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Effect of Storage Temperature on the Antimicrobial Activity of Aqueous Garlic Extract

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Abstract:
Background*: Analgesia, one of the components of triad of anaesthesia, has now extended to relief of postoperative pain, chronic pain and cancer pain. The spinal cord has taken the center stage in analgesia practice and Spinal anaesthesia is the commonly used technique for lower limb surgeries as it is easy to administer, economical and causes less hemodynamic variation than general anaesthesia. Hence different additives can be used to increase the duration of postoperative analgesia. Since there are no studies comparing Buprenorphine and Nalbuphine, we have selected this study to evaluate the effect of intrathecal Bupivacaine with Buprenorphine compared with Nalbuphine for postoperative analgesia.*
Materials and Methods*: In this prospective randomised controlled study, 60 patients of ASA physical status I and II belonging to age group of 18-60years undergoing elective lower limb surgery under sub-arachnoid block were randomly allocated into 2 groups of 30patients each, Group A (Bupivacaine and Nalbuphine) and Group B (Bupivacaine and Buprenorphine). Group A received 2.8ml of 0.5%(H)Bupivacaine+[0.2 ml (2mg) of Nalbuphine (undiluted) taken in 1ml tuberculin syringe 1mg/0.1ml] and group B received 2.8ml of 0.5%(H)Bupivacaine+0.2ml(60µg) of buprenorphine for spinal anaesthesia. The onset and duration of sensory and motor blockade, 2 segment regression, duration of postoperative analgesia, side-effects and haemodynamic parameters were compared between the groups.*
Results*: The mean time of onset of sensory and motor block, 2 segment regression and duration of motor block was comparable and statistically not significant between the two groups. The duration of postoperative analgesia was significantly prolonged with Buprenorphine compared to Nalbuphine with Bupivacaine ($p<0.05$).*
Conclusion: *Intrathecal Bupivacaine with Buprenorphine 60µg caused prolonged duration of postoperative analgesia when compared to intrathecal Bupivacaine with Nalbuphine 2mg.*
Key Word*: Intrathecal; Bupivacaine; Buprenorphine; Nalbuphine; Postoperative analgesia.*

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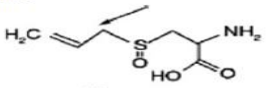
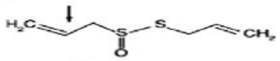
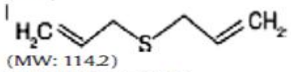
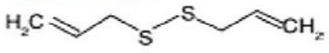
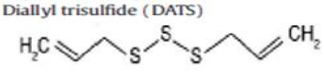
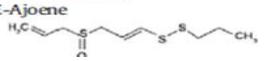
I. Introduction

The development of new antibiotics agents and plant based antimicrobial compounds are effective against the resistance pathogenic microorganism. The utilization of medicinal plant to treat diseases of various kind is part of tradition in the third world countries, for the preservation of health for the rural majority who constitute over 70% of the total population. Despite the advances in human medicine, infectious maladies caused by bacteria, fungi, parasites and viruses are becoming serious threat to public health in developing countries. Recently, due to the increased and indiscriminate use of antibiotics used in treatment of such diseases, multiple drug resistance (MDR) in human pathogenic microorganisms is developing a great in the third world countries (Arya *et al*., 2019). Consequently, there is a critical need to research new antimicrobial agent with promising natural activity to provide an alternative to overcome the mention problem (Baker *et al.,* 2009 and Falodun *et, al.*, 2008)

1.1 Compositional chemistry of garlic

Among the several functional compounds of garlic (Table 1), alliin is the most abundant organosulfur compound in whole garlic. It is a derivative of the amino acid cysteine.Kimbaris*et al.,* (2006) found that fresh garlic contains alliin (6–14 g/g). 25.65–30.03 mg/g alliin was detected in Korean garlic cloves and 6.7 mg/g in Korean garlic bulbs (Yoo *et al.,* 2010). 16.7–21.4 mg/g alliin was also detected in dried garlic cloves and 5.3–9.4 mg/g in fresh Germany garlic cloves (*Allium sativum*). As people become aware that garlic could be of significant medicinal benefit, interest into its compositional chemistry grew and the search for the active principals of garlic began (Yoo*et al.,* 2010).

