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Preparation, Antibacterial Activity, MIC and MBC of Black Soup Against *Staphylococcus aureus* and *Salmonella typhi*

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Abstract: Antibacterial activity of black soup against *Staphylococcus aureus* and *Salmonella typhi* was studied. The reason for this research was to proof the hypothesis which state that the soup has antibacterial property. Black soup was prepared in the lab by the combination of bitter leaf, scent leaf, uziza leaf with culinary effect of salt, knor maggie cube and palm oil. The soup was moltenized to crude extract and placed in a sterile universal container prior use for antibacterial screening. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) was determined. Molecular technique was employed for the identification of *S. aureus* while Analytical Profile Index (API) kit was used for the identification of *S. typhi*. The Zone of Inhibition ZOI was 15.54mm - 12.98mm for *S. aureus*, while *Salmonella typhi* was 11.01mm – 21.05mm. Ciprotab was used as a positive control while Dimethyl Sulfoxide (DMSO) was used a negative control for the sensitivity. Minimum Inhibitory Concentration (MIC) was 250mg/ml for both bacterial isolate. There was no Minimum Bactericidal concentration for both isolate. This result proofs that the nutritive black soup has antibacterial property though bacteriostatic. Higher concentration of the soup against the isolates can be done to validate the pharmaceutical action of the black soup.

Keywords: Black Soup, Bacterial, MIC, MBC

1. Introduction

Black soup popularly called Edo or 'Bini' soup is a medicinal and nutritive meal prepared majorly by southern Nigerians. The soup is called 'black' because of its characteristic dark colour which is as a result of the concussion of selected leaves used for its preparation. The soup is prepared by the mixture of various medicinal leaves such as *Vernonia amygdalina* (bitter leaf), *Ocimum gratissimum* (scent leaf), *Piper guineense* (uziza leaf) in Nigeria and seldom with the addition of curry leaf depending on the West Africa country [1]. The homely preparation of this soup is done by the combination of these medicinal leaves in stock solution of meat, fish, Maggie, pepper, salt and palm oil which gives it a unique soothing and satisfying aftermath taste. Apart from the nutritive value of the soup, the leaves used for its preparation are of medicinal significance because of the presence of secondary bioactive substances that serves as therapeutic purposes i.e. they can serve as precursors for the synthesis of useful drugs [1].

In Nigeria and other African counties, it is a norm to eat

this soup when one is ill, and was suggested to be an elixir for cough, has antibacterial and antifungal, antimalarial, antiviral, anesthetic, antiprotozoal and anthelmintic agent and as such may have therapeutic value. This research is aimed at validating the proof of this by determining the antibacterial properties of the black soup against Gram positive *Staphylococcus aureus* and Gram negative *Salmonella typhi.*

2. Materials and Method

2.1. Isolation of Bacterial Isolate

The selective media used for the isolation of these bacterial were prepared following manufacturer's instruction. Mannitol Salt Agar (MSA) was used for the isolation of *S. aureus*, while Salmonella Shigella Agar (SSA) was used for *Salmonella typhi*. The media was autoclaved at 121°C for 15 minutes at 15 Psi. The media were allowed to cool to 45°C prior use [2].

2.2. Characterization of Bacterial Isolate

Characterization of the bacterial isolates were carried out using conventional method and advanced techniques (Analytical Profile Index and molecular technique).

2.3. Characterization of Salmonella Bacteria Isolate Using API E20

The API 20E system was used for identification of the Gram negative *Salmonella typhi.* This test was conducted following the manufacturer's instructions. The *Salmonella* isolate was picked with a sterile swab and mixed in 2ml sterile and distilled water to make a heavy suspension. Exactly 0.5ml of this suspension was transferred to 5ml sterile distilled water to make turbidity equivalent to 0.5 on the McFarland scale (10^8 cells). Using a sterile pipette, 1ml of the 0.5 McFarland suspension was transferred to an ampoule of API E20 wells medium and homogenized. The strip was incubated for 24hours at 37°C. The test was interpreted as positive (+), and negative (-). The result was recorded on results sheet. Identification was done using the Analytical Profile Index. Numerical profiles were constructed from the reaction patterns and were used to obtain identifications software program on the API catalog or apiweb. The profile for this combination of reactions was therefore 0404540 [3].

2.4. Molecular Identification of S. aureus Isolate

After selective media identification on MSA and conventional screening, the *S. aureus* isolate was subcultured on nutrient agar slant and incubated at 37°C for 24 hours prior molecular characterization [4].

