

In-vitro* Antibacterial Activity of crude Garlic (*Allium sativum*) Extract Against Clinical Isolates of Methicillin Resistant *Staphylococcus aureus

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Methicillin-resistant *Staphylococcus aureus* (MRSA) has gained significant health solicitude globally due to its resistance to nearly almost antimicrobial agents, and garlic is one of nature's most powerful antibiotics that must be used as a pharmaceutical regimen. The current study aimed to determine the In-Vitro antibacterial efficacy of crude garlic extract against MRSA. The aqueous and 70% ethanol crude garlic (*Allium sativum*) extract was prepared. Disc diffusion method was performed to assess the antimicrobial activity for 100 clinical isolates of MRSA collected, the reference standard strain was *Staphylococcus aureus* (ATCC 25923). All MRSA strains assessed were significantly sensitive to 70% ethanolic extract at various concentrations range from 200 to 25%, exhibited inhibitory effects against clinical isolates and *Staphylococcus aureus* (ATCC 25923) with the means of inhibition zones ranging from 17.76- 14.35 mm and 15-13 mm in length, while the aqueous extracts were less in both clinical isolates and *Staphylococcus aureus* (ATCC 25923) ranging from 11.93-8.62 mm and 11-8 mm respectively, methanol and distilled water were not affected on growth. The findings demonstrate that 70% ethanol extract of crude *Allium sativum* has significantly inhibitory effect on methicillin-resistant *Staphylococcus aureus* is better than aqueous extract. This study does not undermine the value of antibiotic use, but instead the probability of using them in low dosage to minimize their negative consequences.

Keywords: *Allium sativum*; Aqueous Extract; Alcoholic Extract; MRSA.

Plant-derived antimicrobials have extremely large therapeutic effects, and they are efficacious in treating infectious diseases even while reducing many of the problems associated with synthetic antimicrobials¹. The different pharmacological effects of plant products

are generally the result of secondary product combinations derived from plants. Such plant compounds are often secondary metabolites such as alkaloids, steroids, and fatty acid gums that have a certain physiological impact on the body^{2,3,4}. There are many natural elements and chemicals in garlic,

including sulfur compounds, numerous enzymes, different minerals, vitamins, and amino acids. Allicin (diallyl thiosulfinate or diallyl disulfide) is among garlic's greatest crucial biologically vigorous substances⁵. Even though allicin is deemed the prime antioxidant compound, other compounds may represent stronger roles; such as polar compounds of phenolic and steroidal origin, that display diverse pharmacological countenance without odor and are furthermore heat-stable⁶.

The efficacy and broad-spectrum antimicrobial activity of garlic versus huge species of bacteria (*Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus fecalis*, *Enterobacter cloacae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Shigella spp*, *Salmonella spp*, *Proteus mirabilis*, and *Helicobacter pylori*), viruses, parasites, and fungi have been documented⁷, also it is much more efficacious than industrial antimicrobials and has low toxicity; as a consequence, can be used as an additional option for the treatment of numerous infections^{8, 9, 10, 11}. The antibacterial activity of garlic has been fundamentally attributed to the entity of allicin generated by the enzymatic activity of allinase on allicin¹². Garlic is also efficacious toward antibiotic-resistant microorganisms, and combining garlic extracts with antimicrobial agents results in partial and total synergism¹³. The emergence of multidrug-resistant strains of Gram-negative and Gram-positive bacteria is troubling for humans and animals, as well as the emergence of epidemic MRSA, as a consequence, garlic is a popular alternative substance for the treatment of MRSA¹⁴. Hence current study aimed to determine the *In-Vitro* Antibacterial Activity of crude Garlic (*Allium Sativum*) extract against clinical isolate of Methicillin-Resistant *Staphylococcus aureus* (MRSA).

MATERIALS AND METHODS

Study design

A cross-sectional study was carried out during the period from April to June 2018 on a clinical isolate of MRSA from patients who were known to have MRSA after full microbiology identification methods, so specimens were collected from, wound, eye, sputum, and blood. Moreover Standard bacterial strains include American type

culture collection (ATCC), *S. aureus* (ATCC 25923) used as control positive. And Sudanese garlic bulbs with small size and strong odor were obtained from the Khartoum Bahri market to determine their antibacterial activity. Study was approved by the College of High Graduate Studies ethical committee/Shendi University.

Sample size

A hundred Isolates were collected randomly, 73 MRSA isolated from wounds, 3 eye swabs, 21 from blood culture, and 3 isolated from sputum samples. Clinical isolates collected according to availability regardless of their type, and all isolates show resistance patterns to Oxacillin were included in the study.

