Antibacterial potency of garlic extract against certain skin pathogenic bacteria

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Abstract

The antibacterial potency of garlic extract (*Allium sativum*) against gram positive and gram negative skin pathogenic bacteria including; *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, and *Pseudomonas aeruginosa* were studied using agar well diffusion and broth dilution assays. Agar well diffusion assay for aqueous garlic extract (AGE) was characterized with zones of inhibition ranging from 4.40 – 3.80cm, 4.13 - 3.57cm, 3.40 – 2.67cm for *S. aureus*, *S. epidermidis* and *Strep. pyogenes*, respectively, however, *Ps. aeruginosa* had lesser zone of inhibition ranging from 2.32 – 1.55cm. Studying the antibacterial potency of AGE against the selected isolates, revealed that it is affected by temperature of storage. Current results showed that storage of AGE at low temperature of -20°C, does not affect its potency, however, its potency was slightly lost at high temperatures above 37°C. The broth dilution test was performed to investigate the Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of the AGE against the bacterial isolates at 37°C. Investigating the activity of AGE loaded on Gel dressing revealed that it can have potency when applied on patients with Staphylococcal skin infections. Findings from this study encourage and support the use of AGE in treating bacterial skin infections especially in developing countries like Africa, as it is available, economic and have no side effects.

Keywords: Garlic extract, Skin pathogens, Antibacterial activity, Gel dressing

1. Introduction

According to Pailler-Mattei *et al*., (2007), skin is the largest organ of the body; it is the principle barrier of the body against external environment. It consists of three layers; epidermis, hypodermis and subcutaneous fat which is subdivided into soft and viscous tissues (Gould, 2009).

Human skin infections, particularly those involving the soft skin and mucosal surfaces constitute a serious problem, especially in tropical and subtropical developing countries (Falahati *et al*., 2005). Methicillin-resistant *S. aureus* (MRSA), *S. epidermidis*, *Escherichia coli (E. coli)*, *Ps. aeruginosa*, and *Candida albicans* were recorded to be the most frequent skin pathogens. MRSA gained much attention in the past decade, as it is a major cause of hospital-acquired infections. The majority of people on this planet still depend on traditional medicine for their daily health care needs. A lot of efforts have been made to discover novel antimicrobial compounds from various species of medicinal herbs (Obeidat *et al*., 2012).

Medicinal herbs are worldwide and heavily used in folk medicine; therefore, screening of such herbs may result in the discovery of novel effective compounds capable of working against pathogenic microorganisms. Compounds that can either inhibit the growth of human skin pathogens or kill them with no toxicity to host cells are considered candidates for developing new antimicrobial drugs. Consequently, there is a critical need to research for new antimicrobial agents with promising natural activities to provide an alternative to common antibiotics. For many decades, garlic has been known for its medicinal activities. Its antibacterial potency is well known against many bacterial species including gram-positive bacteria such as *S. aureus* and gram-negative bacteria such as *E. coli* (Zhang *et al*., 2016).

The aims of the current study were to; evaluate the antibacterial efficacy of aqueous garlic extract, and to design antibacterial dressings such as a gel or cream based upon natural plant materials (AGE) for skin application.

2. Materials and Methods

The antibacterial activity of aqueous and ethanolic garlic extracts was screened against frequent skin pathogens by using the agar well diffusion assay (Bauer *et al*., 1966). The key microorganisms used for all experiments were bacterial cultures of *S. aureus*, *S. epidermidis*, *Strep. pyogenes* and *Ps. aeruginosa*. These bacterial isolates were chosen as representatives of gram positive and gram negative skin pathogens. They were provided from medical culture collection of the University of Wolverhampton, United Kingdom.

