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Synergistic Antibacterial Activity of *Allium sativum (Garlic)* Extracts and Skimmed Milk on Some Bacterial Isolates

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Abstract
As part of the on – going search for potent and resistant – free antimicrobial medicinal plants, the antibacterial and synergistic effects of *Allium sativum* and Skimmed milk on some bacterial isolates were investigated. The antibacterial activity of garlic/ milk extracts was assayed by the agar well diffusion method. The methanol garlic extracts inhibited the bacterial isolates while *E. coli* was resistant. The highest activities were on *K. pnuemoniae* (14mm) closely followed by S. *typhi* (13mm) and *S. aureus* (13mm) and then *S. pnuemoniae (*12mm). The synergistic effect of garlic and skimmed in combination gave a stronger activity on K. *pnuemoniae* being more susceptible with a zone of inhibition of 16mm and *E. coli* (13mm) which was resistant to individual garlic extract. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of garlic/milk combination on *E. coli* were 625µg/ml and 1250 µg/ml respectively. This indicated the potency of the combination. The findings of this study also confirm the use of garlic milk decoction in the treatment of ailments caused by these microorganisms. Therefore this synergistic effect can be used to design good therapeutic approach to combat with bacterial pathogens.
Keywords: Synergistic, Antibacterial activity, *Allium sativum,* Skimmed Milk, Bacterial Isolates.

INTRODUCTION

Garlic (*Allium sativum*) is one of the edible plants which have generated a lot of interest throughout human history as a medicinal universal remedy (Bongiorno *et al.*, 2008). Several studies have confirmed that garlic has antimicrobial properties (Reuter *et al.*, 1996; Martin, and Ernst, 2003; Eja *et al.*, 2007). Chemical analysis of garlic cloves has revealed an unusual concentration of ally sulfides (allicin) (Serge and David, 1999), as the component responsible for the remarkable antibacterial activity of garlic cloves (Serge and David, 1999). Garlic extracts have shown to exhibit invitro antibacterial activity against Gram positive and Gram negative bacteria including species of *Escherichia, Salmonella, Staphylococcus,, Streptococcus, Klebsiella, Proteus* and *Clostridium* (Serge and David, 1999). The application of blends was based on the concept of the effectiveness combination therapy (Synergism) in the treatment of certain bacterial infections involving drug resistant organisms (Duncan *et al.,* 1998). Garlic has been noted to act synergistically with antibiotic (Sivam *et al.*, 1997).

Skim milk powder (SMP) on the other hand, is a product resulting from partial removal of fat and water from pasteurized milk (Misel and FitGerald, 2003). The absence of saturated fat in milk can help prevent weight gain and promote heart health. Besides, the milks is low in cholesterol and thus helpful in maintaining the cholesterol levels in the body (Chi *et al.*, 2004).

In Ayurveda/herbal, a decoction of garlic boiled in milk is considered a wonderful drug for tuberculosis, pneumonia and asthma (Kumar *et al.,* 2010). Also crushed cloves of garlic may be infused in milk and taken for all types of disorders of the digestion. Milk being a coolant in nature, may reduce the hotness and pungency of garlic (Amagase *et al.*, 2001). It has also been reported that drinking garlic milk combination continuously for a week help reduce the low – density lipoprotein (LDL) cholesterol known as "bad" cholesterols thereby increasing high density lipoprotein (HDL) the good cholesterol in body. This in turn enhances circulatory health (Kumar *et al.,* 2010).

Garlic is known for causing halitosis as well as causing sweat to have a pungent "garlicky" smell, which is caused by ally methyl sulfide (AMS). AMS is a gas which is absorbed into the blood during the metabolism of garlic; from the blood it travels to the lungs (and from there to the mouth causing bad breath) and the skin where it is exuded through skin pores (Garlic Encyclopedia, 2010). Studies have shown that sipping milk at the same times as consuming garlic can significantly neutralize bad breath (Garlic Encyclopedia, 2010).

The question and whether a combination of milk and garlic decoction would produce synergistic, antagonistic or antidote effect especially in the therapy of bacterial infection is still yet to be tackled. This study was therefore aimed at investigating the potency of garlic extracts and its mixtures with skimmed milk against some bacterial isolates.

