bacterial groups, which cause food-borne diseases. The emergence of resistant strains has increased progressively, particularly due to the consumption of processed food and agricultural products, which remain in contact with antibiotics (Logue et al., 2003). Salmonella species continue to be a major public health problem in developed and developing countries (Fang, 2005; Lampel et al., 2000). Recently, the number of food-borne diseases due to consumption of contaminated food and water has increased (Sivapalasingam et al., 2004; Tauxe et al., 1997).

In Tunisia, few studies have been addressed the antibiotic resistance of Salmonella and its evolution. In our present study, the in vitro inhibitory activities of aqueous garlic extract have been evaluated against multi-resistant strains of Salmonella, isolated from Tunisian foods, coproculture and wastewater. We have studied the possibility of synergistic effects between some antibiotics and aqueous garlic extract (A.G.E.) and investigated the morphological changes induced by aqueous garlic treatment, using transmission electronic microscopy.

MATERIEL AND METHODS

Aqueous garlic extracts preparation

Garlic cloves collected from the north of Tunisia (Beja), were used for this study. Fresh garlic cloves (70 g) were blended in 35 ml sterile distilled water, centrifuged at 5000 rpm then sterilised by membrane filtration (0.45µm).

Aliquots were stored at –20°C until required, for no longer than 2 weeks.

Allicin Concentration Determination

The concentration of allicin in each preparation was determined spectrophotometrically by reaction with the thio-4-mercaptopyridine.

Varying volumes of garlic extract were incubated with 4-mercaptopyridine (10-4mM) in 50mM phosphate buffer, 2 mM EDTA pH 7.2 which results in the formation of a mixed disulphide, 4-allylmercaptothiopyridine and the consequent shift in absorbance at 324 nm was monitored.

Bacteria and Growth Conditions

A total of six Salmonella serovars, isolated from Tunisian fast food (Merguez), coproculture and wastewater were employed in the present study.

They were listed in table 1: Salmonella cerro, Salmonella enteritidis, Salmonella lindenburg, Salmonella hadar, Salmonella montevideo, and Salmonella nikolaifleet, and they were characterized in our laboratory (Chatti et al., 2007).

Table1: Non-typhoidal Tunisian Salmonella serovars profiles. Long-term storage of Salmonella samples was at –20°C in sterile glycerol (15.)

Salmonella serovars	Isolat	Source	Antibiotic Resistance profile	Antigenic Formula
S. enteritidis	49	One-day-old chick	TET, STR	(9 : gm : -)
S. lindenburg	320	Fresh Milk	TET, AMP, STR	(6.8 : i: 1.2)
S. nikolaifleet	63	Coproculture	TET, AMP, STR	(1.6 : gms : -)
S. cerro	291	Retail ground beef meat (Merguez*)	TET, STR	(1.8 : Z4Z23 : 1.5)
S. montevideo	297	Wastewater	TET, AMP, NEO	(6.7 : gms : -)
S. hadar	287	Turkish meat	TET, AMP, NEO, GEN, STR	(6.8 : Z10/ENX)

A pre-culture was prepared by the transfer from this culture to a fresh sterile liquid medium (Pronadisa, Hispanlab) and cultivated for 18 hours at 37°C with shaking.

A.G.E. Antibacterial activities were tested using the well diffusion assay. 200 µl of the fresh bacterial cell suspension pre-cultures (inoculum, ≈108 CFU/ml) containing Salmonella

serovar was inoculated in the molten nutrient agar (28 g/l) (Pronadisa, Hispanlab) petri plates.

Sterile Whattman n°1 filter paper discs (6mm in diameter) were individually placed on the inoculated plates and impregnated with the different A.G.E concentrations. After 30-min standing in sterile conditions at room temperature, plates were incubated for 24 hrs at 37°C. The diameter of the clear zone shown on plates was accurately measured and expressed in millimetres as its antimicrobial activity. Sterile deionised water was used as negative control; the standard antibacterial agent, ampicillin (10 µg), was used as a positive control. The agar plates were incubated at 37°C/24h. Antibacterial activity was observed as the diameter of the clear inhibitory zones (mm). These assays were done in triplicate. Minimal inhibitory concentration and Minimal bactericidal concentration determination by broth dilutionThe minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) were determined by broth dilution method, in accordance with the Clinical Laboratory and Standard Institute (CLSI, 2006). Serial twofold dilutions of aqueous garlic extract in 5 ml of broth inoculated with 50 µl of fresh precultures (inoculum, 5x108 CFU/ml). The tubes were incubated at 37°C overnight with shaking and the highest dilution where there was no growth was recorded as the MIC. For MBC testing, aliquots (20 µl) of broth from tubes containing no growth were plated onto solid medium and again incubated overnight at 37°C. The highest dilutions in which there were no survivors were recorded as the MBC. In the above method, controls for each organism were performed using the sterile liquid medium without aqueous garlic extract. All MICs and MBCs were confirmed by triplicate assays. The calculated values are the average of the obtained results of the performed assays.

Effects of Garlic Extract on Salmonella Antibiotic Susceptibility

Several potential Enterobacteria active antimicrobial were used to study the effects of garlic extract on Salmonella antibiotic susceptibility. These were: ampicillin (A), oxacillin (Ox), cefalotin (CF), neomycin (N30), erythromycin (E), spiramycin (SP), chloramphenicol (C), vancomycin (VA) and gentamicin (GM). These antibiotics included protein synthesis inhibitors (chloramphenicol, erythromycin, gentamicin, streptomycin and tetracycline) and bacterial cell wall synthesis inhibitors (ampicillin and vancomycin). All antibiotic discs used were supplied by Biomérieux (France). The respective quantities (µg or U/disc) of these compounds were as described in table 2.

Table2: The antibiotic properties list.

Class	CommonName	Code	Charge/ disc (µg or Units)
B-lactamines	Ampicillin	A	10 U
	Oxacillin	OX	1
	Cefalotin	CF	30µg
Aminosides	Neomycin	N30	30
	Gentamicin	GM	10
Macrolides	Erythromycin	E	15
	Spiramicin	SP	100
Phenicols	Chloramphenicol	C	30
Glycopeptides	Vancomycin	VA	30

Ampicillin, CF, Cefalotin, C, Chloramphenicol, E, Erythromycin, FIC, fraction inhibitory concentration, GM, Gentamicin, N30, Neomycin, Ox, Oxacillin, SP, Spiramycin, and VA, Vancomycin. Antibiotic discs were placed on Salmonella–seeded plates in presence or not of garlic extract at a final concentration 12 mg/ml. After overnight incubation at 37°C the diameter of the inhibition zones was measured and compared with that of Salmonella–seeded plates in absence of garlic extract, as antibiogram controls. Experiments were performed in triplicate. The results were interpreted in accordance with the CLSI (CLSI, 2006).

