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IN VITRO ANTIBACTERIAL ACTIVITY OF ALLIUM SATIVUM (GARLIC) EXTRACT AGAINST SALMONELLA TYPHIMURIUM

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ABSTRACT

The inappropriate and irrational use of antibiotics in poultry industry has led to the emergence of resistant microbial populations such as *Salmonella typhimurium*, which is responsible for 26% of cases of enterocolitis in humans. In the fight against this bacterium, *Allium sativum* (garlic) would be an effective alternative. This is because several studies claim the effectiveness of garlic in the prevention and treatment of bacterial diseases. To determine the *in vitro* antibacterial activity of hexane extract of garlic on the growth of *Salmonella typhimurium*, different concentrations of 50, 100, 200, 400 and 800 mg/ml of extract were used. The antibacterial activity was evaluated using agar well diffusion test method. Macro-titer tubes were used to determine the minimum inhibitory concentration (MIC). The minimum bactericidal concentration (MBC) was obtained by streak plate method. Ciprofloxacin was used as a positive control and distilled water as a negative control. The concentrations of 200, 400 and 800 mg/ml showed inhibition zones with diameters ranging from 14 to 24 mm; 29 mm for ciprofloxacin and 0 mm for distilled water. The MIC were between 31,25 to 62,5 mg/ml while MBC was 250 mg/ml. The present work concludes that, to fight *Salmonella typhimurium*, hexane extract of garlic can be used as a potential alternative to antibiotics.

KEYWORDS: Garlic, Medicinal plant extract, Disc diffusion technique, Macro dilution technique, *Salmonella typhimurium* strains, Minimum inhibitory and minimum bactericidal concentration.

INTRODUCTION

The discovery of antibiotics in the last century has reduced mortality and morbidity caused by infectious diseases. However, their widespread and often inappropriate and irrational use has led to the emergence of resistant microbial populations (Ilić *et al*., 2012). The risk of finding itself in a real therapeutic deadlock in the medium term preventing the treatment of some microbial infections is therefore major, unless ambitious and coordinated control actions are put in place (European Commission, 2011). This concern has led international organizations to adopt concerted plans to optimize antibiotic use and promote research for alternative solutions (WHO, FAO and OIE, 2010; WHO, 2015). This recommendation has led to an increased interest in herbal medicine, given that more than 80% of drug substances are either directly derived from natural products or developed from natural compounds (Maridass and John de Britto, 2008).

It has been reported that, the use of phytobiotics such as *Allium sativum* (garlic) extract could be a major asset in the fight against microbial infections (Vidanarachchi *et al*., 2005). In recent years, garlic was found effective as an antibacterial (Belguith *et al*., 2010; Salem *et al*., 2017), antiviral (Mehrbod *et al*., 2009), antifungal, antimicrobial and antioxidant (Javed *et al*., 2011), insecticidal, antiprotozoal and antitumor (Bolton *et al*., 1982; Khorshed *et al*., 2016). Garlic also boosts the immune system (Sheoran *et al*., 2017), improves the body weight gain (Brzoska *et al*., 2015), and augment the meat quality parameters (Rehman and Munir, 2015). Garlic reduces the levels of cholesterol and triglycerides in the serum of broilers, thus helps in improving their lipid profile (Ratika *et al*., 2018). This broad spectrum of activity is attributed to the over 100 phytotherapeutic sulfur compounds present in varying concentrations in garlic. These include allicin and thiosulfinates, which are formed by crushing-induced metabolic action of the enzyme allicinase (a cysteinesulfoxidelyase) on the odorless amino acid allicin (Lawson *et al*., 1991). Other non-sulphur constituents like proteins, saponins and phenolic compounds may also contribute to its anti-microbial activity (Corzo-Martinez *et al*., 2007). Research has demonstrated the in-vitro activity of different garlic extract preparations against bacteria such as salmonella, unlike the hexane extract of garlic which is under-investigated. Salmonella causes diseases called salmonellosis, which are zoonotic. It is a big socioeconomic threat worldwide that causes mortality

and morbidity in both humans and animals (Samad, 2011). *Salmonella enterica* is responsible for foodborne pathogens over the world, with more than a 1 million cases per year in United State (Scallan *et al*., 2011). *Salmonella enterica* serotype Typhimurium is responsible for 26% of enterocolitis cases in humans. This disease has public health significance, as well as being associated with food poisoning in humans (Zhang *et al*., 2019).