Table 1: Chemical structure and quantity of volatile organosulfur compounds garlic.

Sulfur components	Contents (mg/g)	Origin of garlic	Isolation solution
Alliin  alliin (MW: 62.28)	25–30	Korean garlic cloves	Water
	6.7	Korean garlic bulbs	Water
	16.7–21.4	Germany dried garlic	Water
	5.3–9.4	Germany fresh garlic	Water
	22.1	Japanese garlic	Water
Allicin  allicin (MW: 162.27)	2.3–4.6	Korean garlic	Water
	7.7	Australian garlic	Water
	6.1–7.7	USA garlic	Water
	2.4–3.5	Switzerland garlic	Water
	5.1–6.6	Chinese garlic	Water
	5.0–5.3	Japanese garlic	Water
Diallyl sulfide (DAS)  (MW: 114.2)	0.01–0.02	Greek garlic	Diethyl ether
	0.00–0.02	Greek garlic	Ethyl acetate
	0.00–0.02	Greek garlic	Hexane
	0.02–0.23	Korean garlic	Dichloromethane
Diallyl disulfide (DADS)  Diallyl disulfide (DADS) (MW: 146.28)	0.08–0.28	Greek garlic	Diethyl ether
	0.06–0.231	Greek garlic	Ethyl acetate
	0.07–0.26	Greek garlic	Hexane
	0.57–0.89	Korean garlic	Dichloromethane
Diallyl trisulfide (DATS)  Diallyl trisulfide (DATS) (MW: 178.34)	0.00–0.20	Greek garlic	Diethyl ether
	0.01–0.22	Greek garlic	Ethyl acetate
	0.01–0.18	Greek garlic	Hexane
	0.11–0.39	Korean garlic	Dichloromethane
E-Ajoene 	0.17	Japanese garlic	Soybean oil
	0.17	Japanese garlic	Rice oil
	0.12	Japanese garlic	Soybean oil

Investigation into garlic chemistry are centred on its sulphur compounds (initially probably because of their odorous qualities). Garlic has an unusually high sulphur content accounting for approximately 0.3% of its fresh weight (Kimbaris*et al.*, 2006). Many of the sulphur compounds from garlic are reactive in nature, and discovery (along with structural elucidation) of the active principles of garlic occurred in reverse chronological order of the pathways in which they occur in garlic.

II. Material And Methods

2.2 Preparation of Inoculum

All required overnight cultures were pre-ordered and readily available at the General Sani Abatcha Specialist Hospital. In total 2 bacterial strains were used i.e. Staphylococcus strains and Pseudomonas aeruginosa. On use for some of the experiments, adding them to sterile single-strength TSB revived all the cultures however, for other protocols, cultures were used as given.

2.3 Agar Media Preparation

Tryptone Soy Agar (TSA) was obtained from the laboratory and made up following instruction of combining 3.7g of TSA into 100ml of distilled water and 7.4g for 200ml bottles. This was then autoclaved and stored in a 50oC incubator.

2.3.1 Preparation of Broth Media

Tryptone Soy Broth (TSB) was obtained from the Laboratory and up following instructions of combining 3g of TSB into 100ml of distilled water and 6g for 200ml bottles for single- strength TSB. In order to make double-strength TSB, 6g of the TSB powder was combined with 100ml of distilled water. All mixtures were autoclaved and then stored in laboratory cupboards (approximately. 28oC)

2.4 Radial Diffusion Assay Technique

Bacteria were cultured aseptically on Tryptone Soya Agar (TSA) for 24 hours. A flame sterilized platinum loop was used to pick a colony into 50ml sterile conical flask TSB which was subsequently allowed to grow overnight at 37oC in order to yield a turbid culture. Subsequently, on the TSA solidified plate, circular wells were cut in equal spaces using 7mm cork borer, sterilise in 70-90% ethanol for each well. The agar plugs were then removed aseptically using needles and decanted into the waste bins provided. All plates were appropriately labelled.

