**Temporal Extraction, Extracting Solvent Concentration and Extract Concentration Impact on Antibacterial Activity of Garlic**

**Abstract**

Pharmaceutical companies worldwide have celebrated success producing synthetic derivatives of therapeutic plant extracts. These extracts have remained a primary treatment option globally. In this study, the impact of temporal extraction, extracting solvent and extract concentration on antibacterial potency of garlic was evaluated. Garlic extract was prepared according to these parameters; extraction period (1-2;3 days), solvent concentration (50%, 80%, 100%), reconstituted extract concentration (50mg/ml, 100mg/ml) and antibacterial activity established using the well diffusion method. Ethanol is a good extracting solvent but this is concentration dependent. Garlic extract showed varying antibacterial potency based on extraction parameters against Staphylococcus aureus. Empirically, a linear relationship existed between solvent concentration, extraction period (1-2 days) and antibacterial activity of garlic for as concentration of extraction solvent increased (80%-100%), with extraction period (1-2 days) the antibacterial activity also increased. It was also observed that with equal mass of plant material for extraction and equal volume of extracting solvent, 24-hours extraction period empirically produced more bioactive compounds that caused inhibition than 48-hours extraction time. Also with equal mass of material for extraction and volume of extracting solvent over a period of extraction (1-3 days), 80% ethanol produced more bioactive compounds that cause inhibition. From the findings, it appeared that longer extraction time (for fixed quantity of material to be extracted and fixed volume of extracting solvent) allowed other factors to influence bioactive compounds that caused inhibition. In addition, for a fixed volume of extracting solvent, weight of plant material for extraction (eg 20g-30g) within solubility limits is proportional to the availability of bioactive compounds particularly for 1-day extraction. The work also found that there’s extracting solvent concentration dependent reconstituted extract concentration inhibition effect and that if effective solvent concentration is considered a constant, then reconstituted extract concentration would be directly proportional to inhibition zone. Further research on temporal extraction and solvent concentration effect backed by correlation studies and statistical analysis is necessary.

**Key words: Temporal Extraction, Solvent Concentration, Reconstituted Extract Concentration, Garlic, Antibacterial**

**1.0 Introduction**

Garlic is commonly referred to as the “king of herbs'' with reports of its use dating back to 5000 years [1,2]. It is found to have antihypertensive, antioxidant, antiplatelet, antitumor, lipid-lowering actions; it boost the immune system, lessens common cold, aids in human heavy metal detoxification, improves bone health, and is frequently used today for conditions related to the blood system, heart, and various cancers [3,1,4]. Garlic contains sulfur compounds such as allicin, ajoene, allyl methyl trisulfide, diallyl trisulfide and diallyl disulfide that exhibit a range of antibacterial activities including bactericidal, antibiofilm, antitoxin, and anti-quorum sensing activity against a wide range of bacteria, including multidrug resistant strains [5,6]. Fresh garlic contains allicin, which prevents lipid biosynthesis and has been proven to damage the cell walls of *Candida albicans* and inhibit bacterial RNA synthesis [7,8]. Garlic extracts tested for their antibacterial properties against a wide range of microorganisms have inhibited the growth of *Staphylococcus aureus*, *Corynebacterium diphtheriae*, *E. coli*, *B. subtilis*, *Mycobacterium phlei*, *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, *Photobacterium damselae* subsp. *piscicida*, *Streptococcus iniae*, *Staphylococcus saprophyticus* , *P. aeruginosa*, *Klebsiella spp*, *Proteus spp*, *S. marcescens*, *S. typhi*, *Bacillus cereus* and *Helicobacter pylori* [9,10,5,11,12,13].