MATERIALS AND METHODS

Sample Collection and Processing

Three thousand five hundred grams (3500g) of fresh garlic bulbs were purchased in December 2013 from Rimi Market, Kano Nigeria. The bulbs were authenticated by a Botanist, in the Department of Plant Biology, Bayero University, Kano. The Garlic bulbs were air dried at ambient temperature (28±2°C) for 3weeks and then pulverized using mortar and pestle to fine particles. 2000g of garlic powder was weighed and stored in an air tight container and kept at ambient temperature until required.

Bacterial Isolates

Clinical isolates of *Escherichia coli, Salmonella typhi, Klebsiella Pneumoniae, Staphylococcus aureus and Streptococcus pneumoniae* were procured from microbiology department of Aminu Kano Teaching Hospital (AKTH) Kano, Nigeria. The isolates were confirmed using standard biochemical test (Cheesbrough, 2006). The isolates were maintained on freshly prepared nutrient agar slants and kept in a refrigerator at 4°C until required.

Preparation of Media

Nutrient agar and nutrient broth were prepared according to the manufacturers' instructions. The media were sterilized by autoclaving at 121°C for 15min.

Extraction of Plant Materials

Four hundred grams (400g) of garlic powder were separately extracted in 1000ml of solvent (petroleum ether, chloroform, ethylacetate and methanol) for 7 days (Bakht *et al.,* 2010). The garlic powder was placed in a 1.5 litre capacity bottles and the above solvents were added separately in a ratio 4:10. The bottles were agitated twice a day until 7 days. They were then filtered using muslin cloth and concentrated using a rotary evaporator at 65°C, 60°C, 64.7°C and 77°C (petroleum ether, chloroform, methanol and ethylacetate) respectively. The fractions obtained were stored at 4°C until further use (Bakht *et al.,* 2010).

Preparation of McFarland Standard

Barium sulphate standard suspension was used as the turbidity standard. 1% v/v solution of sulphuric acid was prepared by adding 1ml of concentrated sulphuric acid to 99ml of water. 1% w/v solution of barium chloride was also prepared by dissolving 0.5g of dehydrate barium chloride ($Bacl_2$-$2H_2O$) in 50ml of distilled water. 0.6ml of the barium chloride solution was added to 99.4ml of the sulphuric acid solution and then mixed well (Cheesbrough, 2006). The turbid solution that was formed was then transferred into a test tube as the standard for comparison (0.5 McFarland standard) that corresponds to approximately $1.0x10^{-5}$cfu/ml) (Chessbrough, 2006).

Inoculums Standardization

The previously prepared overnight nutrient broth culture of each bacterial isolate was used as innocular by diluting with sterile saline solution. The sterile normal saline was prepared by weighing 0.5g of NaCl and dissolved in 100ml of sterile distilled water. 0.1ml of each overnight broth culture of the test organisms were dispensed into separate test tubes containing sterile normal saline. The suspension was adjusted to match the 0.5McFarland standard which has a similar appearance of an overnight broth culture (El-Mahmood and Amey, 2001). This served as the standard innocular which was used for the antibacterial testing and for determination of MIC and MBC of the extracts.

Determination of Antibacterial Activity of Garlic Extracts

The antibacterial activity was conducted using the method described by Al-Mahmood (2009). The stock was prepared by dissolving 0.3g of each crude extracts of garlic in 3ml of DMSO (dimethylsulfoxide) in different vital bottles to make a stock solution of 100000µg/ml. from the stock, serial dilutions was made from 10^{-1} to 10^{-4}. The sterilized nutrient agar was poured into sterile petridishes and allowed to solidify. A sterile swab stick was dipped into the standardized innocular and spread on the solidified nutrient agar aseptically and labeled. The inoculated plates were allowed to stay for 30minutes to enable the organisms adhere properly to the surface of the agar. Six wells were bored aseptically using a sterile cork borer of 6mm diameter. The wells were then filled with 0.1ml of the serially diluted solution of ethylacetate, chloroform, methanol and petroleum garlic extracts (10.000µg/ml, 5000µg/ml, 2500µg/ml and 1250µg/ml respectively). A 0.1of 30µg/ml ciprofloxacin solution and 0.ml DMSO were used as positive and negative controls respectively. The plates were allowed to dry and subsequently incubated at 37°C for 24hrs. After which zones of inhibition were observed, measured and recorded in millimeter.

