



Antimicrobial resistance of three common molecularly identified pathogenic bacteria to *Allium* aqueous extracts

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ABSTRACT

The aim of this work was to evaluate the *in vitro* bacterial inhibition of different types of garlic on *Escherichia coli* ATCC 25922, *Listeria monocytogenes* and *Staphylococcus aureus*. The bacterial strains were molecularly identified using gen 16S rDNA molecular identification. Four different types of garlicks were used: 1) white, 2) Japanese, 3) elephant and 3) black, and these were evaluated at two different concentrations (0.25 and 0.125 g/mL) per garlic type. Bioactive compounds present in the garlicks were identified using high-performance liquid chromatography coupled to ultraviolet detector (HPLC-UV), and total polyphenols were quantified by the Folin-Ciocalteu technique. The Kirby-Baumber method was used for the bacterial evaluation. Aqueous extract of black garlic had the highest amount of polyphenols 6.26 ± 0.21 mg GAE/mL. The area of inhibition was measured and classified as sensitive, intermediate or resistant. Using the disc diffusion assay, higher concentration (0.25 g/mL) of aqueous extract of white garlic had the highest antibacterial activity area, with 21.46 ± 3.94 mm for *L. monocytogenes*, 20.61 ± 2.47 mm for *S. aureus* and 17.83 ± 2.21 mm for *E. coli*. White garlic had comparable antimicrobial activity as the control (tetracycline at 30 µg) as indicated by the size of the inhibition halos. Based on your results, white garlic can be used as an alternative to synthetic antimicrobials.

1. Introduction

Previously, natural products were used to combat diseases, however, with the discovery of synthetic antibiotics, they were gradually replaced [1,52,53]. Natural products used to be the major ingredient of many drugs but pharmaceutical companies have decreased or even eliminated research on natural products [2,42–46,52]. The focus has been on synthetic antibiotics and their indiscriminate use have reduced their effectiveness against infectious diseases in addition to incidences of antibiotics resistance [3,47–50]. Foodborne diseases are still a public health problem worldwide and the causal agents are mainly *Escherichia coli* O157: H7, *Salmonella* spp., *Listeria monocytogenes*, and *Clostridium botulinum* [4,5]. Bacteria have the ability to adapt to an environment and develop resistance mechanisms, which can be of natural or acquired origin [6,7,51].

Due to the current problems of antibiotics resistance facing the livestock industry, natural products can be viable and economical alternatives [55,57,63]. One of such natural products is garlic (*Allium sativum*) and there is documented literature on its antibacterial effect [8,9]. Therefore, there is a need to look for new medicines of herbal origin as they cannot contribute to antibiotics resistance and they have little or no toxic effect on livestock [10,57,64]. Garlic (*Allium sativum*) is an antimicrobial characterized by its high content of sulfuric compounds [11,12,54] and previous studies reported that garlic has a broad spectrum of antibacterial activity against *E. coli*, *Salmonella* spp. [9,13]. Kamra et al. [14] reported garlic as anti-methanogenic and anti-protozoal agent with the ability to reduce methane emission. They concluded that garlic and plant extract, in general, can be used as an eco-friendly rumen modifier for sustainable production [15,56–62]. During processing, the unstable compounds of fresh garlic, including alliin,

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become stable compounds that include s-allylcysteine (SAC), the water-soluble compound with potent antioxidant effect [16]. The study of medicinal plants is still a work in progress but it has the potential to address some of the major issues such as the antibiotics resistance that have plagued the livestock industry. In the present study, we evaluated two concentrations (0.25 and 0.125 g/mL) of aqueous extracts of white, Japanese, elephant and black garlic for their antibacterial activities on *E. coli*, *L. monocytogenes* and *Staphylococcus aureus*.

2. Materials and methods

2.1. Preparation of the extracts

The study was carried out in the Laboratory of Bacteriology, the Center for Research and Advanced Studies in Animal Health of the Autonomous University of the State of Mexico. Four varieties of garlic were used: white, Japanese, elephant and black, and these were obtained from a local market, (Juarez, Toluca State State of Mexico). For the preparation of the aqueous extracts, 50 g of each garlic per 200 mL of distilled water (0.25 g/mL) and 50 g of garlic per 400 mL of water (0.125 g/mL) were used for the second concentration. The garlic was ground with a commercial laboratory blender and liquefied with water for 5 min. The resulting solutions were filtered twice with Watman® No.1 paper, and the extracts were stored under refrigeration (4 °C) until needed.