Evaluation of the Synergistic Effects of the Combination of Antibiotics and the a.g.e.

This evaluation was done using the tested S. serovars according to The critical dilution method of Muroi and Kubo (1996). Sterile liquid medium containing antibiotic and/or A.G.E. was inoculated with 200 µl of bacterial culture (106 cells/ml) grown in 10 ml of liquid medium for 6 hr. Antimicrobial combinations are considered synergistic if the effect is greater than the sum of the effects of the individual agents and antagonistic if the effect is less than the sum of the effects of the individual agents.

The fractional inhibitory concentration was calculated as follows:

FIC of compound a (FICa) = MIC of compound a in combination/MIC of compound a alone

FIC of compound b (FICb) = MIC of compound b in combination/MIC of compound b alone

The sum of fractional inhibitory concentration (FICs) indices of two compounds in the combination was calculated as follows: FICa + FICb = FICsThey were interpreted as follows: Synergism has traditionally been defined as an FIC index of 0.5 or less, additivity as a FIC index of more than 0.5 and less than 4, and antagonism as FIC index of more than 4 (Braga et al., 2005). Experiments were performed in triplicate.

Electron Microscopy

Samples for examination were collected from the inhibition and the exponential growth phase culture (treated cells) and the exponential growth phase culture (untreated cells). After the supernatant was discarded (10, 000 g for 5 min at 4°C), the Salmonella cells pellet was prefixed with 3% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4).

Samples were post-fixed for 2 h with 1% osmium tetroxide (v/v) in 0.1 M sodium phosphate buffer (pH 7.4). After fixation the sample was centrifuged at 1800 g for 2 min, the pellet washed three times in 0.2 M Na-cacodylate buffer (pH 7.4). Samples were dehydrated in a graded series of ethanol. The TEMs were taken with a JEOL-1010 electron microscope operating at 80 kV.

Statistical analysis

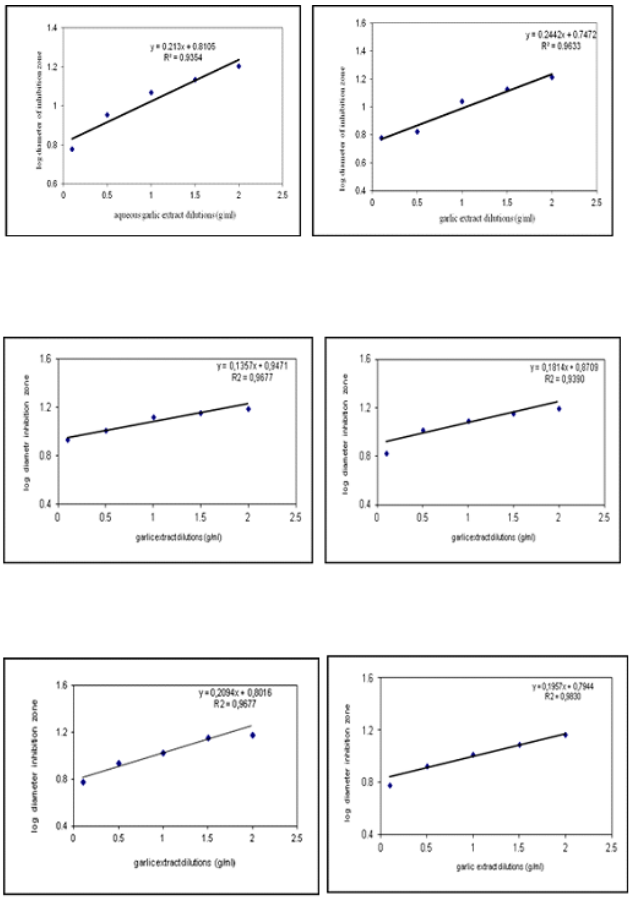
The data collected was analyzed by ANOVA using a SAS system procedure (SAS Institute, Cary, NC, USA). A multiple comparison test (least significant difference) was used to test for significant differences between the treatment means ($P < 0.05$).

RESULTS

Effects of Aqueous Garlic Extract on Salmonella Growth

The aqueous garlic extract (57.1% (w/v), containing 324 µg/ml allicin) inhibited the growth and killed most of the tested Salmonella serovars. In order to study the effects of the A.G.E., different concentrations were tested. No effect on the different cell growth curves was observed when we added an A.G.E. to a final concentration less than 11 mg/ml (mean 89.1 µg/ml of allicin). That's why, we had studied the effect of garlic extract added to a final concentration, which range from 11 mg/ml to 13 mg/ml (data not shown) (Belguith et al, 2010). In order to study the proportionality between the amount of active substance and diameter of inhibition zone, different garlic extract volumes (0.1 to 20µl) or dilutions were applied directly to the surface of the plate or pipetted onto a stack of six mm diameter filter-paper discs cut with a hole-punch. Antibacterial activities of A.G.E. were performed using the well diffusion assay, we have observed clear inhibitory zones (mm) formed around the wells. The diameter of these inhibitory zones was variable from a serovars to another (Figure 1). The size of the inhibition halo was clearly proportional to the A.G.E. applied amount and it showed a linear relationship when plotted against the log of the diameter of the inhibition zone (Figure 1).

Figure1: Regression plot of the log of the diameter of the inhibition zone against the used volume of aqueous garlic extract. Petri plates were seeded with the different Salmonella serovars. (a): S lindenburg, (b): S.enteritidis, (c): S. montivideo, (d): S. hadar, (e): S. cerro, (f): S. nikolaifleet.



This positive correlation between the inhibitory zone diameter and the added A.G.E. concentration was observed in the case of the different Salmonella serovars. Our results are conform to those obtained using the cell suspensions method (Belguith et al, 2010).

These data show that the different Salmonella serovars did not present the same response to the presence of A.G.E. It seems that the sensibility to A.G.E. depends on Salmonella serovars.