2.1. Preparation of ethanol and aqueous garlic extracts

Garlic powder obtained from Cultech Ltd, UK, was soaked in ethanol at the ratio of 1:10 w/v and extracted for 24h at room temperature with shaking at 150rpm. Filtrate of this extract was re-suspended in phosphate buffered saline (PBS) to bring to 500mg/ ml (Obeidat *et al*., 2012). To prepare aqueous garlic extract of 10% (w/v), 9ml of distilled water was added into the sterile UV bottle, and then 1g of powdered garlic was added. The extract was left to stand at room temperature with periodic agitation. After 20 min., the extract was then centrifuged at 4500 rpm for 15 min. Immediately after centrifugation, two filtration steps were carried out using a swinnex filter and 0.45µm membrane filter to obtain sterile extract. Garlic extracts were stored at 4°C till use.

2.2. Preparation of culture media

Tryptic soy agar (TSA) and Tryptic soy broth (TSB) were obtained from the laboratory of University of Wolverhampton, UK, and stored at 4°C till use.

2.3. Agar well diffusion assay

The Antibacterial efficacy of the aqueous and ethanol garlic extracts was screened by using agar well diffusion assay (Perez *et al*., 1990). The bacteria were seeded separately into TSA, the poured into plates. Subsequently, agar discs were cut at equal distances using 6mm cork-borer, and then removed aseptically to make a well. Each well was filled separately with 100µl of each garlic extract, incubated at 37°C for 24h. The zones of inhibition (ZI) were then measured.

2.4. Effect of storage temperature on the antibacterial potency of the aqueous garlic extract

This assay was carried out to evaluate the antibacterial potency of the AGE against tested bacterial isolates after storage at different temperatures of -20°C, -4°C and 37°C. 10% (w/v) garlic extract was prepared and stored at pre-mentioned temperatures for periods of 0, 24, 48, and 72h, respectively, and then tested for antibacterial potency.

2.5. Determination of the minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the AGE were determined following the methods of Nakamura *et al*., (1999); Dulger and Aki (2009); Obeidat, (2011) with slight modifications. A set of tubes were prepared using double strength TSB broth containing; 5%, 2.5%, 1.25%, 0.625%, 0.3%, 0.15%, 0.08 and 0% (Positive control) of the AGE to be tested. Three replicates were made for each concentration with one set as a negative control.

Then 0.1 ml of 1×10^6 cfu of each tested bacteria was inoculated separately into all tubes excluding the negative control tube. All sets were incubated at 37°C for 24h, and then turbidity was recorded. The first tube with no turbidity was recorded as the MIC.

2.6. Determination of the minimum bactericidal concentration (MBC)

The MBC test was performed following results from the MIC in order to confirm if the AGE is actually killing the bacteria or only inhibiting them. 10µl from each concentration of the MIC tubes was dropped as a spot on TSA plate then spread using a sterile glass rod, incubated for 24h at 37°C. All samples were examined in triplicates. The lowest concentration which showed no bacterial growth was indicated as the MBC (Obeidat, 2011).

2.7. Preparation of Gel dressings

In order to make a gel dressing, the methods Fulton and James, (1990) was followed with some modifications. Pure agar was prepared using 3.7g of agar No.2 powder into 100ml of distilled water in a sterile 150ml glass bottle. In order to produce a 2.5% garlic gel, 5ml of 10% (w/v) of AGE was transferred into 5ml of pure agar and gentle swirls were given. This was then immediately poured onto a petri dish and left to solidify at room temperature. Once the gel has set, it was cut out into the desired size. Plates were sealed tightly; stored in the freeze drier for 48h, then tested for antibacterial efficacy.

2.8. Statistical analysis

The experiments were set up in a completely randomized factorial design (bacterial strains × time duration) with three replicates for each. Data recorded were analyzed statistically by the analysis of variance using the statistical software 'SPSS 20' and the differences between means were compared using

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the Least Significant Differences (LSD) at 5% level of probability (P < 0.05).