Laboratory methods

Plant materials collection

The *Garlic bulb* (seeds) was collected from Central Sudan, Khartoum Bahri market in April 2018, and it was checked by the Medicinal and Aromatic Plants professional at Traditional Medicine Research Institute (TMRI), Khartoum, Sudan.

Preparation of crude extracts

Extraction was carried out for the seeds of garlic plant 100 g by using maceration techniques after weighting the grounded material by sensitive balance about 34g round material was macerated equal quantity for both aqueous and 70% ethanol extract.

For preparation of aqueous extraction, about 100 g of the garlic was soaking in 100 ml hot distilled water for about four hours with continuous steering. After cooled, extract was filtered using filter paper and the solvents were evaporated using freeze drier, and the yield percentage were calculated as follow: Weight of extract obtained / weight of plant sample X100.

For obtaining 70% extraction of Ethanol, 100 g of garlic was grinding by mortar and impregnating in 70% ethanol for 5 days with liquidation and steaming on daily basis. David calculated the given percentages after the solvent was evaporated under low pressure to arid and the extract was allowed to air dry completely, then multiply the weight of the extract by the weight of the sample. Finally, it was stored at 4°C in a tightly bottled glass flask, ready for use.

Bacteria strains

Under aseptic conditions, 100 clinical

isolates of MRSA were obtained from El Ribat University hospital's Microbiology laboratory in Khartoum, Sudan. The specimens were isolated from wound swabs, sputum, eye swabs, and blood culture and cultured in suitable media such as blood agar, chocolate blood agar, and McConkey agar and incubated aerobically at 37° C over night.

Identification was achieved depending on colonial morphology, the feature of culture media, gram stain, biochemical reactions (Catalase, coagulase, DNase test, and Mannitol Salt Agar), and susceptibility to Oxacillin discs (1 µg) using Mueller-Hinton agar MHA; when the zone of inhibition is more than 12 mm were identified as sensitive and 11-12mm identified as intermediate^{15, 16}.

Catalase test

A pure of 2-3 ml of hydrogen peroxide solution was added in a test tube, by sterile wooden stick several colonies of test organisms were put in hydrogen peroxide solution. The positive results indicated by immediate bubbling¹⁵

Coagulase test

a colony of the test organism was emulsified in drop of physiological saline to make thick suspension and a drop of plasma was added to and mixed gently by rotating. The positive results indicated by producing clump within 10 seconds¹⁵.

Deoxyribonuclease (DNase) test

The test organism was cultured on a medium which contains DNA. After overnight incubation, the colonies were tested for DNase production by flooding the plate with a weak hydrochloric acid solution. The acid precipitated hydrolyzed DNA. DNase producing colonies were therefore surrounded by clear areas due to DNA hydrolysis¹⁵.

Manitol Salt Agar (MSA)

A portion of colony was inoculated on manitol salt agar containing 75g/l sodium chloride and incubated aerobically at 37oC for 18-24 hours. *S. aureus* ferments manitol producing yellow colonies¹⁵.

Detection of Methicillin Resistance

MRSA identification was carried out using oxacillin screen plates following the guidelines of NCCLS. Briefly, a suspension equivalent to 0.5 McFarland standards, prepared from each strain, was inoculated homogenously on the entire surface of the Mueller-Hinton agar plate (Oxoid-

UK) containing 4% NaCl and 6 ig/mL oxacillin, with the help of sterile swabs. All the plates were incubated at 35° C for 24 hrs. Indication of growth (>1 colony) identified the isolates as oxacillin/methicillin-resistant (Genç *et al.*, 2008) ,when zone of inhibition present above 12mm was identified as sensitive and 11-12mm identified as intermediate.

Antibacterial susceptibility testing for extracts

The paper disc diffusion method was being used to evaluate the antimicrobial property of garlic extracts using MHA. The dilution factor of Bacterial suspension was done with a sterile physiological solution to 10⁸ CFU/ ml (turbidity = McFarland standard 0.5). 100 µl of bacterial suspension swabbed regularly on MHA and was allowed to dry for 5 minutes. Then filter paper discs (Whatman No.1, 6 mm in diameter) was Sterilized in an oven at 80°C for 30 minutes, and placed on MHA and soaked with 20 µl of a solution of each garlic extract with diluted different serial dilution as follow: 25%, 50%, 100%, 200% (0.25mg/ml, 0.5mg/ml, 1mg/ml, 2mg/ml) respectively, next suspended by methanol for 70% ethanolic extract and the same concentration used after re-suspended by sterile distilled water for aqueous extract. The inoculated plates were incubated at 37°C overnight.