Then using a micropipette, each well was filled with 50ul of each extract. A new tip was used for each of the wells and all the plates were then put into the incubator for 24hours. The zone of inhibition (ZI) was measured from the inside of the well edge to the clear area beyond it (figure 1) Picture of zone of inhibition

2.5 **Determination of Minimum Inhibitory Concentration (MIC) And Minimum Bactericidal Concentration (MBC)**

A two-fold dilution was prepared (5ml into 5ml) using autoclaved double strength and single-strength TSB, broth culture and 10% (w/v) of the plant extract to be tested. Three racks of eight tubes (including control set) were labelled. Two replicates were made with one set as a negative control. Then aseptically, 5ml of double-strength TSB was deposited into the first tube only and 5ml of single-strength TSB was deposited into the remaining tubes using a 5-10ml pipette (for all three racks). 5ml of the 10% (w/v) extract was then put into the first test containing 5ml of the double-strength TSB and mixed using a whirly mixer. In order to perform a two-fold dilution, 5ml from test tube 1 was transferred into test tube 2 and again mixed. Then 5ml from test tube 2 was put into test tube 3. The process was repeated until test tube 8, were the 5ml of mixture drawn up from tube 8 was discarded inorder to maintain a uniform volume. A set of percentages was given being: 5%, 2.5%, 1.25%, 0.6%, 0.3%, 0.15%, 0.08 and 0% (Positive control). As tube 8 is kept as a positive control, no plant extract was added to it. Then using a sterile pasture pipette, 1 drop of test bacteria (for example S. aureus broth culture) was aseptically put into all tubes excluding the negative control tubes. All sets were incubated at 37oC for 24 hours and turbidity was recorded. The tube with no turbidity before or after the tube that does have turbidity was recorded as the MIC.

NOTE: Sometimes turbidity can form due to the interaction of plant extract with the proteins in the growth medium as it was observed in Cinnamon extract

The MBC test was performed following results from the MIC. This was done in order to confirm if the garlic extract is actually killing the microorganism or inhibiting them. TSA plates were prepared, and the plates were divided into 4 segments and labelled tube 1- 8. 10ul from each MIC tube was dropped on a spot on the corresponding segment then spread using a plastic sterilized loop ensuring that it was kept in the section and incubated for 24 hours at 37oC; this was repeated for all the replicates. The percentage section that showed no colony growth was indicated as the MBC. The MBC was defined as the lowest concentration at which no growth occurred on the plate.

Statistical Analysis

The experiment was set up in a completely randomized factorial design (bacterial strains × time duration) with three replicates. Data recorded during the course of this experiment were analyzed statistically by the analysis of variance using routines of the statistical software 'SPSS 20' and the differences between means were compared using the Least Significant Differences (LSD) at 5% level of probability ($P < 0.05$).

III. Result

3.1 Against pathogenic bacteria

The aim of this experiment was to evaluate the antimicrobial effect of storage temperature of fresh aqueous garlic extract against *S. aureus*, and *Ps. aeruginosa* bacteria using radial diffusion test. 10% (w/v) extract was prepared and stored at different temperature of 35^{o}C, 30^{o}C, and 37^{o}C for 0, 24, 48 and 72 hours respectively and then tested for antimicrobial activity. The effect of temperature on the antibacterial activity of garlic extracts against different bacteria (Staphylococcus aureus and Pseudomonas aeruginosa) was determined.