The sulphur compounds found in garlic are phytochemicals. Phytochemicals are produced by plants as a defence mechanism against pathogens and are used by humans in numerous cultures around the world to treat various metabolic, immunological and neurological disorders as a part of traditional medicine [14]. The first step in getting phytochemicals from plants is extraction and extraction of phytochemicals is the process of separating active plant materials or secondary metabolites from an inactive material; the purpose of all extraction being to separate the soluble plant metabolites, leaving behind the insoluble residue [15,16,17,18,19,20,21]. Successful extraction begins with the careful selection and preparation of plant samples as well as a thorough review of appropriate literature to identify the protocols that are most suited for a given class of chemicals or plant species [22]. There are generally three types of extractions that are commonly used: liquid/solid, liquid/liquid, acid/base and solvent extraction is the most widely used method [23,15,16,17,18,19].. The commonly employed liquid-solid extraction methods are: maceration, digestion, infusion, lixiviation, decoction, tincture, percolation, steam and hydrodistillation, soxhlet extraction, serial exhaustive extraction and fermentation [24]. Extraction is based on the difference in solubility between the solute, other compounds in the matrix, and the stabilizing solvent used [25]. Typically, extraction methods are selected based on the characteristics of the targeted active phytochemicals, the water content of the plant material and the intended uses of the extracted material [26]. Three fundamental factors that are reported to affect the quality of extraction are the solvent used, the plant part used as starting material and the extraction procedure [27]. The choice of an appropriate extraction method depends on the nature of the plant material, solvent used, pH of the solvent, temperature, and solvent to sample ratio [20]. The extraction efficiency increases with the increase in extraction duration as long as the solute has not reached equilibrium both within and outside of the solid material; also, the extraction yield increases with the solvent to solid ratio but if the solvent to solid ratio is excessively high it can result in excessive extraction solvent that will take a long time to concentrate [28].

The phytochemical composition and biological activities of plant extracts is significantly influenced by the choice and concentration of solvents used during extraction. Several studies have demonstrate that varying solvent polarities and concentrations yield diverse extract compositions and potencies [29,30,31,32,33,34,35,36,37,38]. Solvent concentration particularly in aqueous mixtures affects the extraction efficiency of specific compounds and as such, optimizing solvent concentration is necessary for maximizing the yield of desired bioactive compounds and extract potency [34,38]. Polar solvents are effective at extracting polar compounds while non-polar solvents are effective at extracting non-polar compounds [29,31,33,35]. Since varying polarities allows for the extraction of a broader range of phytochemicals, the selection of a solvent should be based on the target compounds of interest [32,36,37]. The antioxidant activity of plant extract is related to the type of solvent used as different solvents extract compounds with varying antioxidant capacities; polar solvents often yield extracts with higher antioxidant activity due to the extraction of phenolic compounds [29,30,31,33,34,37,38,32,35]. Solvent choice also influences the antimicrobial potential of plant extracts; polar solvents can extract a wider range of bioactive compounds, including alkaloids and flavonoids, which possess antimicrobial properties [30,32,39].

Ethanol has been known as a good solvent for extraction and is safe for human consumption [40]. Ethanol garlic extract of three days had antibacterial activity against *Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis, Staphylococcus aureus, Klebsiella pneumoniae, Shigella sonnei, Staphylococcus epidermidis and Salmonella typhi* [41]. Wolde et. al., 2018 and Tijjani, et. al., 2017 had also shown that garlic extract using ethanol for three and four days also had antibacterial activity against S. *aureus* and E. *coli*. It is already an established knowledge that garlic has antibacterial activity against *Staphylococcus aureus* but the effect of temporal extraction, extracting solvent concentration and extract concentration on its antibacterial activity on S. *aureus* is what this study seeks to establish.

**2.0 METHOD**

**2.1 Sample Collection and Preparation**

Bulbs of garlic were bought from acknowledged dealers in Bori market. The purchased garlic samples were collected in newly bought black polythene bag and transported after purchase to the Microbiology Laboratory of Kenule Beeson Saro-Wiwa Polytechnic, Bori. At the laboratory, the samples of garlic were washed under running tap water, dehusked and washed again.