2.2. Extract characterization and biological properties of active molecules

Aqueous extract was analyzed for its chemical compounds using GC/MS apparatus and the conditions of column oven and temperature program have been previously described in Refs. [17,18]. The identification of chemical compounds was done by matching their retention times and mass spectra with those reported in WILEY 09 [19] and NIST 11 Mass Spectral databases (Table 1).

For the quantification of allicin (by oxidation of diallyl disulfide with hydrogen peroxide), the methodology reported by Ref. [20] was followed, using high performance liquid chromatography coupled to ultraviolet detector (HPLC-UV), a ODS C18 reverse phase column (254 mm × 0.46 mm ID, 5 µm). The analysis conditions were mobile phase Methanol/Water 45/55 at a flow rate of 0.8 mL/min; Analysis temperature 25 °C and UV detection at 230 nm [21] (Table 2).

2.3. Total polyphenols

Quantification of total polyphenols was performed using the Folin-Ciocalteu technique [22]. A standard curve was made with gallic acid and the absorbance was measured at 760 nm. The pH of the extracts was measured with a potentiometer (Conductronic, PC18, Puebla, Mexico).

2.4. Origin and molecular identification of bacterial strains

For comparison of the diameter of the inhibition halo, the bacterial strains used were: *E. coli* (ATCC® 25922™), *L. monocytogenes* (field

Table 2
Allicin content of aqueous extracts.

Type of extract	Allicin content (ppm)
AEWG	2,00 ± 0,007
AEJG	0,02 ± 0,003
AEEG	0,01 ± 0,002
AEBG	0 ± 0

AEWG, Aqueous extract of white garlic; AEJG, Aqueous Japanese garlic extract; AEEG, Aqueous extract of elephant garlic; AEBG, Aqueous extract of black garlic; ppm, Parts per million.

isolate obtained from ovine brain and in a selective medium, *Listeria* Enrichment Broth, Sigma-Aldrich, Inc.) as described by Ref. [23] and *S. aureus* (isolated from a lesion located on the back of a canine; Table 3).

However, *S. aureus* is a member of the microbiota of human, but it is common to isolate it from skin and mucous membranes of canine species [24]. *S. aureus*, is in the group of multiresistant bacteria so they are a challenge for the veterinary profession, since they can cause morbidity and mortality in pets, in addition to being a zoonotic risk [25].

Isolation of *L. monocytogenes* in food creates the need to seek alternatives to prevent the proliferation of this bacterium. In many foods, this is done through the use of chemical preservatives, which has been questioned due to its potentially toxic and carcinogenic effects. These shortcomings have resulted in the push for the use of natural food additives, promoting the exploration of natural antimicrobial compounds as an alternative [26].

The bacterial strains were identified by gen 16S rDNA molecular identification; protocol was developed according to Kavitha et al. [27]. The extracted genomic DNA was subjected to 16S rDNA sequence analysis. The resulting sequences were aligned and analyzed with homologous sequences from other bacteria.

2.5. Experimental design

A completely randomized design with a 4 × 2 × 3 factorial arrangement was used with ten replications for each bacterium. Independent variables were aqueous extracts; white garlic (AEWG), Japanese (AEJG), elephant (AEEG) and black (AEBG), concentrations (0.25 and 0.125 g/mL) and bacteria (*E. coli* ATCC 25922, *L. monocytogenes* and *S. aureus*) and the response/dependent variable was the inhibition of bacterial growth. An analysis of variance and comparison of means was performed by the Tukey Test ($P \geq 0.05$) using the statistical package Statgraphics Plus version 5.0.

2.6. Inhibition capacity

For this test, the Kirby-Bauber method was used and the extracts' ability to inhibit bacterial growth was determined by plaque diffusion. To determine the appropriate control to used, a preliminary test was carried out in which the bacteria were challenged with 7 different antimicrobials (Polydisks for Gram-negative and Gram-positive bacteria, Productos Biológicos de México, S.A.). With the help of a vernier rule, the area of bacterial inhibition around the senses were measured and classified as sensitive (S), intermediate (I) or resistant (R) according to Wayne USA: CLSI (Clinical and Laboratory Standards Institute 2012).

3. Results

Concentrations of total polyphenols in the extracts are shown in Fig. 1. Higher concentration (0.25 g/mL) of aqueous extract of black garlic (AEBG) had the highest total polyphenols (6.26 µg gallic acid equivalents (GAE/mL) and pH of 4.61 ± 0.03; the lowest values were noted for aqueous extract of elephant garlic (AEEG) 0.26 µg GAE/mL and pH of 6.61 ± 0.01.