Determination of Mic and Mbc

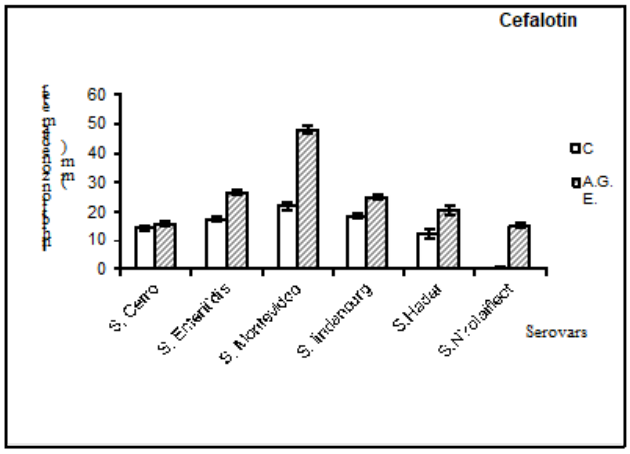
The obtained MIC and MBC values were expressed in terms of garlic extract concentration and the estimated allicin concentrations. The MIC values were determined by broth dilution. We have found that MICs range from 10 mg/ml garlic (8.1 µg/ml of estimated allicin) to 12.5 mg/ml garlic (10.1 µg/ml of estimated allicin). The MBC values range from 13 mg/ml garlic (mean 10.5 µg/ml of estimated allicin) to 15 mg/ml garlic (mean 12.1 µg/ml allicin). We have found that the Salmonella nikolaifleet presented the highest MIC value 12.5mg/ml garlic and the lowest MBC value 13 mg/ml garlic. Whereas,

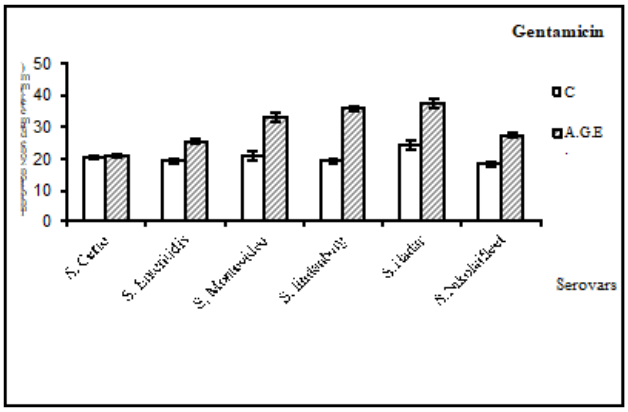
Salmonella lindinburg presented the lowest MIC value 10mg/ml garlic and the highest MBC value 15mg/ml garlic. These data suggest that the A.G.E. sensibility was variable and depends on Salmonella serovars. These MIC values were used to calculate the FIC.

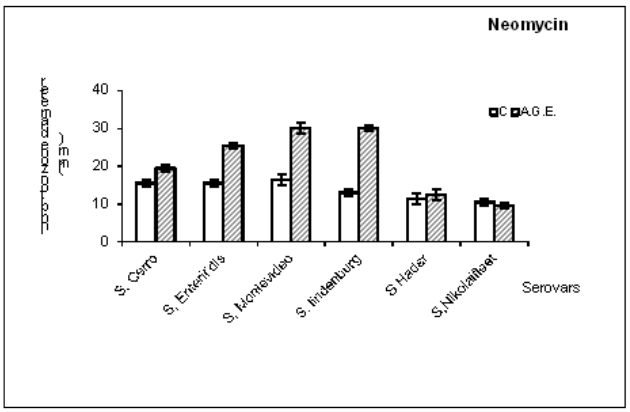
Effects of Garlic Extract on Salmonella Antibiotic Susceptibility

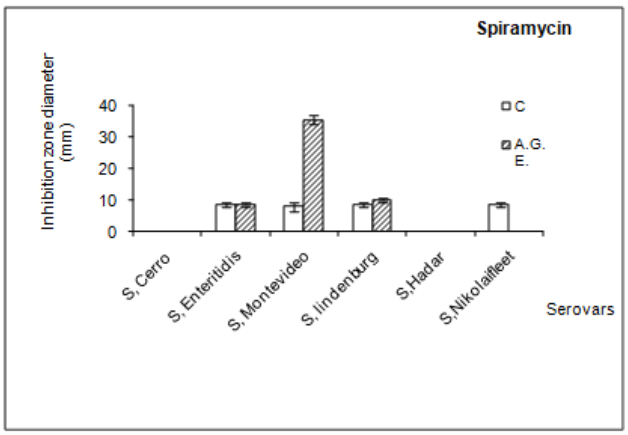
Antibiotic discs were placed on Salmonella–seeded plates in presence and absence of A.G.E. at a final concentration 12 mg/ml. The inhibition zones were measured and compared to that of Salmonella–seeded plates in absence of garlic extract, as antibiogram controls. Figure 2

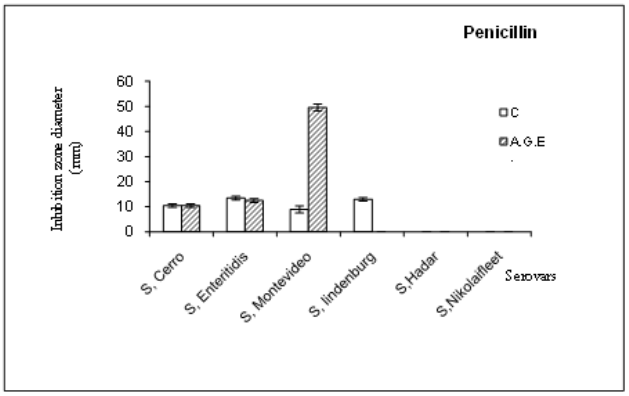
Figure2: Evaluation of the A.G.E. effects on the Salmonella antibiotic susceptibility.12mg/ml of A.G.E. was added in the presence of the tested different antibiotics.