**2.2 Extract Preparation**

Garlic samples were ground using an electric blender into a paste. The extracts prepared were temporal and concentration dependent. The extracts were prepared thus:

1. Six portions of 30g were weighed out. Two of the six portions of 30g were soaked in two portions of 100% ethanol (200ml each), another two in two portions of 80% ethanol (200ml each) and the remaining two in two portions of 50% ethanol (200ml each). The soaking was done for 24 hours (100%; 80%; 50%) and 48 hours. After the soaking period in each case samples were individually filtered through Wathman No.1 filter paper and the filtrates were evaporated to dryness using a water bath. The residues were then reconstituted using Dimethylsulfoxide and for all six extract samples, 100mg/ml was prepared using the formula C1V1= C2V2.

2. Three portions of 20g were weighed out. Each portion was soaked in 200ml of 50%, 80% and 100% ethanol for three days. Each soaked sample was individually filtered through Whatman No.1 filter paper and the filtrates were evaporated to dryness using a water bath. The residues were then reconstituted using Dimethylsulfoxide. Concentration of 50mg/ml and 100mg/ml was prepared using the formula C1V1= C2V2 for each extract residue corresponding to the concentration of extracting solvent (50%, 80% & 100%).

**2.3 Test Organism**

*Staphylococcus aureus* was the test organism. Pure isolates of S. *aureus* were obtained from the stock culture at Microbiology Laboratory of Kenule Beeson Saro-Wiwa Polytechnic and were maintained on fresh nutrient agar slants (in a bijou bottle and kept in a refrigerator at 4°C). A suspension of S. *aureus* was prepared that matched the turbidity of 0.5 McFarland standard.

**2.4 Culture Medi**a

Nutrient Agar (NA) and Muller Hinton Agar (MHA) was used. All agar was prepared with appropriate calculations that matched manufacturer's instructions and sterilised by autoclaving at 120°C for 15 minutes at 15psi.

**2.4 Determination of Antibacterial Activity of Garlic**

Mueller Hinton's Agar that has been allowed to cool was dispensed into twelve sterilized petri dishes with the following notations; one petri dish for 100% 24 hours extraction time (ET), 1 for 100% 48 hours ET, 1 for 80% 24 hours ET, 1 for 80% 48 hours ET, 1 for 50% 24 hours ET, 1 for 50% 48 hours ET, 1 for 50mg/ml 100% ethanol extract (EE), 1 for 100mg/ml 100% EE, 1 for 50mg/ml 80% EE, 1 for 100mg/ml EE, 1 for 50mg/ml 50% EE and 1 for 100mg/ml 50% EE. The agar was then allowed to set. After the agar has been observed to set in the respective dishes, their surfaces were dried in a hot air oven for 5 minutes at 60°C and allowed to cool in preparation for seeding organisms to them.

The well diffusion method was used to test for antibacterial activity. *Staphylococcus aureus* from the prepared suspension were seeded onto the freshly dried agar in the twelve petri dishes and distributed evenly in each dish by means of sterile swab stick. With sterile cork borers, wells were made in the agar medium. The wells were filled with the respective plant extracts and allowed to stay for a while for extracts diffusion into the agar. Plates were thereafter incubated in an incubator for 24hrs.

**2.5 Measurement of Zone of Inhibition**

In each petri dish, clear zone of inhibition around the bored well that indicated the antibacterial activity of garlic extracts against *Staphylococcus aureus* was measured using a transparent

meter rule. The measurements were recorded in millimeters (mm).

All statistical analysis and charts or figures was done using Excel application.

**3.0 Results and Discussion**

The results of temporal extraction, solvent concentration and extract concentration are presented below in figures and tables with associated explanations and discussion. Figures 1 to 3 shows how solvent concentration and extraction period affects the antibacterial activity of garlic.

**Figure 1: Solvent Concentration, Extraction Time (24hrs) and Inhibition Zone**

Figure one has shown that for an extraction period of 24 hours using the same volume of extracting solvent and an equal weight of plant material to be extracted, as solvent (ethanol) concentration increases the zone of inhibition (antibacterial activity/potency of garlic on *S. aureus*) tends to increase as well.

**Figure 2: Solvent Concentration, Extraction Time (48hrs) and Inhibition Zone**

Figure two also shows that for an extraction period of 48 hours using the same volume of extracting solvent and an equal weight of plant material to be extracted, as solvent concentration increases the measured zone of inhibition also tends to increase as well.