2.4.1. Genomic Extraction

The DNA of the overnight grown *S. aureus* isolate was isolated using phenol chloroform technique. Phosphate buffer was used to detach the colonies from the nutrient agar slant and transferred into sterile emperdoff tubes. This was centrifuged at 14000Xg for 2 minutes and the supernatant was discarded. The obtained pellets were re-suspended with 300ul of lysis buffer and 5ul of proteinase K. This mixture was gently vortexed in few seconds to lyse the cell. Incubation of the mixture was done at 60°C for 1hour. Exactly 400ul of phenol chloroform was added and vortexed. This was centrifuged for 5mins at 15000Xg. The supernatants were transferred into sterile emperdoff tubes, precipitated using 100ul of ethanol and refrigerated overnight.

2.4.2. Polymerase Chain Reaction (PCR) of the Bacterial 16S rRNA Gene

The PCR amplification of the 16R rRNA gene was carried out using universal primers. The reaction was carried out in 20ul reaction mixture containing 1X PCR buffer, 1.5Mm MgCl, 0.2 mM of each d NTP, 2 ul Taq DNA polymerase 20 pMol of each primer and sterile water was used to make up the reaction mixture. PCR was carried out in an Eppendorf Nexus thermal cycler with the following cycling parameters; an initial denaturation step at 95°C for 5 minutes, followed by 30 consecutive cycles of denaturation at 95°C for 1 minute and annealing at 55°C for 1 minute. After this, a final extension at 72°C for 10 minutes was carried out.

2.4.3. Gel Electrophoresis of Bacterial DNA Fragments

Following PCR, the fragments were separated on 1% agarose gel. A broad range DNA ladder was used as DNA molecular weight marker. Electrophoresis was done at 80V for 1 hour 30 minutes (90 min). The gel was viewed under UV light after fluorescent dye staining was employed. The ladder on the agarose gel indicated the base pair fragment of the DNA of the bacterial.

2.4.4 Sequencing of DNA Nucleotides

Amplified PCR fragments were purified and sequenced with universal primers using Dye Terminator Cycle sequencing kit. Electropherogram of the sequence generated were observed.

2.4.5. BLAST (Basic Alignment Algorithm Search Tool)

Sequence identification was performed using GenBank's Basic Alignment Search Tool (BLAST) algorithm of National Centre for Biotechnology and Information (NCBI).

2.5. Preparation of Black Soup

Black soup was prepared by the combination of the different medicinal leaves. Clean water of 800ml was initially poured into a clean pot on hot plate. Washed and diced meat was put in water to boil at 100°C before the addition of pitch of salt, pepper, knor maggie cubes to add savour and taste. The stock was allowed to stay in pot for a few minutes to allow its cooking. The leaves were weighed in ratio of 2:2:1:1 and pre-mixed. The mixture of medicinal leaves were introduced into the cooked meat stock and stirred to obtain homogeneity of the soup. Final 300ml of sterile water was added to cook the soup. The prepared soup was

allowed to dry on hot plate to obtain crude extract. At cool, the cooked black soup was transferred into a sterile container and refrigerated at 4°C till use [1].

2.6. Determination of Antibacterial Activity of Black Soup Against S. aureus and S. typhi.

2.6.1. Preparation of 0.5 Mcfarland Standard

Mcfarland 0.5 turbidity standard was prepared by adding 0.6ml of 50% barium chloride ($BaCl_2$) solution to 99.4ml of 1% Sulphuric acid (H_2SO_4) and mixed thoroughly. Small volume estimate of the turbid solution was transferred into a screw capped test tube of the same types that was used to compare with the test inoculum. Turbidity standard equivalent to 0.5 McFarland was used to adjust the turbidity (concentration) of the inoculum for antimicrobial susceptibility tests. The reaction between these two chemicals results in the production of a fine precipitate called barium sulfate ($BaSO_4$). When shaken well, the turbidity of this McFarland standard is visually comparable to an approximate bacterial suspension of 1.5 x 10^8 colony forming units/ml [5].

2.6.2. Inoculum Preparation

Inocula for standard antimicrobial susceptibility tests were prepared by aseptically picking four to five colonies of identified pure bacteria culture with a sterile wire loop into sterilely prepared 9ml of nutrient broth and incubated at 35°C for 8 hours. The nutrient broth culture was compared to the turbidity of 0.5 Macfarland standard.

2.6.3. Preparation of Antibiotic Control

A broad spectrum antibiotics (Ciprofloxacin) was used as positive control while DMSO and was used as negative control. Exactly 500mg was dissolved in 1ml of Di-methyl sulfoxide (DMSO), to obtain a concentration of 500mg/ml each in each plate. Subsequent concentrations were attained by dissolving serially 0.5ml of each into 1ml of DMSO. The subsequent concentrations achieved include 250mg/ml, 125mg/ml and 62.5mg/ml [6].