Data analysis

All data were analyzed using (SPSS) software version 21, descriptive statistics (e.g., frequency and percentage), chi-square test, were used and the threshold for statistical significance was ($p < 0.05$).

RESULTS

A total of 100 clinical isolates were collected from wound swab 73%, eye swab 3%, sputum 3%, and blood for culture 21%. All isolates were positive for catalase, coagulase, Deoxyribonuclease (DNase) test , and MSA as well as all isolates were showed growth on the Oxacillin disc, which indicates resistance. 70% ethanol extract and their inhibition effect between MRSA clinical isolates and ATCC (25923) reference strains was significantly stated in higher concentration of ethanol 200%, and 100% (P-value=0.001, 0.038) respectively. No significant difference in aqueous extraction, result displayed in table 2 and 3. Different concentrations of 70% ethanol extract and aqueous extract and

their inhibition effect related to gender, there is no significant P. value” 0.05 association between sex with different concentrations of ethanol extracts and their inhibition effect. All data are summarized in table 4.

Figures 1, 2 display the antibacterial activity of *A. sativum* 70% ethanol and aqueous extract tested on clinical isolates and ATCC (25923) *Staphylococcus aureus* reference strain after re-suspended with distilled water as negative control respectively. The 70% ethanol extracts in all concentrations were sensitive when matched with oxacillin susceptibility test on MRSA strain, intermediate only in 200% (2mg/ml) aqueous extract, and other concentrations were resistant. The activity of *A. sativum* in both extracts increased with the concentration of it, Figure 3 display plates (a and c) showed antibacterial activity of 70% ethanol, and aqueous extract of *A. sativum* extract as well on clinical isolates, plate (b and d) showed antibacterial activity of 70%

ethanol, and aqueous extract of *A. sativum* extract STD ATCC strain.

DISCUSSION

Antibiotics’ massive use in the treatment of infectious diseases has resulted in the emergence and development of resistant strains, which is now a significant cause of failure in the treatment of infectious diseases¹⁷. In this study, 70 % ethanol extract of crude *A. sativum* showed remarkable antibacterial activity against standard strains of *S. aureus* and clinical isolates methicillin-resistant that agreed with Janan *et al*¹⁸, while aqueous extract showed little activity, these were different from that obtained by Hadir *et al*¹⁹, may be referred to using the homogenization aqueous extraction technique. However, the opposite results of extract do not indicate less activity of bioactive constituents because the active ingredient (s) could be existent in inadequate amounts. , but the use of boiled water

Table 1. Aqueous and 70% Ethanol Extraction

Sample No	Name of plant	Weight of plant in g	Weight of extract in Gram	Yield %
1	Garlic	100 g	6.8 g	6.8 %
2	Garlic	100 g	7.2 g	7.2 %

Table 2. 70% ethanol extract and their inhibition effect between MRSA clinical isolates and ATCC (25923) reference strains

Con%	MRSA Clinical isolates Mean± SD	ATCC reference strain Mean± SD	P. value
200%	17.59 ± 0.880	14.56 ± 1.095	0.001
100%	17.96 ± 1.095	12.24 ± 0.632	0.038
50%	15.41 ± 0.714	13.62 ± 0.805	0.761
25%	14.33 ± 0.614	12.25 ± 0.47	0.798

Table 3. Aqueous extract and their inhibition effect between MRSA clinical isolates and ATCC (25923) reference strains

Con%	MRSA Clinical isolates Mean± SD	ATCC reference strain Mean± SD	P. value
200%	11.91 ± 0.293	10.88 ± 0.206	0.329
100%	10.65 ± 0.555	9.51 ± 0.465	0.642
50%	9.59 ± 0.659	9.44 ± 1.501	0.571
25%	8.57 ± 0.716	8.12 ± 0.474	0.422

Table 4. Different concentrations of 70% ethanol and aqueous extract and their inhibition effect related to gender

Con%	Male n=54 Mean± SD	Female n=46 Mean± SD	P. value
70% Ethanol Extract			
200%	17.59 ± 0.880	17.96 ± 1.095	0.74
100%	17.96 ± 1.095	16.54 ± 0.836	0.388
50%	15.41 ± 0.714	15.41 ± 0.805	0.971
25%	14.33 ± 0.614	14.37 ± 0.771	0.798
Aqueous Extract			
200%	11.91 ± 0.293	11.96 ± 0.206	0.329
100%	10.665 ± 0.555	10.70 ± 0.465	0.642
50%	9.59 ± 0.659	9.46 ± 1.501	0.571
25%	8.57 ± 0.716	8.67 ± 0.474	0.422