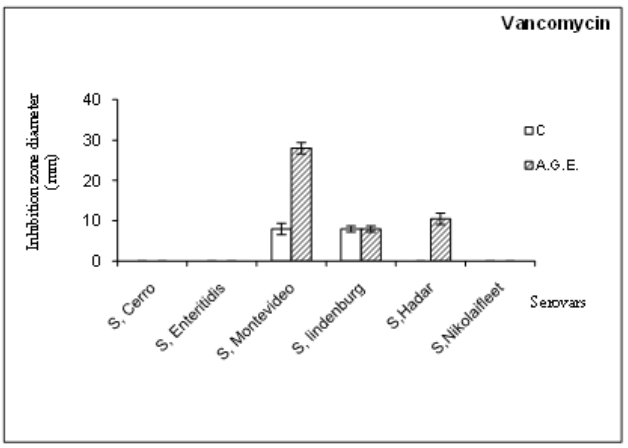


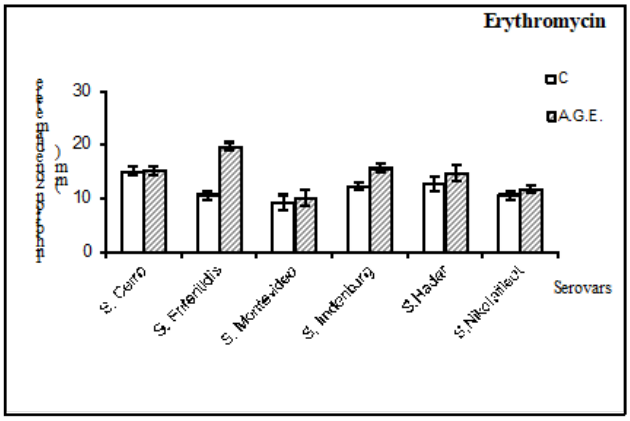


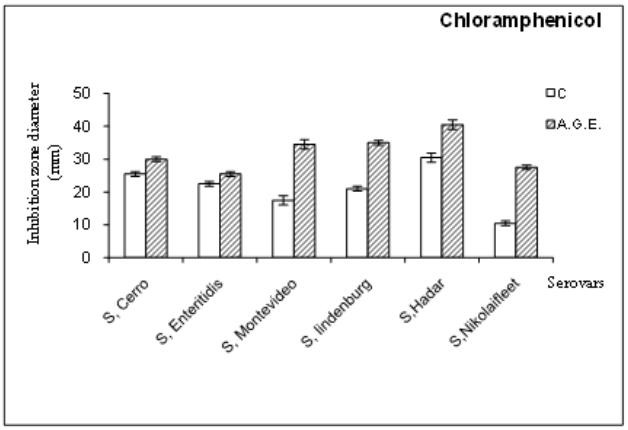


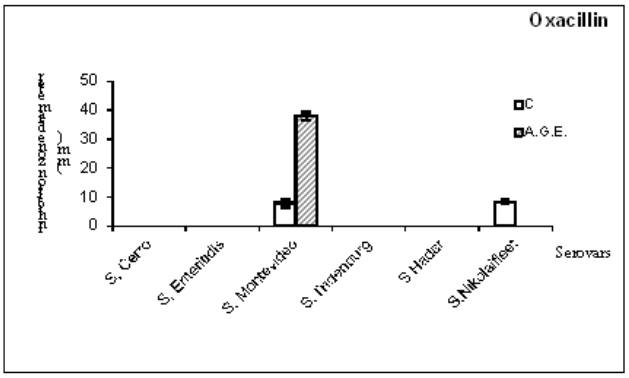






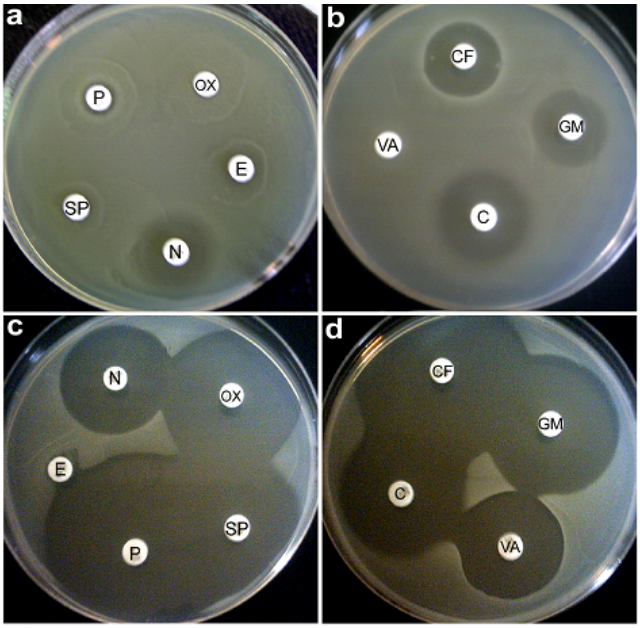






Shows the evaluation of the A.G.E. effects on the Salmonella antibiotic susceptibility. Comparison of the antibiotic inhibition diameter zone obtained on a Salmonella–seeded Petri plate treated or not of garlic extract, shows that the size of the inhibition halo was clearly affected, this result was observed for the different tested Salmonella serovars. In the case of S. montevideo, (Figure 3)

Figure3: Susceptibility of Salmonella montevideo to the combination ofA.G.E. and antibiotics. 12 mg/ml (97.2 µg/ml estimated allicin) of A.G.E. was added to the nutriment agar Petri plates.



a and b: Control antibiogram (untreated bacteria)

c and d: Antibiogram in the presence of A.G.E.

Tested antibiotics: Amp: Ampicillin, CF:Cefalotin, C: Chloramphenicol, E: Erythromycin, GM: Gentamicin, N30: Neomycin, OX: Oxacillin, SP:Spiramycin, VA: Vancomycin. we observed a significant enlargement of the diameter of all the antibiotic inhibition zones (p 0,001). However, an increase of the sensitivity to the cefalotin, gentamicin and chloramphenicol was observed in all cases. These results suggest a synergic or additive effect between garlic extract and these antibiotics. We observed that in presence of A.G.E., a vancomycine sensitivity was developed in the case of S. hadar and a cefalotine sensitivity in the case of S. nikolaifleet . The oxacillin resistance developed by S. cerro, S. enteritidis, S. lindenburg and S. nikolaiflee seems to be due to an antagonist effect between A.G.E. molecules and antibiotics. No effect on the penicillin resistance was observed, exceptionally in the case of S. montevideo.

Evaluation of the synergistic effects of antibiotics and a.g.e. against different salmonella serovars

Although the antimicrobial activity of A.G.E. extract was very strong against the different tested Salmonella, synergism with other antimicrobial products was considered possible and various antibiotics were therefore evaluated (Table 3). The action modes and inhibitory mechanisms of the used antibiotics ranged from inhibition of protein synthesis to interference with cell wall synthesis.