3. Results

3.1. Agar well diffusion assay to detect the antibacterial efficacy of AGE against skin pathogenic bacteria

Results observed in Fig. 1. demonstrate that the aqueous and ethanol extracts were effective against all tested bacteria. However, the AGE gave much higher diameters of inhibition zones than ethanol extract. Consequently all sort of further research were performed using AGE only.
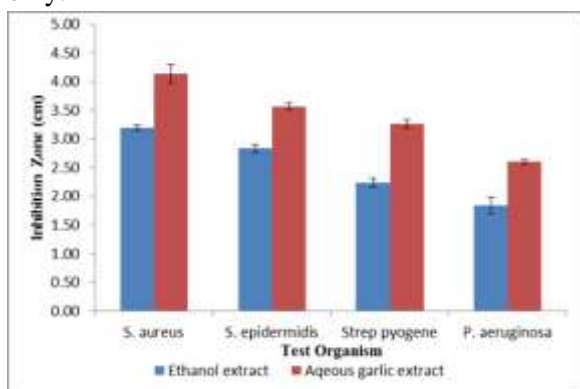

Fig. 1. Comparison of diameters of inhibition zones (antibacterial activities) of the 10% aqueous and ethanol garlic extracts. Error bar represent the SD of the mean (n=3).

3.2. Effect of different storage temperatures on the antibacterial activity of the AGE

Results in Fig.'s 2. and 3. showed that *S. aureus* had the highest diameter of inhibition zones ranging from 4.40 – 3.80 cm, followed by *S. epidermidis* ranging from 4.13 - 3.57cm, and then *Strep. pyogenes* with zones ranging from 3.40 – 2.67cm. However, least inhibition zones of about 2.32 – 1.55cm were observed in *Ps. aeruginosa.* The diameter of inhibition zones decreased slightly on increasing the storage periods from 0- 72h. There was least significant difference (LSD = 0.09) between the *S. aureus* and *S. epidermidis* isolates at both storage

temperatures of -20°C (Fig. 2.) and -4°C (Fig. 3.), however, *Strep. pyogenes* and *Ps. aeruginosa* showed high significant difference (P>0.02) at both temperatures. Thus, *S. aureus* and *S. epidermidis* were highly more sensitive to the AGE than *Strep. pyogenes* and *Ps. aeruginosa* at both of these storage temperatures.
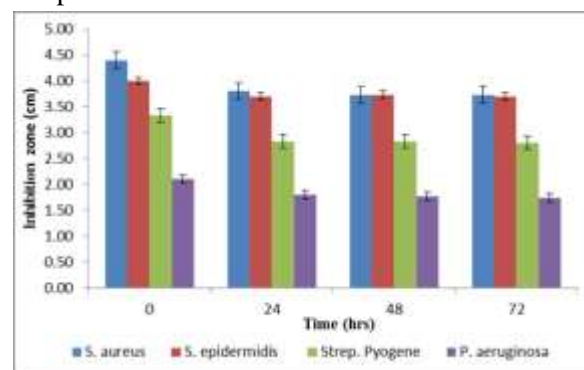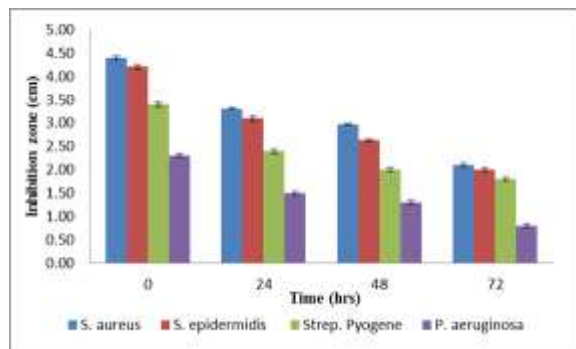