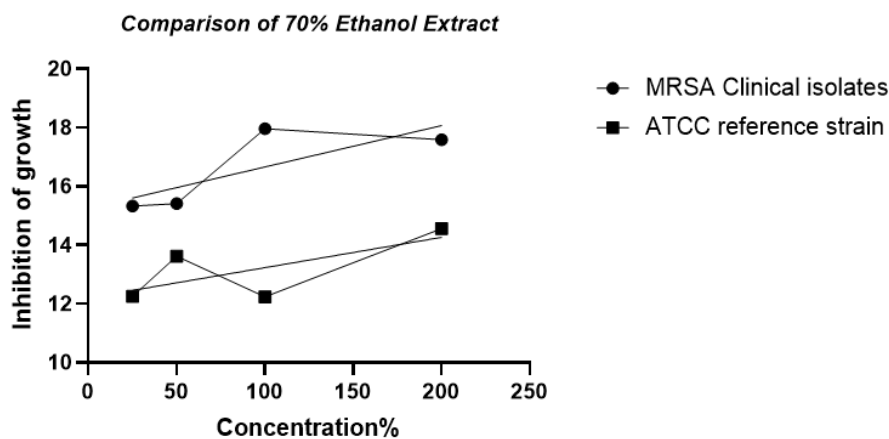


Fig. 1. Mean of 70% of growth inhibition of clinical isolates of MRSA and ATCC references strain

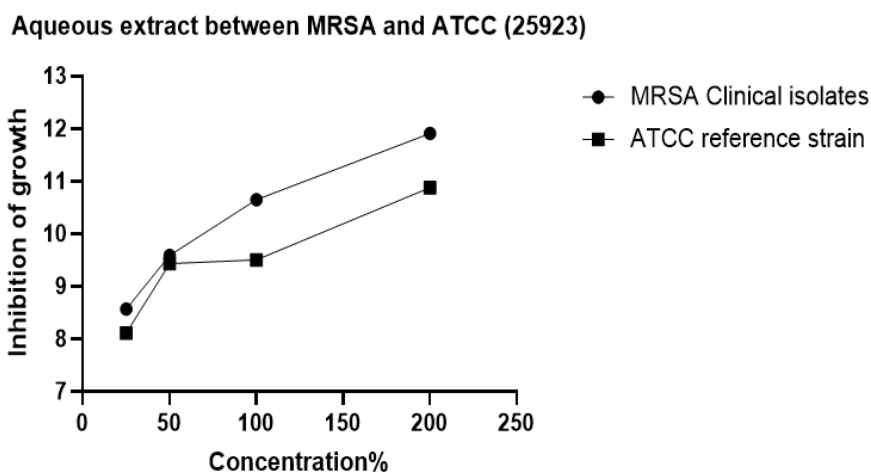


Fig. 2. Mean of aqueous growth inhibition of clinical isolates of MRSA and ATCC references strain

in the maceration method of the extract for garlic may be an effect on the stability of allicin and other bioactive components that agreed with Strika *et al*²⁰. Besides that, this could be due to various main: hydrogen bonding of water to the reactive oxygen atom in allicin can minimize its instability; and/or there could be water-soluble elements in minced

garlic that destabilize the chemical compound that Lawson mentioned²¹.

The limitation of this strategy is that allicin can develop diallyl di-sulfide once it reacts with water^{22, 23}, which may not have the same degree of antimicrobial effect as allicin. The yield percentage of ethanolic extract was noted