Determination of Synergistic Antibacterial Effects of Garlic Mixed with Skimmed Milk

Fifty grams (50gw/v) of powdered skimmed milks was dissolved in 100ml of DMSO to make 50% stock solution. From the stock, serial dilutions was made to obtain 5% and 10% solutions. The 5% and 10% solutions were further serially diluted with DMSO from 10^{-1} - 10^{-4}. Equal volumes of the extracts and milk were combined by measuring 1ml each from different dilutions into 16 test tubes (4 concentrations x 4 extracts). This was used for the synergistic test following the same procedure for the antibacterial activity.

Determination of Minimum Inhibitory Concentration (MIC) of Garlic Extracts

The Minimum Inhibitory Concentration (MIC) of the extracts was determined according to the methods described by Shahidi (2004) and Kabir *et al.,* (2005). Extracts were diluted to concentrations ranging from 312. 50µg/ml to 5000µg/ml (for garlic and a mixture of garlic with milk). To each dilution of garlic and a mixture of garlic and milk in a nutrient broth tubes were seeded with a loopful of standard bacterial inoculums. Negative controls tubes with no bacterial inoculation, were simultaneously maintained. Tubes were incubated aerobically at 37^{o}C for 24hrs. The lowest concentration of extract that produced no visible bacterial growth (turbidity) was recorded as MIC.

Determination of Minimum Bactericidal Concentration (MBC) of Garlic Extracts

The minimum bactericidal concentration of garlic extracts were determined from the broth dilution test resulting from the minimum inhibitory concentration tubes by inoculating the content of each test tube with no visible turbidity on a nutrient agar plates. The plates were then incubated at 37^{0}C for 24hours. The lowest concentration of the extract that showed no growth was noted and recorded as the minimum bactericidal concentration (Aliyu *et al.,* 2015).

RESULTS

The physical characteristics of garlic extracts are presented in Table 1. The result revealed variations in final weight, colour and texture. The highest yield of 11.0g was recovered from methanol while the least yield recovery was from chloroform (1.0g).

The antibacterial activities of extracts of *A. Sativum* against bacterial isolates are presented in Table 2. Methanol extracts at 1000µg/ml produced zones ranging from 12mm – 14mm against the bacterial isolates apart from *E. coli* which was resistant. This indicates an appreciable antibacterial activity. Also chloroform extracts at 1000µg/ml had a minimal effect (8mm – 10mm) against all the bacterial isolates.

Tables 3 and 4 shows the antibacterial activities of garlic extracts mixed with 5% and 10% skimmed milk respectively. Methanol and chloroform extracts combined with 5% skimmed milk produced synergy especially against *E. coli* (7 – 13mm). While there was antagonist effect in milk combination with petroleum and ethylacetate extracts (Table 3).

However, the ethyl acetate and petroleum ether extracts with 10% skimmed milk (Table 4) showed an additive effect on *K. pneumoniae* (12mm – 16mm).

The minimum inhibitory concentration (MIC) of garlic extracts and in combination with skimmed milk (Table 5) ranged from 312.5 - 5000µg/ml. The combination of extracts with milk had antagonistic effect on *S. typhi* and *K. pneumoniae* at the above concentrations. This indicates that the two organisms were not inhibited and therefore turbidity was observed. *E. coli* was inhibited at 625µg/ml indicating a synergy.

The minimum bactericidal concentration (MBC) of extracts and combination with skimmed milk (Table 6) were observed at the range of 1250µg/ml - 2500µg/ml. *E. coli* was inhibited at these concentrations indicating no growth on nutrient agar plate.