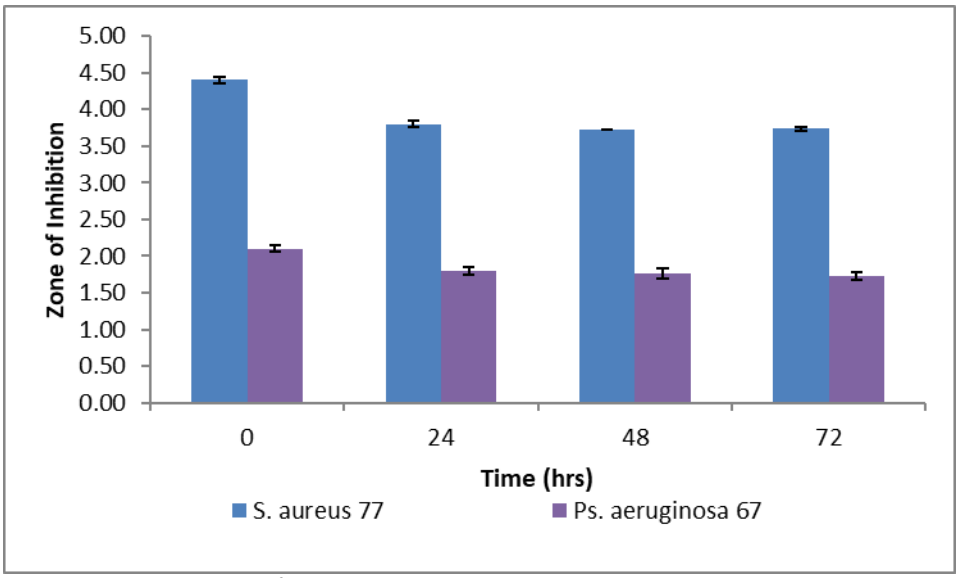


Fig. 3.1 Effect of storage at 25°C on efficacy of Cinnamon extract on antimicrobial activity measured at different time duration. The vertical error bars represent the standard error based on 3 replicate of the mean (n=3)

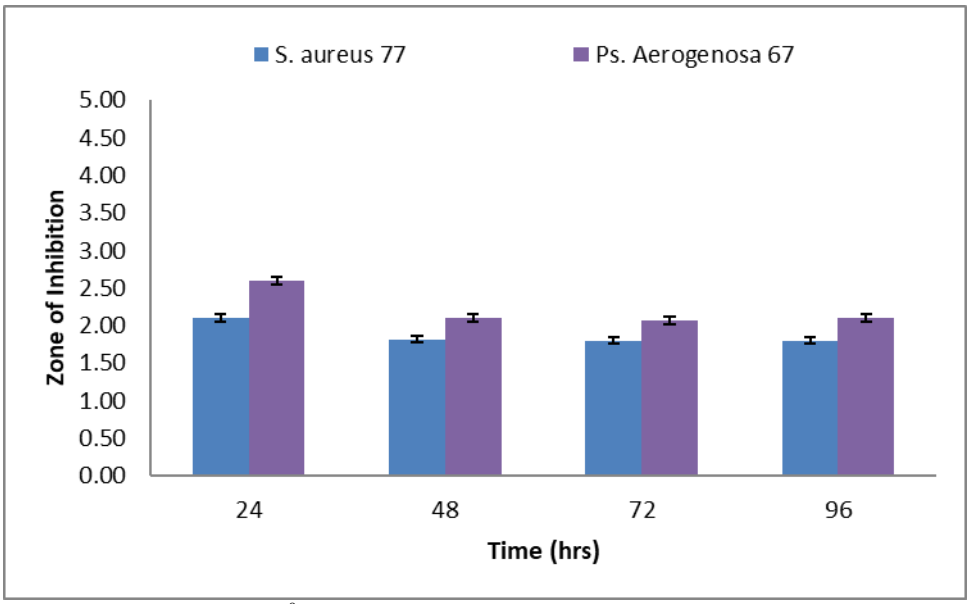


Fig. 3.2 Effect of storage at 30°C on efficacy of garlic extract on antimicrobial activity measured at different time duration. The vertical error bars represent the standard error based on 3 replicate of the mean (n=3)

The aqueous garlic had significance effect ($P < 0.001$) on the zone of inhibition of *S. aureus.* However *P. aeruginosa* is more sensitive to the fresh garlic extract than *S. aureus* at all the storage temperature of 25°C (Fig. 3.1), 30°C (Fig. 3.2) and 37°C (Fig. 3.3).