Fig. 2. Effect of storage of AGE at -20°C for different periods on its efficacy against bacterial isolates measured using diameters of inhibition zones. The vertical error bars represent the standard error based on 3 replicates of the mean (n=3).
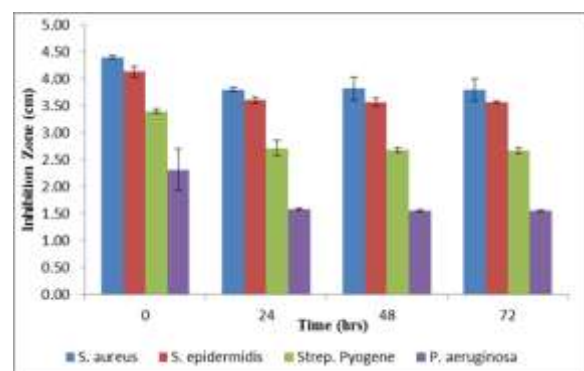
Fig. 3. Effect of storage of AGE at - 4°C for different periods on its efficacy against bacterial isolates measured using diameters of inhibition zones. The vertical error bars represent the standard error based on 3 replicates of the mean (n=3).

Fig. 4. demonstrated high decrease of antibacterial activities of the AGE stored at 37°C. *S. aureus*, *S. epidermidis* and *Strep. pyogenes* were significantly more sensitive (P < 0.05) to the AGE than *Ps. aeruginosa* upon storage at 37°C. Following the results in Fig. 4., there is no significant difference between *S. aureus* and *S. epidermidis* in the extract stored at

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37^oC, however, there was main difference (LSD = 0.15) between *Strep. pyogenes*. and *Ps. aeruginosa*. These observed results implies that AGE can retain its activity both at low storage temperatures of (-20^oC, -4^oC) and at higher temperature of (37^oC).

Fig. 4. Effect of storage of AGE at 37^oC for different periods on its efficacy against bacterial isolates measured using diameters of inhibition zones. The vertical error bars represent the standard error based on 3 replicates of the mean (n=3).

3.3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the AGE on different skin pathogenic bacteria

This assay was done to determine the MIC and MBC of the garlic extracts against *S. aureus*, *S. epidermidis*, *Strep. pyogenes* and *Ps. aeruginosa* in a broth medium, to determine the lowest concentration which inhibit or prevent the bacterial growth thus could be incorporated into the gel.

Results presented in Table 1. demonstrate that, *Ps. aeruginosa* had the highest MIC value followed by *Strep. pyogenes* and *S. epidermidis*, while *S. aureus* had the least MIC value. The MBC value for *Ps. aeruginosa* was 5%, while *S. aureus*, *S. epidermidis* and *Strep. pyogenes* had the MBC values of 2.5%. These results implies that *S. aureus* was the most sensitive to 10% AGE followed by *S. epidermidis* and *Strep. pyogenes*, while *Ps. aeruginosa* was the least

sensitive. Thus high concentration of the AGE is needed to prevent the growth of *Ps. aeruginosa*.

3.4. Evaluation of antibacterial efficacy of AGE on clinical dressing

This assay was carried out in order to assess the efficacy of the AGE of 2.5% clinical dressing, as it showed its potency on both solid (agar well diffusion assay) and broth (broth dilution assay) media.

According to results observed in Fig. 5., AGE showed its antibacterial efficacy upon incorporation into both fresh gel and freeze dried gel against all bacterial isolates tested in this study. However, fresh gel seems to have a higher efficacy compared to freeze dried one. *S. aureus* was highly sensitive to the extract; on the contrary, *Ps. aeruginosa* was more resistant to both gels.
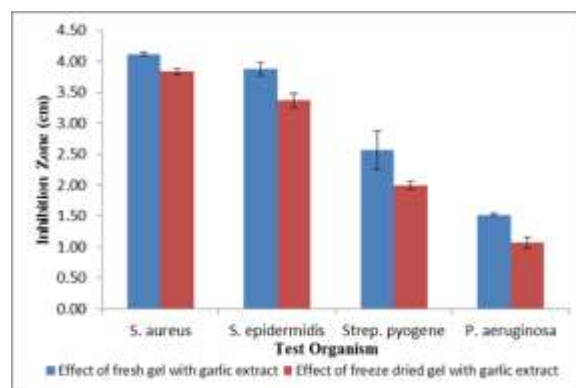
Fig. 5. Effect of fresh and freeze-dried 2.5% agar gels containing AGE (10% w/v), upon its antibacterial activity. Freeze-dried gels were rehydrated prior to testing. Error bars represent the standard error based on 3 replicates of the mean (n=3).