Based on the FIC index, their inhibitory strength against Salmonella in the presence of A.G.E. was found to be variable inside the different serovars. We have found that in the case of S. montevideo it was in the following order: erythromycin > gentamicin > neomycin = chloramphenicol > ampicillin >oxacicllin> = vancomycin >cefalotin. For S. enteretidis it was in the following order: gentamicin >cefalotin> erythromycin > neomycin. For S. nikolifleet it was in the following order: gentamicin >spiramycin>oxacicllin> vancomycin >cefalotin>

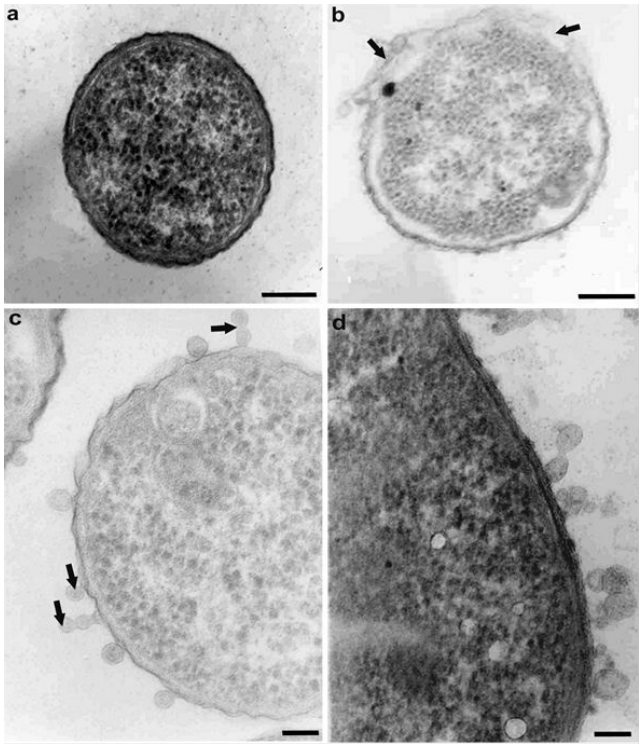
erythromycin = chloramphenicol. For S. cerro it was in the following order: ampicillin > neomycin > erythromycin > vancomycin > gentamicin > chloramphenicol >cefalotin. For S. lindengurg it was in the following order: erythromycin > vancomycin > ampicillin > neomycin > chloramphenicol >cefalotin> gentamicin. For S. hadar it was in the following order: vancomycin > chloramphenicol > gentamicin > erythromycin >cefalotin.

The analysis of table 3 shows that the combination effects of A.G.E. and the different tested antibiotics were variable according to the antibiotic and the serovars. Two effects were observed: synergism and additive effects. The synergistic effects of A.G.E. with the different antibiotics against the tested Salmonella serovars varied from 32% to 76%. However, the observed additive effect ranged from11%to46%.

Garlic Stress-Associated Morphological Changes

Electron microscopic examination images of untreated Salmonella revealed regular rod-shaped cells with a continuous uniform cell wall (Figure 4-8, a). The general morphology and the ultrastructure of the cell envelope of untreated bacteria (Figure 4-8, a) are quite similar to those of Gram-negative rods in general, which is characterized by a regular rod-shaped cell with a continuous cell wall. Contrary, A.G.E-treated cells (at 12 mg/ml) had visible cell wall damage (Figure 4-8, b, c and d). In fact, the cytoplasm was disorganized and the inner and the outer membrane were separated, the integrity of the outer membrane, however was not maintained in some cases. We observed some rod-like projections that appeared on the surface of the OM. These projections seemed to involve the outer membrane of the cell wall.Furthermore, micrographs of the damaged cells showed disintegrated cells exhibiting the normal rod-shaped morphology (Figure 4-7, a).

Figure4: S. Montevideo



While A.G.E. kills this organism completely (Figure 6-8, b, c and d), with the most drastic effects on the membrane. Effect of Aqueous garlic extract on the ultra-structure of the different Salmonella as demonstrated by Transmission electron microscopy. Observation of the disintegrated cell membrane and the rodlike projections that appeared on the surface of the OM (arrows).Cells from inhibition growth phase and exponential growth phase were analyzed. a: Control samples, from exponential growth phase; b-d:cells treated with garlic extract 12 mg/ml, from inhibition growth phase. Fig. 4. S. montevideo: a and b: Scale bar 0.1 μ; c and d: Scale bar 0.05 μ;The outer membrane appeared to disintegrate, perhaps causing the fused appearance of the inner and outer membranes in the micrographs.We observed some rod-like projections that appeared on the surface of the OM. These projections seemed to involve the outer membrane of the cell wall.These projections become more numerous and longer with increasing concentrations of A.G.E. (Figure 4-8, b, c and d) and they probably reflect the breakdown of the integrity of the cell envelope. The overall structure of the projections (Figure 4-8, b, c and d) was that of a thin rod. Their diameter, length, number and the size were variable. Cross sections (Figure 4-8, b, c and d) showed that the projections were cylindrical rather than cuts from longitudinal folds. There was no consistent internal structure visible in the projections, and in this respect, they resembled simple lipid micelles.

Figure5: S. cerro :a and b: Scale bar 0.1 μ; c: Scale bar 0.05

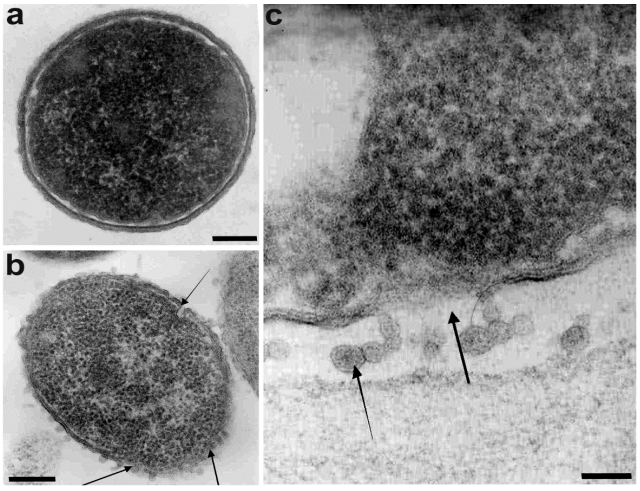


Figure6: S. nikolaifleet: a, b and c: Scale bar 0.1 µ;