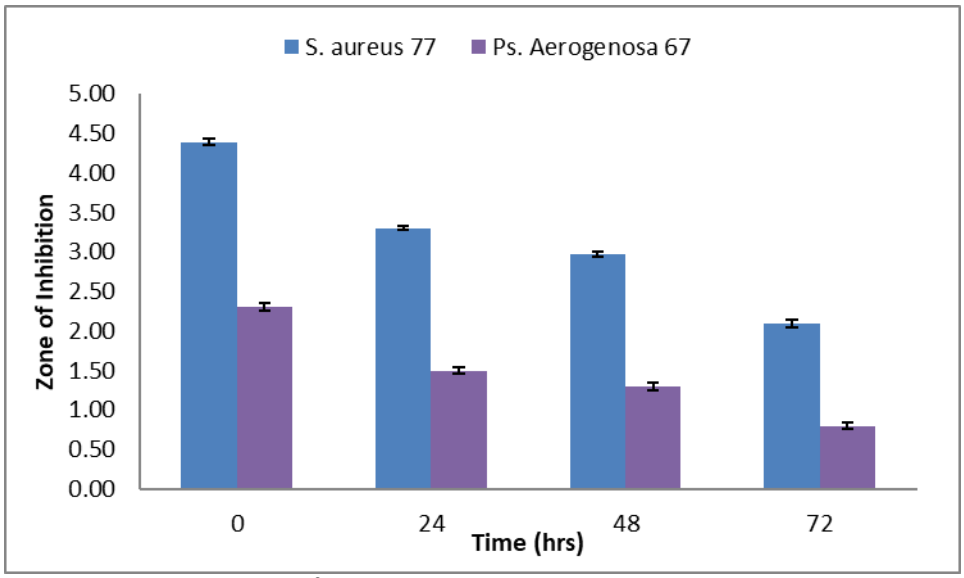


Fig. 3.3 Effect of storage at 37°C on efficacy of garlic extract on antimicrobial activity measured at different time duration. The vertical error bars represent the standard error based on 3 replicates of the mean (n=3)

The Antimicrobial properties of aqueous garlic extract at storage temperature of 25°C (Fig. 3.1) and 30°C (Fig. 3.2) showed that there was a slight loss of antimicrobial activity after 24 hours storage with an average zone of inhibition diameter ranging from 0.7 – 0.5cm. Further storage for 48 and 72 hours maintained antimicrobial properties. In contrast, the extract stored at 37°C (Fig. 3.3) of aqueous garlic extract loss antimicrobial properties with time.

Figure 3.1 and 3.4 indicate that *S. aureus* and *S. epidermidis* were significantly more sensitive to aqueous garlic extract than *Strep. pyogenes* and *P. aeruginosa* at both the storage temperature of 25°C and 37°C. There was least significant difference (LSD = 0.09) in the sensitivity of *Staphylococcus* strain to the 10% aqueous garlic extract between the *S. aureus* and *S. epidermidis* zone of inhibition diameter at -20°C.

Figure 3.4. Indicate that, there was slight loss of antimicrobial properties of dried garlic extract stored at 37°C. The result also showed that *S. aureus* were significantly more sensitive ($P < 0.05$) to fresh garlic extract than *Ps. aeruginosa* at 37°C storage temperature. Following the results in Figure 3.2 and 3.4 There is no significant different between *S. aureus* and *Ps. aeroginosa* in fresh garlic at 37°C. However, there was a mean significant difference (LSD = 0.15) between *S. aureus* and *Ps. aeroginosa* in the dried garlic extract stored at 37°C. This implies that garlic extract can retain its activity both at low temperature (-25°C) and higher temperature (37°C). However a wider range of temperature testing and a day to day variation would have performed in order to provide enough evidence to support the hypothesis.