Table 1
Chemical constituents of aqueous extract.

Peak	Compound name	Area(%)
1.	2-Ethylidene [1,3]dithiane	0.39
2.	3-Vinyl-1,2-dithiacyclohex-4-ene	12.62
3.	3-Vinyl-1,2-dithiacyclohex-5-ene	21.47
4.	Diallyl disulfide	3.16
5.	Dimethyl trisulfide	1.23
6.	Methyl 2-propanol trisulfide	2.49
7.	Di-2-propenyl trisulfide	5.90

Table 3
Origin of bacterial strains used during the evaluation.

Origin	Anatomical site description of the lesion	Biochemical	Bacterial species
Creole canine Male 5 years	Presence of dermatitis of wet appearance, in the area of the chin, left flank of the thigh and rump Presence of boils, ulcers and superficial scabs Pyoderma in the left groin No treatment Two weeks with the problem	Gram staining: Cluster Grammar Coconut Coagulase: Positive Blood agar hemolysis: Positive β Catalase: Negative Oxidase: Negative L-Arabinose: negative Sucrose: Positive Maltose: Positive D-Ribosa: Positive Hyaluronidase: Positive Nitrate reduction: positive DNase: Positive O/F: F	<i>Staphylococcus aureus</i>
Suffolk sheep Male 2 years	Incoordination, sudden death. Sample: Cerebellum/medulla oblong Sample: silo	Gram staining: Gram positive bacillus Growth in BD PALCAM Listeria Agar®: Positive Catalase: Positive Oxidase: Negative Motility test: 4 °C Negative 37 °C Positive (surface) Mannitol: Negative Rhamnose: Positive Xilosa: Negative Indole: Negative Urea: Negative Methyl Red: Positive Voges-Proskauer: Positive Nitrate Reduction: Negative	<i>Listeria monocytogenes</i>

For *E. coli*, higher concentration (0.25 g/mL) of white garlic (AEWG) had the highest inhibition with 17.83 mm compared with aqueous extract of Japanese garlic (AEJG) and AEEG with 9.80 and 2.93 mm, respectively. Similar trend was noted for the lower the concentration (0.125 g/mL) of the extracts, with the highest inhibition halo noted for AEWG with 13.11 mm. No halo was noted for AEBG. In the case of *S. aureus*, AEWG had 20.61- and 17.87-mm halos for the higher and lower concentrations, respectively. The zone of inhibition for AEJG and AEEG were smaller with values of 8.12 and 0.82 mm and 9.01 and 3.13 mm, respectively. Higher concentration of white garlic had the biggest zone of inhibition (21.46 mm) on *L. monocytogenes*, followed by Japanese garlic with 5.88 mm and elephant garlic with 4.32 mm. As noted in the other two bacterial, AEBG showed no antibacterial activity in both concentrations.

The inhibition zones of the seven commercial antimicrobials used in the preliminary study are shown in Table 4. Based on the result, Tetracycline (30 μ g) was selected as a positive control and nitrofurantoin (300 μ g) as a negative control in the present study. The zone of inhibition on bacterial growth by the different garlic treatments is shown in Table 5. With the exception of AEWG, no halo was noted for the other treatments.

4. Discussion

The analysis identified 2-ethylidene[1,3]dithiane, 3-Vinyl-1,2-dithiacyclohex-5-ene, 3-Vinyl-1,2-dithiacyclohex-4-ene, diallyl disulfide, dimethyl trisulfide and methyl 2-propanol trisulfide. Allicin represents 70–80% of the thiosulphinates formed and previous reports