**Figure 3: Solvent Concentration, Extraction Time and Inhibition Zone Comparison**

Figure 3 clearly shows the differences in the measured zone of inhibition in relation to solvent concentration and time of extraction.

In the two instances, there's correlation between the solvent concentration and the inhibition zone. Figure 4 and 5 shows this correlation in 24 hours and 48 hours of extraction while figure 6 shows a comparative correlation.

**Figure 4: Inhibition zone to solvent concentration correlation for 24 hours**

**Figure 5: Inhibition zone to solvent concentration correlation for 48 hours**

**Figure 6: Comparison of correlations**

As shown particularly in figure 6, there's an observed difference in the trend of the correlation. Table 1 below shows this difference in trend form the correlation coefficients:

**Table 1: Correlation Between Temporal Extraction and Antibacterial Activity of Garlic**

|  |  |  |
| --- | --- | --- |
| **Extraction Time** | **Correlation Coefficient** | **Interpretation** |
| **Twenty four Hours** | **0.993** | **Extremely strong positive linear relationship** |
| **Forty Eight Hours** | **0.990** | **Extremely strong positive linear relationship** |

The contents of table 1 shows clearly that both time periods show nearly perfect positive correlations (≈0.99), meaning higher ethanol concentration strongly predicts a larger zone of inhibition. The correlation, though with minimal difference, is slightly stronger at 24 hours than at 48 hours despite the fact that the measured zone of inhibition for 48 hours (0, 10, 21) is greater numerically than that for 24 hours (0, 8, 16). A possible explanation for this slight difference could be that over the longer period of extraction (48 hours), other factors might start to influence the growth inhibitors (bioactive compounds) to a greater extent, potentially affecting inhibition which has slightly caused deviation from a perfectly linear relationship with the initial ethanol concentration. The correlation has however suggested that ethanol concentration is a highly reliable predictor of antimicrobial effectiveness across both time frames.

From the foregoing with respect to measured outcomes, the concentration of the extracting solvent (at a specific volume) and the time it takes for extraction (of a specific weight) affects the antibacterial activity of garlic: the 24 hours extract comparatively producing greater inhibition and the 80% ethanol making more bioactive compounds available for inhibition.

Results for the effect of reconstituted extract differential concentration is presented in tables 2, 3 and 4 with table 5 having a comparison.

**Table 2: 50% Solvent Concentration, Extract Concentration and Inhibition zone**

|  |  |  |
| --- | --- | --- |
| **Test Organism** | **Reconstituted Extract Concentration (mg/ml)** | **Inhibition Zone; mm** |
| **S. aureus** | **50** | **0** |
| **S. aureus** | **100** | **0** |

**Table 3: 80% Solvent Concentration, Extract Concentration and Inhibition zone**

|  |  |  |
| --- | --- | --- |
| **Test Organism** | **Reconstituted Extract Concentration (mg/ml)** | **Inhibition Zone; mm** |
| **S. aureus** | **50** | **0** |
| **S. aureus** | **100** | **8** |

**Table 4: 100% Solvent Concentration, Extract Concentration and Inhibition zone**

|  |  |  |
| --- | --- | --- |
| **Test Organism** | **Reconstituted Extract Concentration (mg/ml)** | **Inhibition Zone; mm** |
| **S. aureus** | **50** | **8** |
| **S. aureus** | **100** | **14** |

**Table 5: Solvents Concentration, Extract Concentration and Inhibition zone Comparison**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Test Organism** | **Reconstituted Extract Concentration (mg/ml)** | **Inhibition Zone for 50%** | **Inhibition Zone for 80%** | **Inhibition Zone for 100%** |
| **S. aureus** | **50** | **0** | **0** | **8** |
| **S. aureus** | **100** | **0** | **8** | **14** |

From tables 2 to 5, the following can be deduced:

1. All extracts reconstituted from 50% ethanol as extracting solvent showed no inhibition.

2. Only the 100mg/ml of the 80% produced inhibition; 8mm.

3. All extracts reconstituted from 100% produced inhibition; 50mg/ml = 8mm and 100mg/ml = 14mm.

4. There’s extracting solvent concentration dependent reconstituted extract concentration effect.

5. If effective solvent concentration is considered a constant, then as reconstituted extract concentration increases, the inhibition zone also increases proportionally.