3.2 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of garlic extract on the two different organisms

This experiment was done to determine the MIC and MBC for Fresh garlic and Dried garlic extracts against *S. aureus* and *Pseudomonas aeruginosa* in a liquid medium following the evaluation on the solid agar medium order to determine the lowest concentration to inhibit and prevent growth which can then be incorporated in gel. Table 3.1 indicate that, *P. aeruginosa* had the highest MIC value while *S. aureus* has the least MIC value. The MBC value for *P. aeruginosa* was 5% while *S. aureus* has the MBC value of 2.50%. The result in Table 3.1 implies that *S. aureus* is the most sensitive to fresh garlic extract while *Ps. aeruginosa* is the least sensitive because it has the highest MIC value. This implies that high quantity of dried garlic extract is needed prevent the growth of *P. aeruginosa.*

Table 3.1 Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of 10% aqueous garlic extract against different organism

	MIC and MBC Value for Garlic Extract			
Organisms	**MIC**	**MIC Range (%)**	**MBC**	**MBC Range (%)**
S. aureus	0.625%	(1.25 - 0.625%)	2.50%	(1.25- 2.5%)

P. aeruginosa	2.50%	(2.50 - 5.0%)	5%	(5.0 – 10%)

IV. Discussion

The study has confirmed the antimicrobial activity of garlic against gram positive (S. aureus) and gram negative (Ps. aeruginosa) bacteria. This finding correlates with the report of Sivam (2001), and Ross et al., (1993) that garlic has antimicrobial activity against a wide range of bacteria. However, Pseudomonas aeruginosa was less sensitive to the 10% aqueous garlic extract used, although only one-gram negative bacteria was tested but what was found in the experiment is confirmed by the report of Godden and Keynes, (2005). Another research done by Abubakar, (2009) showed that the MBC of garlic extract for Ps. aeruginosa was the highest (150mg/ml) compared to S. aureus (75mg/ml), S. pneumoniae (100mg/ml), E. coli (125mg/ml) implying that a higher concentration of garlic extract is needed to inhibit the Ps. aeruginosa. Poople et al., (1993) identified an efflux operon in Ps. aeruginosa which is apparently involved in pyoverdine secretion, and this operon encodes an outer membrane protein whose overproduction is responsible for the multi-drug resistance. Contrarily, studies of Iwalokun et al., (2004) reported that out of the 18 isolates of Ps. aeruginosa tested with aqueous garlic extract, only 9 of them were sensitive. This therefore gives more evidence for the resistance of some of Ps. aeruginosa and hence possible resistance of the isolate tested in this study. Similarly, the findings from figure 3.3 showed that P. aeruginosa was insensitive to fresh garlic extract when using WPD and this resistance is confirmed by the report of Godden and Keynes, (2005) reported that garlic has antimicrobial activity against all the bacteria tested. The finding in figure 3.1 and 3.2 and table 3.4 showed that, gram positive bacteria (*S. aureus*) were more sensitive to the 10% aqueous garlic extract used than the gram-negative bacteria (*P. aeruginosa*) which is in line with the observation of previous studies done by Kumar and Sharma, (2010). This finding disagrees with the studies of Bakri and Douglas, (2005) who showed that the MIC value of garlic extract was lower for gram negative bacteria (garlic MIC range 35.7 – 1.1 mg/ml) than for gram positive bacteria (garlic MIC range 142.7 – 35.7). Similar to the findings in these studies, Agaglu *et al*., (2005) reported that, the aqueous garlic extract displayed a variable degree of antimicrobial activity on different microorganism. *S. aureus* was more sensitive than the *S. epidermidis.* Eja *et al.,* (2007) revealed in their experiment that the gram-negative bacteria were more sensitive than the gram-positive bacteria isolates. Eja*et al.,*(2007) also explained that, the difference in their sensitivity to be due to the nature of the cell wall structure. The cell wall of gram-negative bacteria is made up of 15 – 20% polysaccharides and 10 – 20% lipid, while the gram-positive bacteria consist of 35-60% polysaccharide and only 0.2% lipid. The permeability of the allicin and other garlic constituents is affected by the polysaccharide and the lipid component of the cell wall and thus the aliicin is more permeable through the gram-negative cell wall than through gram positive cell wall (Eja*et al.,* 2007). Miron *et al*., (2000) also observed that alliicin can permeate freely and easily though phospholipids bilayers and interact with the SH group.