Table 1: Physical Characteristics of Garlic Extracts

Solvent	Initial eight(g)	Final weight(g)	Colour	Texture	Odour
Petroleum	400	2.0	Light yellow	Oily	Pungent
Chloroform	400	1.0	Yellow	Sticky	Pungent
Ethyl acetate	400	1.5	Light yellow	Sticky	Pungent
Methanol	400	11.0	Brown	gummy	Pungent

Table 2: **Antibacterial Activities of Garlic Extracts against bacterial isolates.**
Diameter of zone of inhibition (mm)/ Extract Concentration (µg/ml)

Isolates	ET				CH				MET				PET			
	1250	2500	5000	10000	1250	2500	5000	10000	1250	2500	5000	10000	1250	2500	5000	10000
S. pneumoniae	00	00	00	00	07	08	09	10	00	00	09	12	00	00	00	11
S. aureus	00	00	09	11	00	00	00	10	00	08	12	13	00	00	00	09
E. coli	00	00	00	00	00	00	00	08	00	00	00	00	00	00	00	00
S. typhi	07	08	09	11	00	00	00	08	09	10	11	13	00	00	00	13
K. pneumoniae	00	00	00	09	00	00	08	09	08	10	12	14	00	00	00	00

Key: ET = ethylacetate, CH = chloroform, MET = methanol, PET = petroleum ether

Table 3. Antibacterial Activities of Garlic Extracts mixed with 5% Skimmed milk against bacterial isolates.
Diameter of zone of inhibition (mm)/Extract / milk concentration (µg/ml)

Isolates	ET				CH				MET				PET			
	1250	2500	5000	10000	1250	2500	5000	10000	1250	2500	5000	10000	1250	2500	5000	10000
S. pneumoniae	00	00	00	00	00	00	00	10	00	00	00	00	00	00	00	07
S. aureus	00	00	07	08	00	00	00	00	00	00	00	00	00	00	00	00
E. coli	07	08	09	11	08	09	11	13	00	07	08	10	00	00	07	08
S. typhi	00	00	00	00	00	00	00	00	00	00	00	07	00	00	00	00
K. pneumoniae	00	00	00	00	00	00	00	12	00	00	08	10	00	00	07	08

Key: ET = ethylacetate, CH = chloroform, MET = methanol, PET = petroleum ether

Table 4. Antibacterial Activities of Garlic Extracts mixed with 10% Skimmed milk against bacterial isolates.
Diameter of zone of inhibition (mm)/Extract / milk concentration (µg/ml)

Isolates	ET				CH				MET				PET			
	1250	2500	5000	10000	1250	2500	5000	10000	1250	2500	5000	10000	1250	2500	5000	10000
S. pneumoniae	00	00	00	09	00	00	00	08	00	00	00	00	00	00	00	09
S. aureus	00	00	00	07	00	00	10	12	00	00	00	00	00	00	00	08
E. coli	07	08	09	12	09	10	11	13	00	08	09	11	00	00	09	10
S. typhi	00	00	07	08	00	00	00	00	00	00	00	07	00	00	00	10
K. pneumoniae	00	00	08	16	00	00	00	00	00	00	00	07	00	00	00	12

Key: ET = ethylacetate, CH = chloroform, MET = methanol, PET = petroleum ether,

Table 5: Minimum inhibitory Concentrations of crude garlic, garlic/5% skimmed milk and Garlic/10% skimmed milk

Isolates/Garlic Concentration	MIC / Different Solvents			
	ET	CH	MET	PET
Crude Garlic				
S.pneumoniae	0	625	5000	0
S. aureus	5000	0	2500	0
E. coli	0	0	0	0
S. typhi	312.5	0	312.5	0
K.pneumoniae	0	5000	312.5	0
Garlic/5% skimmed milk				
S.pneumoniae	0	0	0	0
S.aureus	2500	0	0	0
E. coli	625	625	2500	5000
S. typhi	0	0	5000	0
K.pneumoniae	0	5000	2500	2500
Garlic/10% skimmed milk				
S.pneumoniae	5000	5000	0	5000
S.aureus	5000	2500	0	5000
E. coli	625	625	1250	2500
S. typhi	2500	0	5000	5000
K.pneumoniae	2500	0	5000	5000

Key: ET = ethylacetate, CH = chloroform, MET = methanol, PET = petroleum ether, MIC = Minimum inhibitory Concentrations