6. There’s ineffective solvent concentration for extraction of plant extract.

**Figure 7: Comparative correlation of Solvent Dependent Reconstituted Extract Concentration to Inhibition Zone**

For 50%, there’s no zone of Inhibition but for 80% and 100% there’s inhibition. The correlation for 80% and 100% individually is perfect since there are just two variables but when all three (50%, 80% and 100%) are compared as presented in figure 7, a remarkable interest already shown in table 5 is highlighted. For 100mg/ml for both 80% and 100%, the movement of inhibition zone for 80% is from 0 to 8 (8 points) while it is from 8 to 14 (6 points) for 100%. There’s a numerical difference of 2 which is empirically higher meaning there are more inhibitors in 100mg/ml of 80% than the 100mg/ml of the 100% but this cannot be shown statistically.

Inferences derived from observed findings are the following:

**1. Ethanol is a good extraction solvent: this is however concentration dependent for there's ineffective solvent concentration for extraction of plant extract.**

Ethanol has been known as a good solvent for extraction according to Yi-Hsu *et. al*., 2014 and the findings of this work has shown this.

**2. Garlic extract shows a range of antibacterial activity against *Staphylococcus aureus*.** According to Viswanathan *et al.,* 2014 and Bhatwalkar *et al*., 2021 garlic contains compounds that exhibit a range of antibacterial activities  against a wide range of bacteria while Guo et al., 2015, Cahayani et al., 2019, Viswanathan et al., 2014, Lionel et al., 2020, Chand, 2013, Elsom, 2000 have all found that garlic extracts tested for their antibacterial properties did shown inhibition against a wide range of microorganisms including *Staphylococcus aureus*. Gull et. al., 2012, Wolde et. al., 2018 and Tijjani, et. al., 2017 have also shown that ethanol garlic extract of three days and four days had antibacterial activity against  *Staphylococcus aureus*. The results of this research work is in line with the findings of the stated works.

**3. Empirically, there's linear relationship between solvent concentration, extraction time/period (1-2 days) and the antibacterial activity of garlic for equal weight of plant material to be extracted (eg. 30g) and equal volume of extracting solvent (eg. 200ml). As the concentration of extraction solvent increases (80%-100%), with extraction period (1-2 days) the antibacterial activity of garlic tends to increase.**

To Akullo et al., 2023, Borges et al., 2020, Dirar et al., 2019, El Mannoubi, 2023, Gonfa et al., 2020, Sultana et al., 2029, Thouri et al., 2017, Tourabi et al., 2023, Truong et al., 2019 and Chatepa et al., 2024 the phytochemical composition and biological activities of plant extracts is significantly influenced by the choice and concentration of solvents used during extraction with varying solvent polarities and concentrations yielding diverse extract compositions and potencies. According to Sultana et al., 2009 and Chatepa et al., 2024, solvent concentration affects the extraction efficiency of specific compounds and that optimizing solvent concentration is necessary for maximizing extract potency. In this work, varying solvent concentrations have produce various potencies of garlic antibacterial activity with higher concentration of solvent producing higher inhibition zone.

**4. With equal mass of plant material for extraction (eg. 30g) and equal volume of extracting solvent (eg. 200ml), the 24 hours extraction time empirically produces more bioactive compounds (primary plant materials and secondary metabolites) that cause inhibition than 48 hours extraction time.**

**5. With equal mass of plant material for extraction (eg. 30g) and equal volume of extracting solvent (eg. 200ml) over a period of extraction (1-3 days), 80% ethanol produces more bioactive compounds that cause inhibition.**

**6. It appears that longer extraction time (for fixed quantity of plant materials to be extracted and fixed volume of extracting solvent) allow other factors to influence to a greater extent the bioactive compounds that cause inhibition, potentially affecting inhibition.**