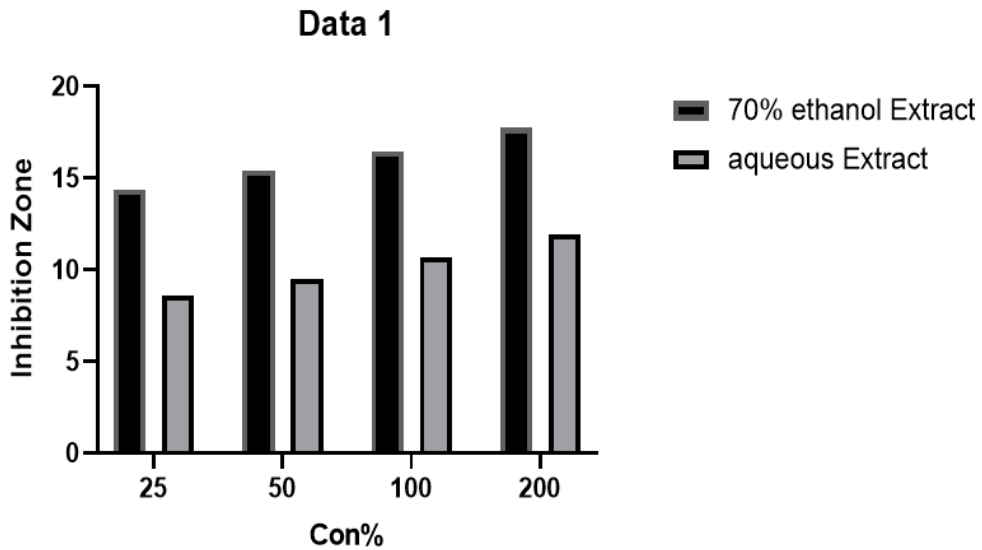


Fig. 3. The antibacterial activity of *A. sativum* aqueous extract opposite with 70% ethanol extract against clinical isolate MRSA



Fig. 4. Plates (a and c) shows an antibacterial activity of 70% ethanol and aqueous *A. sativum* extract on clinical isolate MRSA strains, and plate (b and d) display antibacterial activity of 70% ethanol *A. sativum* extract on STD ATCC strain

7.2 % slightly increased than aqueous 6.8 %, and that due to the time of extract according to the methodology of technique, in aqueous 4 hours only needed and then filtrated the final product in one day, but the ethanolic extract needed 5 days with daily filtration. This effect of time on yield and in sufficient quantity has a role in antibacterial activity. The most probable explanation for these differences between results of zone inhibition mm in this study compared with other studies may be due to the different bacterial strains tested, and the antibacterial activity methods, method of extract, and time, plus the concentration of organo sulfur compounds, which vary greatly between garlic species around the world²⁴. A study by Bayan L et al²⁵ noted that one negative aspect in ascertaining the antimicrobial property of Garlic Extract is the inability to detect Methicillin Resistance results, the scientists used standardization in their methodologies, and this results in significant differences in the outcomes. As a result, it is crucial to establish policies and procedures for all guidelines used to assess the antimicrobial property of garlic extract. One major benefit of garlic is that bacteria need not appear to emerge resistant to it, as they do to several advanced antibiotics: “garlic does not seem to produce such resistant strains” Erdogrul,^{26, 27}. With greatly increased extract concentration, the mean diameter of the growth inhibition zone of standard and clinical isolates of bacteria enhanced. This outcome is in contact with the report of Mahfuzul Hoque et al. MD²⁸. The best inhibition zone obtained by 70% ethanol extract of crude garlic was 20 mm in diameter against clinical isolates of MRSA strains at the concentration of 4 mg\ 2ml, (200% conc). And the minimum inhibition zone was 13 mm in diameter at 0.5 mg/ml (25% conc) on aqueous. Despite the fact that garlic has been postulated to have a synergistic or additive impact with regular antimicrobial agents against *E. coli* and *S. aureus* just a few studies have been conducted to demonstrate the effect of garlic when used in combination with standard antimicrobials^{29, 30}.

Limitations of study and Prospective view

The extract's active garlic ingredient (Allicin) compounds are crucial for its antimicrobial properties. More research is required to verify these results and for evaluating the combination of garlic with other commercial antibiotics. so Broad

extraction projects for garlic plant components is crucial to determine the best active ingredients by an ideal standard methods. Alternative methods to extract processing by solvents must be more used and tested as a squeezing technique to avoid the loss of bioactivity of components. one of the limitation of current study is that a commercial / clinical antibiotic) to show the efficacy of the garlic extracts were not used in this study.

CONCLUSION

The findings demonstrate that 70% ethanol extract of crude *Allium sativum* has significantly inhibitory effect on methicillin-resistant *Staphylococcus aureus* is better than aqueous extract. From this, we can conclude that the potential of using alcohol - related extract of garlic as potential antimicrobial agents. This study does not undermine the value of antibiotic use, but instead the probability of using them in low dosage to minimize their negative consequences.

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Conflict of interest

The authors declare that there is no conflict of interest.

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None

Authors' contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

Data Availability

All datasets generated or analyzed during this study are included in the manuscript.

Ethical statement

This article does not contain any studies with human participants or animals performed by any of the authors.

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