During an epidemic, not all individuals exposed to the same infectious agent develop the disease with the same severity. This would depend, not only on the genetic polymorphism of each individual, but also on the environment and the quality of the food consumed. This is because all nutrients (carbohydrates, fats, amino acids, vitamins and minerals) are able to modulate gene expression differently (Walker and Blackburn, 2004). The location of these genes in chickens that have resisted *Salmonella typhimurium* infection, under the effect of garlic, can be used to improve poultry resistance. Indeed, the identification of birds with superior phenotype has been a major step forward in improving breeding. Many traits such as genetic disorders, body weight, meat quality, and disease resistance are under the control of several genetic loci, which contributes to the variation of the trait (Wakchaure *et al*., 2015).

The loci associated with the phenotypic variation of a trait is called quantitative trait loci (QTL). The genetic markers of these QTLs linked to the trait gene can be used to select animals for selective breeding programmes (Williams, 2005; Moniruzzaman *et al*., 2014). Marker-assisted selection could therefore be used to detect disease resistance genes and consequently be a promising approach to improve the efficiency, health and welfare of farm animals for healthy consumption (Wakchaure *et al*., 2015).

The aim of this *in vitro* study was to evaluate the antibacterial activity of hexane extract from garlic against *Salmonella typhimurium*, determined the minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC). This work will enable the establishment of the concentration of hexane extract of garlic at which, the effects are more beneficial for *in vivo* use in QTL analysis of *Salmonella typhimurium* resistance genes in chickens fed garlic. This work will enable the establishment of the concentration of hexane extract of garlic with more beneficial effects, for *in vivo* use in QTL analysis of *Salmonella typhimurium* resistance genes in chickens.

MATERIALS AND METHODS

Bacteria strain

A standard strain of *Salmonella typhimurium* ATCC 14028 was generously received from the Bacteriology Laboratory of National Institute of Hygiene of Togo.

Preparation of *Allium sativum* hexane extract

The extraction method described by Handa *et al*. (2008) was adopted with a few modifications.

Fresh pinkish garlic (*Allium sativum*), imported from northern Nigeria, was bought at the Grand Market in Lomé-Togo.

The garlic bulbs were washed with clean water and 70° ethanol to remove impurities. Two hundred grams (200 g) of garlic was crushed in a mortar and macerated in 1 L of methanol for 48 hours. The macerate garlic was filtered with Whatman paper No 3. The filtrate was evaporated under vacuum using a rotary evaporator at 40°C. The lipid compounds were removed using hexane. The non-fat fraction was then evaporated under the vacuum using a rotary evaporator at 40°C.

Bacterial culture

Antibacterial activity was determined by agar disc diffusion method (Heatley, 1944). Hundred (100) milliliters of Mueller Hinton (MH) agar was autoclaved and cooled in a water bath. About 25 ml of MH agar was aseptically dispensed to each Petri dish, sized 60 x 15 mm and allowed to solidify. Bacterial suspensions were prepared at 0.5 McFarland (equivalent to the concentration of 1-2 $x10^8$ CFU/ml) with the latex equivalence turbidity standards (Fisher Scientific, Waltham, Massachusetts, USA) in 2 ml of sterile 0.85% NaCl solution. Approximately 1 ml of bacterial inoculum was uniformly spread over the plates using a sterile cotton swabs. Seven pores of 6 mm in diameter were prepared. Known concentrations of extracts (50, 100, 200, 400 and 800 mg/ml) were placed respectively inside each corresponding well. Distilled water was used as a neutral control and commercial ciprofloxacin (5µg/µl) as the standard antibiotic. The plates were incubated at 37°C for 24 hours. At the end of the incubation, inhibition zones formed around the discs were measured with Himedia zone scale.

Determination of minimum inhibitory concentrations (MICs)

The macro dilution method was used to determine the MICs of plant extract using empty tubes without anticoagulants (Iwalokun *et al*., 2004).

As shown in Table 1, the 10 tubes used were distributed as follows: 8 labelled test tubes (T_1, T_2, T_3, T_4, T_5, T_6, T_7 and T_8) and the other 2 tubes (T_9 and T_{10}) as negative and positive controls respectively. Using a pastry micropipette, 2 ml of the broth was added to all tubes (except T_1 which contained pure plant extract), starting with (T_9) negative control so as to avoid contamination. Subsequently, 2 ml of the extract contained in tube 1 (6 ml) was collected for the other 7 test tubes (T_2, T_3, T_4, T_5, T_6, T_7 and T_8) using bifold dilution method (Quinn *et al*., 2004). The 2 ml of the eighth tube remaining was discarded. The liquid MH culture medium (0.2 ml) was introduced into each tube except the T_9 negative control.