Extraction is based on the difference in solubility between the solute, other compounds in the matrix, and the stabilizing solvent used according to Omeroglu et al., 2019. To Li et al., 2008, Li et al., 2014, Yi et al., 2012, Zhou et al., 2012, Du et al., 2011 and Azwanida, 2015 extraction is the separation of active plant materials or secondary metabolites from an inactive materials while Abubakar and Haque, 2020 acknowledged that appropriate extraction amongst other things depends on solvent to sample ratio. Zhang et a., 2018 acknowledged that the extraction efficiency increases with the increase in extraction duration as long as the solute has not reached equilibrium both within and outside of the solid material. From Zhang et al., 2018 then duration is defined by the “time the solute reached equilibrium both within and without”  and not just a length of time. Extraction is based on the difference in solubility between the solute, other compounds in the matrix, and the stabilizing solvent used and solubility is affected by solid to sample ratio; the “compounds in the matrix” above may refer to “insoluble plant materials”. Perhaps a longer time duration for extraction may provide interaction between primarily plant material and secondary metabolites or increase the adsorption of primary plant materials and secondary metabolites to “insoluble plant materials” thus reducing inhibitors. In this work, there's empirical evidence that longer duration of extraction (1-2 days) produce greater inhibition zone but correlation studies didn't support this.

**7. For a fixed volume of extracting solvent (200mg/ml), the weight of plant material for extraction (eg 20g to 30g) within solvent volume complete solubility is proportional to the availability of bioactive compounds particularly for one day extraction.**

**8. There’s extracting solvent concentration dependent reconstituted extract concentration inhibition effect. All extracts reconstituted from 50% ethanol as extracting solvent showed no inhibition; for the 80%, only the 100mg/ml produced inhibition while all extracts reconstituted from 100% produced inhibition.**

**9. If effective solvent concentration is considered a constant, then as reconstituted extract  concentration increases, the inhibition zone also increases proportionally.**

**4.0 Conclusion**

Ethanol is a good extracting solvent particularly of garlic’s antibacterial compounds but its extraction potency is related to its concentration; for a fixed weight of plant material to be extracted and volume ethanol, there’s ineffective ethanol concentration for extraction. Garlic extract showed varying antibacterial potency based on extraction parameters: for effective solvent concentration, extracts obtained after 48 hours showed greater inhibition zones (80% = 10mm, 100% = 21mm) than that obtained after 24 hours (80% = 8mm, 100% = 16mm); for reconstituted extract, as effective solvent concentration dependent reconstituted extract concentration increases the inhibition zone also increased (80%: 50mg/ml = 0mm, 100mg/ml = 8mm; 100%: 50mg/ml = 8mm, 100mg/ml = 14mm).

Of interest, this work also inferred that with equal mass of plant material for extraction (eg. 30g) and equal volume of extracting solvent (eg. 200ml), the 24 hours extraction time empirically produces more bioactive compounds (primary plant materials and secondary metabolites) that cause inhibition than 48 hours extraction time. Also with equal mass of plant material for extraction (eg. 30g) and equal volume of extracting solvent (eg. 200ml) over a period of extraction (1-3 days), 80% ethanol produces more bioactive compounds that cause inhibition. It appeared from the findings that longer extraction time (for fixed quantity of plant materials to be extracted and fixed volume of extracting solvent) allow other factors to influence to a greater extent the bioactive compounds that cause inhibition, potentially affecting inhibition. In addition, for a fixed volume of extracting solvent (200mg/ml), the weight of plant material for extraction (eg 20g to 30g) within solvent volume complete solubility is proportional to the availability of bioactive compounds particularly for one day extraction. Importantly, the work also found that there’s extracting solvent concentration dependent reconstituted extract concentration inhibition effect.

A more elaborate research on temporal extraction that considers in addition to time solute equilibrium in solvent, solvent concentration effect back by correlation studies and statistical analysis. These will help both pharmaceutical companies and local consumers of direct plant based therapeutic materials.

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