Table 6: Minimum Bactericidal Concentrations of crude garlic, garlic/ 5% skimmed milk and Garlic /10% skimmed milk

Isolates/Garlic Concentration	MIC / Different Solvents			
	ET	CH	MET	PET
Crude Garlic				
S.pneumoniae	0	1250	5000	0
S. aureus	5000	0	2500	0
E. coli	0	0	0	0
S. typhi	1250	0	1250	0
K.pneumoniae	0	5000	2500	0
	Garlic/5% skimmed milk			
S.pneumoniae	0	0	0	0
S.aureus	2500	0	0	0
E. coli	1250	1250	1250	2500
S. typhi	0	0	5000	0
K.pneumoniae	0	5000	2500	2500
	Garlic/10% skimmed milk			
S.pneumoniae	5000	5000	0	5000
S.aureus	5000	2500	0	5000
E. coli	1250	1250	1250	2500
S. typhi	2500	0	5000	5000
K.pneumoniae	2500	0	5000	5000

Key: ET-ethylacetate, CH – chloroform, MET – methanol, PET – petroleum ether, MBC - Minimum Bactericidal Concentrations

DISCUSSION

Results obtained in this study have revealed that garlic extract mixed with skimmed milk have a minimal antibacterial activity (mean zone of inhibition 7 – 16mm) against the test organism. It has been reported that garlic extract has antimicrobial potency (Cai *et al.,* 2007; Eja *et al.,* 2007).

The antimicrobial potency of garlic has been attributed to its phytochemical component, allicin whose removal completely renders garlic ineffective against microorganisms (Hughes and Lawson, 1999; Cai *et al.,* 2007). Plants antimicrobial have been found to be synergistic enhancers in that though they may not have any antimicrobial alone, but when they are taken concurrently with standard drugs they enhance the effect of that drug (Rakholiya and Chanda, 2012). It has been known that one of the effective approaches to overcome bacterial resistance is restoration of antibiotic activity through the synergistic action of antibacterial materials from natural and synthersized agents (Betoni *et al.,* 2006).

However, not much research has been carried out on the antimicrobial properties of garlic/milk combination. The methanolic extracts of garlic exhibited the highest antibacterial activity against the test organisms. However, *E. coli was* not susceptible to the methanol extract. This could be due to presence of thick murine layer in their cell wall which prevents the entry of inhibitors (Martins, 1995). Similar to our results, Saravanan *et al., (*2010) and DeBoer *et al*., (2005) reported that methanol extracts of garlic posses significant antibacterial activity against bacterial pathogens.

Chloroform extract of garlic was found to be effective against all the bacterial isolates. This may be related to the fact that the solubility of phytochemicals in different solvents decides which will be most effective (Rawat, 2015).

In the combination of extracts of garlic and skimmed milk it is apparent that there is greater antimicrobial efficacy when used synergistically against *E. coli* which was resistant to individual garlic extract. On the other hand, there was increased effectively against *K. pnuemoniae*. This might be due to resultant effect of the active agents in garlic and milk. This also agrees with the work of Rawat (2015) who reported that combination of extracts showed synergistic effect with antibiotic in exhibiting antimicrobial potential against bacterial pathogens.

The result of the MIC revealed that the combination of garlic skimmed milk were able to inhibit the growth of *E. coli*. While the MBC result showed that the combination was able to kill *E. coli* at a low concentration. These concentrations are important because it is usually used to evaluate the efficacy of agents such as antiseptics, disinfectants and chemotherapeutic (Aliyu *et al.,* 2015.

Conclusion and Recommendations

The study's emphasis was on a possible combination of garlic and milk which showed enhanced antimicrobial effect on *E. coli* and an improved bactericidal activity against *K. pnuemoniae* which was concluded as synergism. Therefore, the combination was found to be effective when compared to individual antibacterial activity of garlic. This can act as promising strategy to combat the increasing spread of drug resistance. With these, there is need for toxicity studies of garlic/milk combination to determine the safety indices of the mixtures. Also clinical trials should be carried out to explore the potentials of these combinations in the treatment of diseases caused by susceptible pathogens.

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