Table 1: Dilution protocol.

Tube N°	1	2	3	4	5	6	7	8	9 (Positive control)	10 (Growth Control)
Stock solution of garlic (1000 mg/ml)	6 ml of pure garlic extract	2 ml	2 ml	2 ml	2 ml	2 ml	2 ml	2 ml	-	-
Bacterial suspension	0	2 ml	2 ml	2 ml	2 ml	2 ml	2 ml	2 ml	2 ml	2 ml
Mueller Hinton medium	0	0.2 ml	0.2 ml	0.2 ml	0.2 ml	0.2 ml	0.2 ml	0.2 ml	0.2 ml	-

The optical density (OD) of both the experimental and control tubes were determined by spectrophotometer at 620 nm. The control and experimental tubes were then incubated at 37°C for 24 hours. At the end of the incubation period, the OD of each tube was determined as before. The difference between the final and initial readings was calculated and interpreted as the growth of bacteria whereas the comparison of the final readings with the control reading depicted the inhibitory effect of garlic. Various concentrations of extract (7.8 - 1000 mg/mL) were used. The minimum inhibitory concentration was defined as the lowest concentration able to inhibit any visible bacterial growth (Andrews, 2001).
The tubes were examined for visible growth and was recorded growth as (+) and no growth as (-).

Determination of Minimum Bactericidal Concentration (MBC)
To determine minimum bactericidal concentration (MBC), all the tubes which showed no visible signs of growth / turbidity (MIC and higher dilutions) or loopfuls were inoculated onto sterile Mueller Hinton agar plates by streak plate method. The plates were then incubated overnight at 37°C. The least concentration that did not show any growth of *Salmonella typhimurium* was considered as the MBC value.

Statistical analysis: The experiments were replicated three times (n = 3). Data obtained were analyzed by one way analysis of variance and significant means were separated using Duncan new multiple range test (SPSS 20.0 version). Differences were considered significant at $P \leq 0.05$.

RESULTS AND DISCUSSION

Anti-bacterial activity of hexane extract of garlic
Inhibitory activity of hexane extract of garlic more obviously decreased the growth of *Salmonella typhimurium* that was subjected to different concentrations of these extracts. It was noted that as the concentration of garlic extract increased, the effect was more significant. In fact, the sizes of inhibition zones were proportional to the increase of the concentration of garlic (Figure 1 and Table 2). These results are similar to those of Hannan *et al*. (2012) who showed that the more concentrated the garlic extract was, the larger the inhibition diameter of *S. typhimurium*. Azu and Onye-agha (2007) also reported that the efficacy of most plant extracts is concentration dependent, the susceptibility of the test organisms increased with increasing extract concentration.

The results in Table 2 and Figure 2 which showed the cumulative percentage of *S. typhimurium* which was 0, 42, 52, 76 and 100% at concentrations of 100, 200, 400, 800 and 1000 mg/ml respectively. This is believed to be due to a high concentration of allicin (antibacterial component) content in garlic (Jabar and Al-Mossawi, 2007). Previous studies have revealed major chemical constituents, which are likely to be responsible for the antimicrobial properties of plants. *A. sativum* has been reported to contain enzymes (alliinase, myrosinase, peroxidase and a volatile oil of about 0.1-0.4%), sulphur compounds (allicin, diallyldisulphide, diallytrisulphide, ajoene) and other related compounds such as allylcystanesulfoxide, mythulallylthio-sulfinate (Borlinghaus *et al.*, 2014).