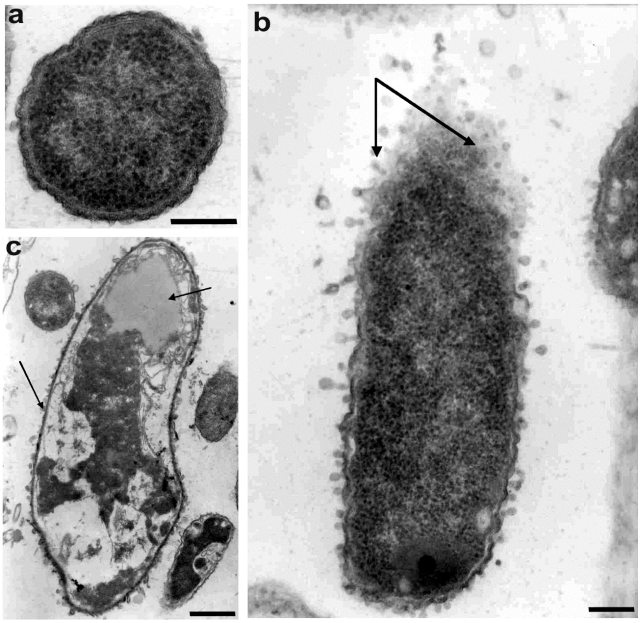
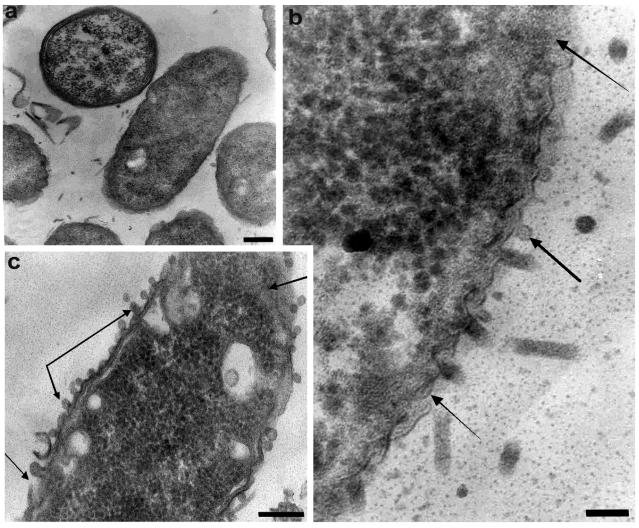
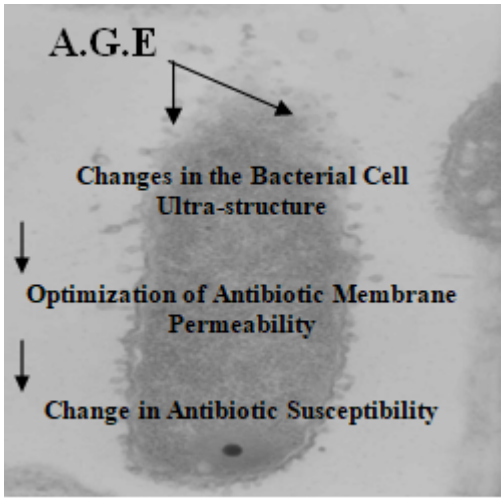


Figure7: S. enteretidis: a, b and c: Scale bar 0.1 µ.



These morphological changes were observed in all A.G.E. treated Salmonella serovars, and they confirm what we had observed in the case of Salmonella hadar (Belguith et al., 2009).

Toc graphic



DISCUSSION

The increasing antibiotic resistance exhibited among common pathogenic microorganisms encountered in wounds and other infections is a serious current public health issue, which will have a major impact on antimicrobial treatments in the future (Bakri et Douglas, 2005). For our safety there is a prompted renewed interest in the use of natural alternatives and antimicrobial agents such as bacterial interference (probiotics), specially, plant antimicrobial peptides (Altieri et al, 2005).In the present work, all of the non-typhoidic Salmonella serovars used were multi-resistant. Salmonella hadar, presented the highest rates of resistance (five antibiotic resistance) compared to the other serovars (Chatti et al, 2007). The disc diffusion method allowed us to observe a marked inhibitory effect of A.G.E. against the different Salmonella serovars. We have found a notably high in vitro antibacterial activity against Salmonella. Aqueous garlic extract was effective in all cases, and even acted against those strains that are antibiotic multi-resistant. This result confirms what we have obtained using the cell suspension culture (Belguith et al, 2010).

The comparison of the obtained Salmonella serovars inhibition zone diameters shows that the size of the inhibition halo was clearly variable, from a serovar to another. Examination of the effects of A.G.E. concentration on the different Salmonella serovars, using the cell suspension growth method revealed that the duration of the inhibition phase varied according to the tested Salmonella serovars and it was clearly proportional to the applied amount of A.G.E. (Belguith et al, 2010). A good proportionality between A.G.E. amount and the antibacterial activity was observed and seems to be a rapid and reliable way of determining the antibacterial activity.We have found that the duration of the growth inhibition phase was positively correlated to the added A.G.E. concentration. The resumed growth rate, expressed as a percentage of uninhibited growth rates, was proportional to A.G.E. concentration and it was different on the tested Salmonella serovars. The lowest resumed growth rate was observed in the case of Salmonella enteritidis using 13 mg/mL of A.G.E. Our results demonstrate that the different Salmonella serovars did not present the same duration of

inhibition and resumed growth rate (Braga et al., 2005). The observed growth inhibitory effect has been described in case of other bacteria.

Our data demonstrate that the different Salmonella serovars did not present the same growth inhibition intensity. It seems that the sensibility to A.G.E. depends on Salmonella serovars.The positive correlation observed between the inhibitory zone diameter and the added A.G.E. concentration; using the two Salmonella serovars culture methods, confirms that the A.G.E. antimicrobial activity was dose-dependent. The A.G.E. appears to offer benefits such as selective inhibition and low risk of bacteria resistance development, as well as being bactericidal in some cases.Feldberg et al. (1988) described that the in vitro mechanism of Salmonella typhimirium inhibition growth by allicin at bacteriostatic concentrations (0.2 to 0.5 mM) was found to be due to a delayed and partial inhibit of DNA and protein synthesis and an immediate inhibition of RNA synthesis, suggesting that this is the primary target of allicin action. The obtained values of MIC and MBC were very close to those described in the literature for other Gram-negative strains, but they are lower than the Gram-positive strains (Bakri et Douglas, 2005).However, the relatively higher MICs of A.G.E. render it as a valid alternative for the treatment of antibiotic resistant bacterial infections when compared to the clinically impractical concentrations of antibiotics required to obtain the same effect.