There is a high discrepancy in the sensitivity of gram positive and gram-negative bacteria to aqueous garlic extract as different researchers have different MIC and MBC of garlic against the same bacteria tested. This probably because of the difference in the type of bacteria strain or isolates used, as some may be more sensitive than others and not necessarily because they are gram positive or gram-negative bacteria as seen in the studies of (Iwalokun*et al*., 2004). Also, the method of testing the antimicrobial activity is often different as some researchers may choose to use Agar well diffusion (Iwalokun*et al.,* 2004), while others would use Disc diffusion method (Kumar and Sharma, 2010) which could also influence the efficacy of garlic extract. The source of garlic also contributes to the amount of alliicin. The findings from The radial diffusion test showed that the aqueous garlic extract (AGE) produced zone of inhibition diameter ranging from 4.40 – 3.57cm comparing the antimicrobial activity of 10% dried garlic extract (Fig. 3.2 and 3.3), the level of sensitivity is seen in the order: *S. aureus* > *Ps. aeruginosa*. However, this order of sensitivity has agreed with the MIC and MBC result in table 3.1. The order of sensitivity of the strain to 10% garlic extract is *S. aureus* > *Ps. aeuginosa*. Therefore, there is a difference in the sensitivity of *Staphylococcus* strain to garlic depending on the method used because the radial diffusion method. However, since only *S. aureus* had an MBC value of 1.25% (50mg/ml), it is most likely to be the most sensitive strain. It is not possible to conclude that *S. aureus* is the most likely to be most sensitive because, replicates of this experiment were performed on a single day.

V. Conclusion

The sensitivity of the tested bacteria to the 10% garlic Aqueous extract in this study is temperature dependent as decrease in temperature increases the sensitivity of the strain to the garlic extract (Fig. 3.1 and 3.3). With this finding, it's possible to infer that, garlic can work in both high and low temperatures. Hence it can work in tropical climates having an environmental temperature as high as 37^{o}C as well as in cold and temperate climate like having an environmental temperature of less than 20^{o}C. However, there is not much research that has not been done to assess the effect of incubation temperature on the sensitivity of bacteria to

garlic extract which showed that, increase in concentration of garlic extract had a positive effect on its potency against *S. aureus and Ps. aeroginosa* strain tested. This is in consistency with the analysis of Deresse, (2010) who stated that garlic extract having concentration between 15 – 16 mg/ml inhibited the growth of *S. aureus* isolated from different samples. While garlic extract having concentration of 0.75mg/ml did not show any inhibition of growth for all the *S.aureus* isolates. Al-Astal, (2003) explained that, allicinase need about six hours to attain the optimal time to act on alliin thereby producing the antibacterial material allicin. He went further to explain that the efficacy of the AGE is lost because allicin changes to entirely different compounds (mainly, diallyldisulfide and diallyltrisulfide). As observed in this study, the temperature of storage did not influence the efficacy of the garlic extract meaning that garlic can still retain its antimicrobial activity at higher temperatures of 37^{o}C and lower temperatures of 4^{o}C. However due to the wider range of storage temperature variation in this study, it's possible to infer that garlic can still retain its efficacy after storage at temperatures greater than 37^{o}C. Nonetheless, Al-astal, (2003) also showed that, the efficacy of garlic disappears at high tempearature (70 – 100^{o}C) and explained that, the main in-effective compound aliin or allinase enzyme will be destroyed at these temperature range.

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