The antibacterial efficacy against all tested bacteria was not temperature dependent; this implies that the gel can be effective on human skin having temperatures between 20^oC and 37^oC. Thereby, AGE clinical gel dressing can be an effective drug of choice for treating bacterial skin infections, especially in the tropical regions like Africa; where it will be a better choice there because it's economic and available.

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Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of 10% (w/v) AGE against different tested skin pathogenic bacteria

Bacterial isolates	MIC and MBC of AGE			
	MIC (%)	MIC range (%)	MBC (%)	MBC range (%)
S. aureus	0.625%	(1.25-0.625%)	2.5%	(1.25-2.5%)
S. epidermidis	1.25%	(1.25-2.5%)	2.5%	(1.25-2.5%)
Strep. pyogenes	1.25%	(1.25-2.5%)	2.5%	(1.25-2.5%)
Ps. aeruginosa	2.5%	(2.5-5.0%)	5%	(5.0-10%)

-Results are averages of three replicates.

4. Discussion

Garlic (*Allium sativum*) has attracted too much attention because of its durability during shipping and storage characteristics. For centuries, it has been known as a flavoring agent with important medicinal characteristics (Martins *et al*., 2016). Many authors (Sung *et al*., 2014; Li *et al*., 2016; El-Sayed *et al*., 2017) proposed the use of garlic because of its antibacterial and antifungal efficacy, in addition to its active ingredients present in the essential oil.

In this study, the AGE gave largest diameters of inhibition zones compared to ethanol extract against all the tested skin pathogenic bacteria. The antibacterial activity of garlic extract were against some gram positive (*S. aureus*, *S. epidermidis*, and *Strep. pyogenes*) and gram negative (*Ps. aeruginosa*) bacteria, correlating with the report of Cutler and Wilson, (2012) that garlic has antibacterial activity against a wide range of bacteria. The antibacterial potency of fresh garlic extract against many species of pathogenic bacteria has been well-documented (Curtis *et al*., 2004), even against species having acquired resistance to antibiotics, such as *S. aureus* (Marchese *et al*., 2016).The order of sensitivity of the bacterial isolates to 10% garlic extract was; *S. aureus* > *S. epidermidis* > *Strep. pyogenes* > *Ps. aeruginosa*. Thus, *Ps. aeruginosa* was less sensitive to the AGE used, in agreement with the report of Godden and Keynes, (2005). In addition, the study of Abubakar, (2009) showed that the MBC of AGE for *Ps. aeruginosa* was the highest (150mg/ml), compared to *S. aureus* (75mg/ml), *S. pneumoniae* (100mg/ml) and *E. coli* (125mg/ml), implying that a higher concentration of AGE is needed to inhibit *Ps. aeruginosa*, in accordance with our results.

On the contrary, the study of Bakri and Douglas, (2005) showed that the MIC value of garlic extract was lower for gram negative bacteria (MIC range 35.7 – 1.1 mg/ml) than for gram positive bacteria (MIC range 142.7 – 35.7 mg/ml). Eja *et al*., (2007) explained this difference in their sensitivity due to the nature of their cell wall structure. The cell wall of gram negative bacteria is made up of 15 – 20% polysaccharides and 10 – 20% lipids, while the wall of gram positive bacteria consist of 35-60% polysaccharide and only 0.2% lipids. The

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permeability of the allicin and other garlic constituents are affected by the polysaccharide and the lipid component of the cell wall. Thus allicin component is more permeable through the gram negative cell wall than through gram positive cell wall. In addition, the method of testing the antibacterial efficacy of the garlic extract was often different, as some researchers chose to use Agar well diffusion assay (Iwalokun *et al*., 2004), while others used Disc diffusion assay (Praba and Kumaresan, 2014), which could accordingly influence testing the efficacy of this extract.