The calculated FIC index shows that it was variable according to the antibiotics and the tested Salmonella serovars. The FIC indexes indicate a synergistic effect in the case of oxacillin, cefalotin, neomycin, spiramycin, chloramphinical, vancomycin and ampicillin, whereas, an additive effect was observed in the case of Gentamicin and Erythromycin. Our results suggest that garlic extract induce an increase of the antibiotic sensibility by disturbing the bacteria walls. These findings are supported by the electron microscopy examinations and they might be due to the outer membrane damage, which contributes to the optimization of the antibiotic membrane permeability. Our findings show that the combination effect of the aqueous garlic extract and the antibiotics against Salmonella, was variable inside the tested serovars and the same antibiotic family. These garlic extract effects might be mediated by changes in bacterial cell wall structure or permeability, as it was described by Belguith et al. (2010). Yoshida et al. (1987) had reported that morphological changes such as thickening of cell wall and destruction of cell organelle of Aspegillus niger and Candida albicans was induced by ajoene.The aqueous garlic extract seems to increase the effectiveness of some antibiotics against Salmonella. Our results suggest that the observed synergetic or additive garlic effects are related to the presence in the aqueous garlic extract of other active molecules such as plant antimicrobial peptides, which allow to the garlic to have the greatest antibacterial activities. In the most cases of the tested Salmonella serovars, we have observed synergistic or additive effects of the combination of A.G.E. and antibiotic. These results are encouraging and may enhance the complementary use of medicinal plant products, especially in food bio-preservation industry.Davis et al. (1994) had shown an in vitro synergism of concentrated Allium sativum extract and amphotericin B against Cryptoccus neoformans. So the combination of antibiotics and A.G.E. appears to be efficient to limit the spared of multi-resistant bacteria. Knowledge of the mechanism of action of A.G.E. might lead to better use of antibiotics against pathogenic microbes. Antimicrobials are substances that inhibit the growth of, or kill, microorganisms with little or no damage to the host. The three main mechanisms of action for antimicrobials to achieve this goal are: inhibition of cell wall synthesis, inhibition of protein synthesis, and inhibition of DNA synthesis. In addition to these three targets, antimicrobials may also have an indirect method of action by blocking folic acid synthesis and subsequently inhibiting nucleic acid development (Zasloff , 2002).

The cell wall acts as a mechanical means of protection, as a surface for proteins and appendages for cell adhesion, for motility, host infection, and horizontal gene transfer (Dufour et al., 2003). In Gram-positive bacteria, the cell wall is thick and composed of multiple layers of cross-linked glycan and peptide strands crossed by molecules of teichoic and teichuronic acids. In Gram-negative bacteria, the peptidoglycan wall is thinner and surrounded by a lipopolysaccharide. This lipopolysaccharide layer, in Gram-negative bacteria, decreases permeability and therefore affects the uptake of certain antimicrobials such as glycopeptides (Dufour et al., 2003).Bacterial DNA synthesis permits the replication of the bacterial chromosome during cell division, and RNA synthesis allows gene expression and protein synthesis by transcription of DNA into RNA (Dufour et al., 2003). Quinolones target two enzymes involved in the early stages of this process including topoisomerase II or DNA gyrase and topoisomerase IV, and exert their effect through interaction with theenzyme bound DNA complex (Dufour et al., 2003). The assumption that SH enzymes seems to be the targets for the attack of allicin is further confirmed by the observed structural differences of the various bacterial strains. The different bacterial species differ in cell membranes lipid content. For example, the E. coli cell membrane contains 20% lipid, while that of Staphylococcus aureus contains only 2% lipid (Salton, 1964). These structural differences probably cause varying membrane permeability to allicin and other garlic constituents. These cell membranes difference, may explain the observed variable garlic extract effects on the treated bacteria. Elsayed et al. (2011) have found that combination of imipenem and green tea extract seems to be significantly synergistic and may be eligible for further evaluation in vivo against MRSA infections.The use of medicinal plants and herbal biomolecules and products is increasingly being explored worldwide as a potential therapeutic alternative in primary healthcare, for addressing food bio-preservations and multi-bacterial infections (Groppo et al., 2002).

Hammami et al. (2009) described that plant antimicrobial peptides have been found to be excellent candidates for the development of a new generation of antimicrobial agents. These antimicrobial peptides, which constitute a heterogeneous class of low molecular mass peptides, are recognized as important components of the natural plant defense system (Talas-Ogras, 2004). A peptide from O. Africana seeds extract was purified (Hammami et al., 2009), which exhibit a very broad spectrum of activity including Gram-positive and Gram-negative bacteria,

fungi and yeast. This extract appeared to increase the effectiveness of at least two antibiotics against a bacterial genus that includes a known pathogen. Our results are encouraging, and may enhance the complementary use of medicinal plants in the treatment of multi-resistant bacteria and infectious diseases. Future studies on the chemical characteristics of the active molecules of the aqueous garlic extract should be carried out. The knowledge of their molecular action mechanism might lead to better use of antibiotics against pathogenic microbes.The authors extend their appreciation to the Tunisian Ministry of Higher Education, Scientific Research and Technology and the Carthage and El-Manar University, Tunisia. We thank Professors Monji Gaja and Ahmed Aissi for the critical reading and revision of the manuscript.The author(s) declare(s) that there is no conflict of interests regarding the publication of this paper.