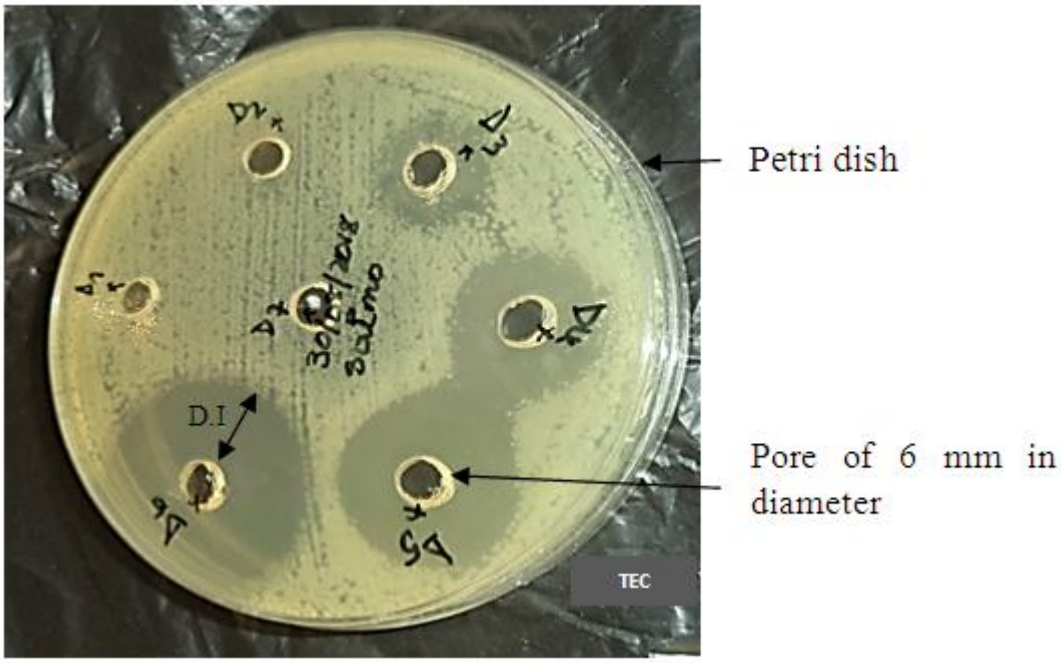

Figure 1: Antibiogram with the hexane extract of garlic on the growth of *S. typhimurium*.

The different concentrations of extract used are respectively: D1=50 mg/ml, D2=100 mg/ml, D3=200 mg/ml, D4=400 mg/ml and D5=800 mg/ml; D6=Ciprofloxacin, D7= Distilled water.
D.I= diameter of inhibition.

Table 2: Anti-bacterial activity of hexane extract of garlic in agar well diffusion assay (Zone of inhibition).

Hexane concentrations (mg/ml)	**800**	**400**	**200**	**100**	**50**	**ciprofloxacin**	**Water**
Diameters of inhibition (mm)	24±0.58[b]	20±1.15[c]	14±0.58[d]	00±00	00±00	29±0.58[a]	00±00

Data shown are Mean±S.E; each experiment consisted of three replicates.
[abcd] *Means followed by the same letter are not significantly different using Duncan new multiple range test.*

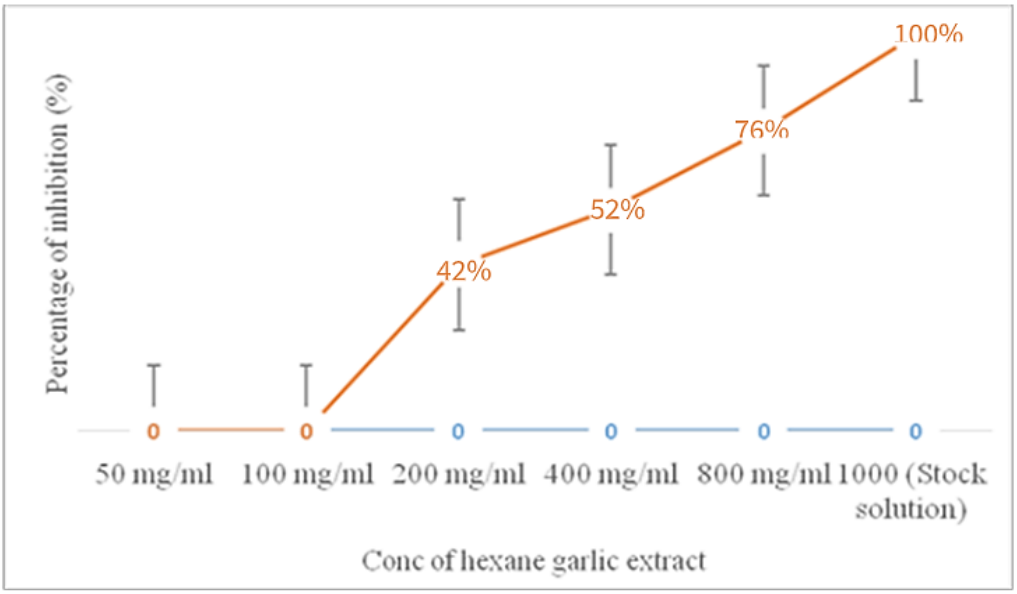

Figure 2: Cumulative percentage of inhibition of *Salmonella typhimurium* at different concentrations of hexane garlic extract.