Moreover, the growth mediums may also affect the susceptibility of the bacteria to antibiotics and to the garlic extract, as some bacteria can grow faster in certain media such as TSA or TSB, while others cannot. The source of garlic may affect the amount of allicin. This hypothesis was confirmed by Praba and Kumaresan, (2014) who tested the antimicrobial activity of fresh cloves and leaves of garlic collected from agricultural land of Haridwar, Uttarkhand (U.K.) and Bulandshahr, Uttar Pradesh (U.P), and they found that the U.K sample exhibited higher antimicrobial activity compared to the U.P samples. In addition, the ability of garlic to release organo sulphur compounds such as allicin depends on the method of extraction of garlic. The MBC value of the AGE for gram negative bacteria (*Ps. aeruginosa*) was high 5% (50mg/ml) which is similar with the studies of Abubakar, (2009), who observed that the MBC of garlic extract for *Ps. aeruginosa* was 150mg/ml.

In the current study, the sensitivity of the tested bacteria to the 10% AGE was temperature dependent. However, garlic can still retain its antibacterial activity at higher temperatures of 37°C and lower temperatures of -20°C on storage for 0, 24, 48 and 72h. Atsamnia *et al*., (2017) stated that increasing the temperature above $42°$C causes a reduction of the

antibacterial potency of garlic extract, marked by a reduction of diameters of inhibition zones. Accordingly AGE can work in tropical climates having an environmental temperature as high as 37°C, as well as in cold and temperate regions having an environmental temperature of less than 20°C.

In agreement to the findings of our study, Al-Astal, (2003) observed that there is an increase in efficacy of garlic extract against *S. aureus* on storage from 0-6h and a decrease in its efficacy from 12 – 72h. Al-Astal, (2003) explained that, allicinase needs about six hours to attain the optimal time to act on alliin thereby producing the antibacterial material allicin. He added that, the efficacy of the AGE is lost upon further storage because allicin changes to entirely different inactive compounds mainly; diallyldisulfide and diallyltrisulfide.

There are several numbers of pharmaceutical dressing products for treatment of skin diseases, mainly being topical application such as cream, gel and ointment. Our results indicated that gel incorporated with AGE can therefore be applied onto clinical dressing before applying onto the skin surface of individuals infected with bacterial skin infections. This is in agreement with the results of Rajan *et al*., (2017), who reported that allicin incorporation to fabric exhibits good antimicrobial activity against the inoculated pathogens. They added that allicin nano-composite have good bactericidal property against the two pathogenic isolates of *S. aureus* and *E. coli.*

According to Chen *et al*., (2018), changes in permeability of cell membranes and protein leakage detected by scanning electron microscopy suggested that the antibacterial potency of the AGE might be due to destruction of the structural integrity of bacterial cell membranes; leading thus to its death.

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Conclusion

This study has moved beyond laboratory work to making of clinical dressing which could be applied directly onto the skin of people infected pathogenic bacteria. AGE on the gel was not temperature dependent, this implies that it can work in tropical climate such as Africa where people have high skin temperature, as well as in Europe where people have low skin temperatures. The aims of this study have therefore been achieved as garlic could be a drug of choice in Africa where people are poor and cannot buy expensive drugs such as antibiotics; however, garlic extract is economic and available.

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Fresh AGE in a gel

Freeze dried AGE in a gel

Freeze dried AGE stored aseptically for use as gel on bacterial skin infections