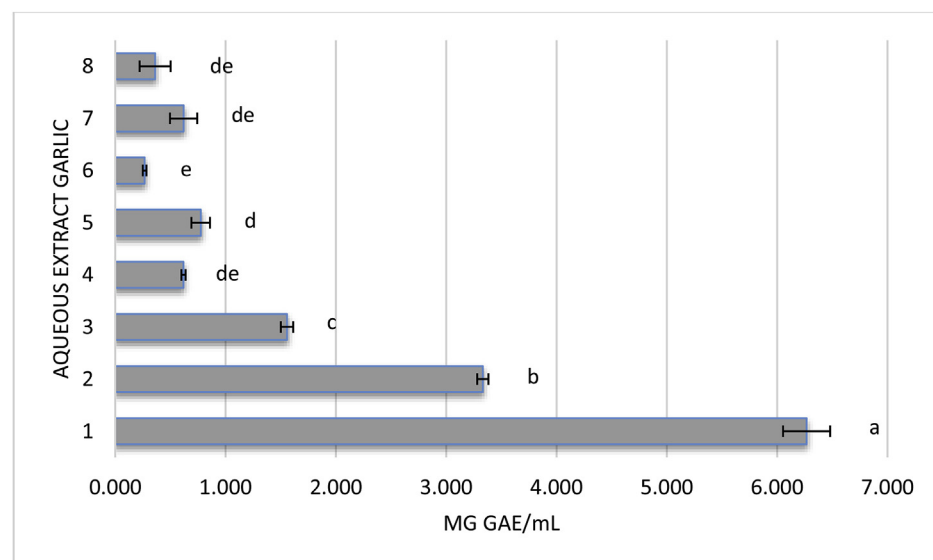


Fig. 1. Content of total polyphenols (expressed in μ g equivalents to gallic acid) of the aqueous extracts of garlic.

1, Aqueous extract of black garlic (AEBG) at 0.25 g/mL; 2, AEBG at 0.125 g/mL; 3, Aqueous extract of white garlic (AEWG) at 0.25 g/mL; 4, AEWG at 0.125 g/mL; 5, Aqueous extract of elephant garlic (AEEG) at 0.25 g/mL; 6, AEEG at 0.125 g/mL; 7, Aqueous extract of Japanese garlic (AEJG) at 0.25 g/mL; 8, AEJG at 0.125 g/mL.

Table 4Inhibition halos (mm) *in vitro* for commercial antimicrobials and their classification according to the CLSI (Clinical and Laboratory Standards Institute).

Bacteria	CF 30 µg	CIP 5 µg	FO 50 µg	GM 10 µg	MAC 300 µg	TE 30 µg	TSX 25 µg	SEM	P value
<i>E. coli</i> ATCC 25922	14.95 ± 0.86 ^c	21.39 ± 2.36 ^b	18.20 ± 1.32 ^{bc}	19.87 ± 0.94 ^b	0 ^d	26.68 ± 0.84 ^a	25.46 ± 1.25 ^a	1.86	0.001
CLSI	I	S	S	S	R	S	S		
<i>S. aureus</i>	24.58 ± 1.42 ^a	19.36 ± 1.36 ^b	0 ^d	12.11 ± 2.13 ^c	0 ^d	28.12 ± 1.30 ^a	26.03 ± 1.22 ^a	2.48	0.001
CLSI	S	I	R	R	R	S	S		
<i>L. monocytogenes</i>	24.26 ± 1.11 ^a	21.14 ± 1.89 ^b	25.66 ± 0.84 ^a	19.34 ± 0.72 ^b	0 ^c	25.39 ± 1.03 ^a	24.47 ± 1.21 ^a	1.90	0.001
CLSI	S	S	S	S	R	S	S		

CF, Cephalotin; CIP, Ciprofloxacin; FO, Fosfomicin; GM, Gentamicin; MAC, Nitrofurantoin; TE, Tetracycline; TSX, Trimetoprim/Sulfamethoxazole; R, Resistant; I, Intermediate; S, Sensitive. The values are expressed as the mean ± standard deviation. Means with similar letters are not statistically different at a level of P < 0.05 according to the Tukey test.

documented the presence of allicin in garlic extracts [28,29]. They mentioned that allicin is converted into vinyl dithiols and into other compounds such as methyl 2-propanol disulphide, Dimethyl trisulphide and Diallyl tetrasulphide. Wang et al. [30] concluded that these compounds are the main sulfur components of fresh garlic which decompose and form other compounds. The black garlic is SAC [31] – (Table 1).

Contrary to expectation, greater concentration of total polyphenols in black garlic did not have any effect on the bacteria. The antibacterial property of garlic is attributed to allicin, which is produced by grounding garlic. Allicin inhibits sulfhydryl enzymes and has been reported to reduce inflammation [8]. Cáceres et al. [32] highlighted the importance of the synergy between phenolic and sulfur compounds by blocking the activity of reactive oxygen on proteins, lipids and DNA. Another mechanism of action is the inhibition of RNA, DNA and protein synthesis [33].