Minimum inhibitory concentration (MIC) of hexane extract of garlic

The minimum inhibitory concentration (MIC) of hexane extract of garlic was within the range between 31.50 to 62.50 mg/ml. The spectrophotometer reading at 620 nm showed a decrease in turbidity of the test tubes with introduction of garlic extract and at a higher concentration a much more decrease in absorbance. T9 (Positive control) showed the highest absorbance reading (1.40) because there was no garlic extract added. This was followed by test tubes (T8 to T1) which subsequently decreased in absorbance reading with the introduction of different concentrations of garlic extract. It was noted that the higher the garlic concentration, the lower the absorbance (Table 3). This garlic-inhibiting activity has also been shown by the findings of Iotsor *et al*. (2019) and Airaodion *et al*. (2020).

Table 3: Effect of hexane garlic extract on the growth of *Salmonella typhimurium*.

		Different concentrations of hexane garlic extract (mg/mL)							
Tubes	T_9 Positive control	T_8 1/128 (7,812)	T_7 1/64 (15,625)	T_6 1/32 (31,25)	T_5 1/16 (62,5)	T_4 1/8 (125)	T_3 ¼ (250)	T_2 ½ (500)	T_1 1 (1000)
Turbidity reading (OD620)	1.40 ± 0.06^{a}	1.19 ± 0.02^{b}	0.80 ± 0.2^{c}	0.41 ± 0.02^{d}	0.31 ± 0.02^{de}	0.260 ± 0.02^{e}	0.180 ± 0.01^{ef}	0.025 ± 0.001^{fg}	00 ± 00^{g}

OD= optical density; T_1, T_2, T_3, T_4, T_5, T_6, T_7 and T_8= test tubes.
Data shown are Mean±S.E; each experiment consisted of three replicates.
^{abcdefg} *Means followed by the same letter are not significantly different using Duncan new multiple range test.*

In Table 4 *S. typhimurium* showed turbidity after 24 hrs of incubation in nutrient broth with different concentrations of garlic. It has been observed that the more concentrated the extract, the less the tube was disturbed.

Table 4: Growth pattern of *S. typhimurium* using different concentrations of garlic in broth after 24 h incubation at 37°C.

				Different concentrations of hexane garlic extract (mg/mL)							
Tubes	Growth Control	Positive control	Ciprofloxacin	1/128 (7,812)	1/64 (15,625)	1/32 (31,25)	1/16 (62,5)	1/8 (125)	¼ (250)	½ (500)	1 (1000)
Turbidity in broth	-	+++	-	+++	++	+	-	-	-	-	-

+ = Growth; - = no growth.

After reading the MIC and incubating, the tubes showed no visible growth of *S. typhimurium* after 24 hrs at 37°C, the MBC was determined and its value was 250 mg/ml. The tubes were equally observed after 48, 72 and 96 hrs and there was no sign of *S. typhimurium* growth. This indicated that the bioactive compounds contained in garlic completely inhibited the *S. typhimurium* growth. These results are similar to those of Tayel and El-Tras (2012) who showed that plant extracts have the potential to completely inhibit *Salmonella typhimurium* growth. In 2019, a qualitative phytochemical analysis of hexane extract of garlic, was carried out by Iotsor and collaborators. They found that flavonoids, saponins, alkaloids and triterpenes are compounds responsible for the antibacterial activity of hexane garlic extract. These compounds are therefore behind the inhibitory power of the plant extract against bacteria.

CONCLUSION

The use of medicinal plants for the treatment of diseases has been widely applied worldwide. This has been intensified particularly because of the emergence of more resistant microbial populations. This study showed that hexane extract of garlic could be used as a treatment against *Salmonella typhimurium*. Regarding the minimum inhibitory concentrations, the results demonstrate that, the more concentrated the extract, the lower the turbidity. For minimum bactericidal concentration, it was observed that even after four days of incubation, there was no growth in the tubes containing garlic. More precisely, at a concentration of 800 mg/ml, the antibacterial effect was the most pronounced. This antibacterial effect probably will makes garlic a potential plant for the genetic improvement of farm animals, including their resistance to salmonellosis.

CONFLICT OF INTERESTS

The authors have no conflict of interest to report.

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