REFERENCES

1. Altieri, B.Speranza, M.A. Del Nobile and M.Sinigaglia. Suitability of bifidobacteria and thymol as biopreservatives in extending the shelf life of fresh packed plaice fillets. Journal Applied Microbiology 99: 1294-1302.
2. Ankri S.and Mirelman D, Antimicrobial properties of allicin from garlic. Microbes and Infection 1: 125–129.1999
3. Bakri IM. et Douglas CW. Inhibitory effect of garlic extract on oral bacteria , Archive of Oral Biology 50(7): 645-651.
4. Belguith H, Kthiri F, Chatti A, Abu Sofah A, Ben Hamida J.and Landoulsi A. Inhibitory effect of aqueous garlic extract (Allium sativum) on some isolated Salmonella serovars. African Journal of Microbiology Research 4 (5): 328-338. (2010)
5. Belguith H, Kthiri F, Chatti A, Abu Sofah A, Ben Hamida J.and Landoulsi A. Morphological and Biochemical changes of Salmonella hadar exposed to aqueous garlic extract. International Journal of Morphology 27(3): 705-713.2009.
6. Braga LC,.Leite AAM,.Xavier KGS, Takahashi JA,.Bemquerer MP, Chartone Souza E.And. Nascimetto AMA . Synergic interaction between pomegranate extract and antibiotics against Staphylococcus aureus. Canadian Journal of Microbiology 51: 541-547. (2005).
7. Chatti A, Daghfous D.and Landoulsi A. Acid resistance of Salmonella isolated from animals, food and wastewater in Tunisia. Annals Saudi Medicine 27(3): 195-198. (2007).
8. Cellini L, Campli ED, Masulli M, Bartolomeo SD, and Allocati N . Inhibition of Helicobacter pylori by garlic extract (Allium sativum), FEMS Immunology and Medical Microbiology 13: 273 – 277.1996.
9. Clinical Laboratory and Standard Institute. Performance Standards for Antimicrobial Susceptibility Testing: Sixteenth Informational Supplement. 16th Ed., Clinical and Laboratory Standards Institute, Wayne PA., USA, 183(2006).
Cutler RR and Wilson P. Antibacterial activity of a new, stable, aqueous extract of allicin against methicillin-resistant Staphylococcus aureus. British Journal of Biomedical Sciences 61(2): 71-74.2004.
10. Curtis H, Noll U, Stormann1 J and Slusarenko AJ. Broad-spectrum activity of the volatile phytoanticipinallicin in extracts of garlic (Allium sativum) against plant pathogenic bacteria, fungi and oomycetes. Physiological and Molecular Plant Pathology 65: 79-89.2004.
11. Davis LB, Shen E and Rover RE. In vitro synergism of concentrated Allium sativum extract and amphotericin B against Cryptoccus neoformans, Planta Medica 60: 546 -549.1994.
12. Dufour M, Simmonds RS and Bremer PJ. Development of a method to quantify in vitro the synergistic activity of natural antimicrobials, International Journal of Food Microbiology 85: 249–258.2003.
13. Elsayed A, Al-Mohammed HI and Al-Badry S. Synergism and post antibiotic of green tea extract and imipenem against methicillin-resistant Staphylococcus aureaus, Microbiology Journal 1(3):. 89-96.2011.
14. Fang FC. Sigma cascades in prokaryotic regulatory networks. Proceeding of the National Academy of Sciences 102 (14): 4933-4934.2005.
15. Feldberg RS, Chang SC, Kotik AN, Nadler M, Neuwirth Z, Sundstrom DC and Thompson NH. In vitro mechanism of inhibition of bacterial growth by allicin. Agents Chemotherapy 32: 1763-1768.1988.
16. Groppo FC, Ramacciato JC, Simoes RP, Florio FM and Sartoratto A. Antimicrobial activity of garlic, tea tree oil, and chlorhexidine against oral microorganisms. International Dental Journal 52(6): 433-437.2002.
17. Gupta K, Hooton T M and Stamm WE. Increasing antimicrobial resistance and the management of uncomplicated community-acquired urinary tract infections. Annals of Internal Medicine 135(1): 41-50.2001.
18. Hammami R, Ben Hamida J, Vergoten G, Lacroix JM, Slomianny MC, Neffati M and Fliss I. A new antimicrobial peptide isolated from Oudneya africana seeds. Microbiology and Immunology 53: 658-666.2009.
19. Hemaiswarya S, Kruthiventi AK and Doble M. Synergism between natural products and antibiotics against infectious disease. Phytomedicine (15): 639-652.2008.
20. Jonkers D, VandenBroek E, VanDooren I, Thijs C, Dorant E, Hageman G and Stobberingh E. Antibacterial effect of garlic and omeprazole on Helicobacter pylori. Journal of Antimicrobial Chemotherapy 43: 837-839.1999.
21. Lampel KA, Orlandi PA and Kornegay L. Improved template preparation for PCR-based assays for detection of food-borne bacterial pathogens. Applied Environmental. Microbiology 66(10): 4539-4542.2000.
22. Logue CM, Sherwood JS, Elijah LM, Olah PA, and. Dockter MR. The incidence of Campylobacter spp. On processed turkey from processing plants in the Midwest United States. Journal of Applied Microbiology 95:234–241.2003.
23. Miron T, Rabinikov A, Mirelman D, Wilchek M And Weiner L. The mode of action of allicin: its ready permeability through phospholipid membranes may contribute to its biological activity. Biochimica and Biophysica Acta 1463: 20–30.2000.
24. Murio H.and Kubo I.Antibacterial activity of anacardic acid and totaral, alone and in combination with mithicillin, against methicillin-resistant Staphylococcus aureaus. Journal of Applied Bacteriology 80(4): 387-394.1996.
25. Reuter HD, Koch HP and Lawson DL . Therapeutic effects and applications of garlic and its preparations. In: Garlic: The Science and Therapeutic Applications of Allium sativum L. and Related Species, 2sd Ed. William et Wilkins, Baltimore, MD: 135–212. (1996).
26. Sivapalasingam S, Friedman CR, Cohen L and Tauxe RV. Fresh produce: a growing cause of outbreaks of food borne illness in the United States, 1973 through 1997. Journal Food Protection 67(10): 2342-2353.2004.
27. Talas-Ogras T. Screening antimicrobial activities of basic protein fractions from dry and germinated wheat seeds. Biologia Plantarum 48:.583-588.2004.
28. Tauxe R, Kruse H, Hedberg C, Potter M, Madden J and Wachsmuth K. Microbial hazards and emerging issues associated

with produce: a preliminary report to the national advisory committee on microbiologic criteria for foods. Journal Food Protection 60: 1400–1408.1997.

29. Uchida Y, Takahashi T and Sato N . The characteristics of the antibacterial activity of garlic. Japanese Journal of Antibiotics 28: 638-642.1975.
30. Yang L, Weiss TM, Lehrer RI and Huang HW. Crystallization of antimicrobial pores in membranes: magainin and protegrin. Biophysics Journal 79: 2002-2009.2000
31. Yoshida S, Kasuga S, Hayashi N, Ushiroguchi T, Matsuura H and Nakagawa S. Antifungal activity of ajoene derived from garlic. Applied Environmental Microbiology 53: 615-617.1987.
32. Zasloff M. Antimicrobial peptides of multicellular organisms. Nature 415: 389-395.2002.
33. World Health Organization (2001). WHO Global Strategy for the Containment of Antimicrobial Resistance. World Health Organization, Geneva, Report: WHO/CDS/CSR/DRS, vol.2b.