Results showed that only the higher concentration of white garlic was comparable to the control treatment (tetracycline) in inhibiting *S. aureus* and *L. monocytogenes*. [34], reported a linear relation between garlic concentration and the diameter of inhibition halos, which they attributed to the effect of allicin and other sulfur compounds. García Rico et al. [35] evaluated the antibacterial activity of aqueous extract of three species of the Allium family (*Allium sativum*, *Allium fistulosum* and *Allium cepa*) on five bacterial strains, concluding that *A. cepa* showed greater effectiveness against *E. coli* with inhibition halos of 15 mm in diameter. Similarly, Salazar Córdova [36] reported a zone of inhibition of 14.3 mm against *E. coli*, with 25% concentration of aqueous extract of *A. sativum*. A previous study by Jiménez [2] reported that a range of 15.5–24.5 mm inhibition zones were established against *E. coli* 25922, *S. aureus* Methicillin Resistant and *S. aureus* ATCC 25923 using a garlic concentration of 250 mg/mL.

The allicin precursor enzyme, allinase, is inactivated by heat, so that the desirable bioactive is not formed if it is heated before cell disruption

[37]. This agrees with Pérez [38], who reported that during cooking, sulfur losses occur due to high temperature; and this could be the reason why black garlic extract showed no antibacterial activity. This reaction could either be enzymatic degradation or Maillard reaction responsible for changes in taste, smell and color [39,40]. Ryu and Kang [41] reported that this process increases the concentration of flavonoids, pyruvate, total phenol, SAC, free sugars and minerals.

In conclusion, higher concentration (0.25 g/mL) of aqueous extract of white garlic had the best antibacterial activity against *Escherichia coli* ATCC 25922, *Listeria monocytogenes* and *Staphylococcus aureus* compared with the aqueous extracts of Japanese garlic and elephant garlic. Aqueous extract of black garlic had no antimicrobial action on the bacteria studied. According to the results from the present study, the antimicrobial action of the aqueous extract of white garlic was comparable to tetracycline and can be used to replace synthetic antimicrobials.

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CRedit authorship contribution statement

Héctor D. Arzate Serrano: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing - original draft. **María A. Mariezcurrena-Berasain:** Funding acquisition, Supervision, Project administration. **Adriana Del Carmen Gutiérrez Castillo:** Writing - review & editing. **Benjamín Valladares Carranza:** Writing - review & editing. **Alberto Barabosa Pliego:** Writing - review & editing. **Martín Talavera Rojas:** Writing - review & editing. **Uchenna**

Table 5Inhibition of bacterial growth in millimeters (mm) of two concentrations of aqueous extract of white, Japanese, elephant and black garlic on *E. coli*, *S. aureus* and *L. monocytogenes*.

Bacteria	0,25 g/mL				TE 30 µg	MAC 300 µg	SEM	P value
	AEWG	AEJG	AEEG	AEBG				
<i>E. coli</i> ATCC 25922	17.83 ± 1.49 ^a	9.65 ± 1.59 ^b	0 ^c	0 ^c	26.24 ± 0.84	0	1.20	0.001
<i>S. aureus</i>	20.78 ± 1.53 ^a	9.78 ± 2.23 ^c	13.28 ± 1.52 ^b	0 ^d	24.64 ± 1.05	0	1.46	0.001
<i>L. monocytogenes</i>	21.57 ± 3.25 ^a	11.79 ± 0.56 ^b	12.48 ± 1.16 ^b	0 ^c	25.52 ± 0.61	0	1.68	0.001
Bacteria	0,125 g/mL				TE 30 µg	MAC 300 µg	SEM	P value
	AEWG	AEJG	AEEG	AEBG				
<i>E. coli</i> ATCC 25922	13.10 ± 1.28 ^a	0 ^b	0 ^b	0 ^b	26.24 ± 0.84	0	0.91	0.001
<i>S. aureus</i>	18.06 ± 1.60 ^a	0 ^b	0 ^b	0 ^b	24.64 ± 1.05	0	1.41	0.001
<i>L. monocytogenes</i>	16.48 ± 2.52 ^a	0 ^b	0 ^b	0 ^b	25.52 ± 0.61	0	1.15	0.001

AEWG, Aqueous extract of white garlic; AEJG, Aqueous Japanese garlic extract; AEEG, Aqueous extract of elephant garlic; AEBG, Aqueous extract of black garlic; TE, Tetracycline; MAC, Nitrofurantoin. The values are expressed as the mean ± standard deviation. Means with similar letters are not statistically different at a level of P < 0.05 according to the Tukey test.

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Declaration of competing interest

The authors declare no conflict of interest.

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