2.6.4. Antibacterial Screening of Black Soup on Bacteria Isolates

Agar well diffusion technique was employed to determine antimicrobial activity of black soup. Exactly 20ml of nutrient agar was poured in sterile Petri dish and was allowed to solidify. Then, 1ml of the test culture was poured on the solidified agar and the characterized bacteria was spread all over the surface of the agar using a sterile spreader. Wells of 6mm in diameter were made on the surface of the agar medium using a sterile cork borer and the plates were allowed to stay. Then, the inoculated plates were inverted and the plates labeled with a marker. Each well was filled with 0.2ml of the black soup. The plates were incubated aerobically at 37°C for 18-24 hours. Sensitivity of the bacteria isolates to the extract and soup showed zone of inhibition (ZOI) while resistance of the selected bacteria would show no zone of inhibition [7].

2.7. Determination of Minimum Inhibitory Concentration (MIC) of Black Soup on Bacteria Isolates

Broth dilution technique was employed for the determination of the MIC of the black soup the on the selected bacteria isolates following the procedure of Tojola et al. [7].

Four (4) tubes labeled 500mg/ml, 250mg/ml, 125mg/ml and 62.5mg/ml were used for the black soup. The first contained 5mls of double strength of nutrient broth while the remaining contained 5ml of single strength of nutrient broth. Five (5mls) of the black soup in the aforementioned concentration were introduced into tube one, thoroughly mixed and serially diluted. To each of the test tubes, 0.1mls of broth cultures (equivalent of 10^8 CFU/ml) of the test organisms were added and incubated at 37°C for 24 hours.

The minimum inhibitory concentration (MIC) was determined as the lowest concentration of the black soup that was able to inhibit the growth of the selected bacteria.

2.8. Determination of Minimum Bactericidal Concentration (MBC) of the Black Soup

Minimum Bactericidal Concentration (MBC), was done following the method of [8]. With a sterile wireloop, a loopful from the MIC test tubes were streaked on prepared nutrient agar. The plates were labelled accordingly and further incubated at 37°C for 24 hours. After the incubation period, the plates were observed for growth. The absence of growth indicates the MBC of the black soup against the selected bacterial isolates.

3. Result

Table 1. *Conventional Screening/Characteristics of the Bacterial Isolates.*

Category of Test	Test and Assessment	Staphylococcus aureus	Salmonella typhi
	Colour on plate	Yellow	Black
	Texture	Opaque	Translucent
Microscopy	Shape	Cocci	Rod
Gram Staining	Gram reaction	+	-
Biochemical	Catalase	+	NT
	Coagulase	+	NT
Molecular Identification	Nature of DNA	Condensed white thread like	NT
	Electrophoretic Band	800kbp	NT

Key: + (Positive) - (Not detected) NT (Not Tested)

Table 2. *Identification of Staphylococcus aureus and its' Closest GenBank Relatives.*

Bacterial Isolate	Closest Genbank Relative	Accession Number	Maximum Identity (%)
Staphylococcus aureus	S. aureus	MG557810.1	99

Key: % = percentage

Table 3. *Identification of Salmonella typhi and its Closest on API Web.*

Bacterial Isolate	API Number	Maximum Identity (%)
Salmonella typhi	4004500	99

Key: % = percentage

The antibacterial activity against *S. aureus* and *S. typhi* was assessed in terms of inhibition zone at concentrations of 62.5mg/ml, 125mg/ml, 250mg/ml and 500 mg/ml. The concentration tested produced significant ($p<0.05$) dose except at the concentration of 62.5mg/ml and 125mg/ml. The zones of inhibition increased as the concentrations increased. The highest activity was recorded at 500 mg/ml where inhibition zone range of 11.01 ± 0.53^{a} mm to 21.05 ± 0.45^{a} mm for *S. typhi,* while 12.98 ± 0.21^{a} mm to 15.54 ± 0.52^{a} mm was observed for *S. aureus*.

Table 4. *Sensitivity Test Showing Zone of Inhibition (mm) of Black Soup on S. aureus and S. typhi.*

Bacteria Isolate/agent	500mg/ml	250mgm/l	125mg/ml	62.5mg/ml
S. aureus	15.54 ± 0.52^{a}	12.98 ± 0.21^{a}	0	0
Ciprotab	21.34±0.06	20.50±0.05		
DMSO	0			
S. typhi	21.05 ± 0.45^{a}	11.01 ± 0.53^{a}	0	0
Ciprotab	31.32±0.03	30.45±0.05	0	0
DMSO	0			

abc-(Mean with difference superscript are significantly different) ($P<0.05$).

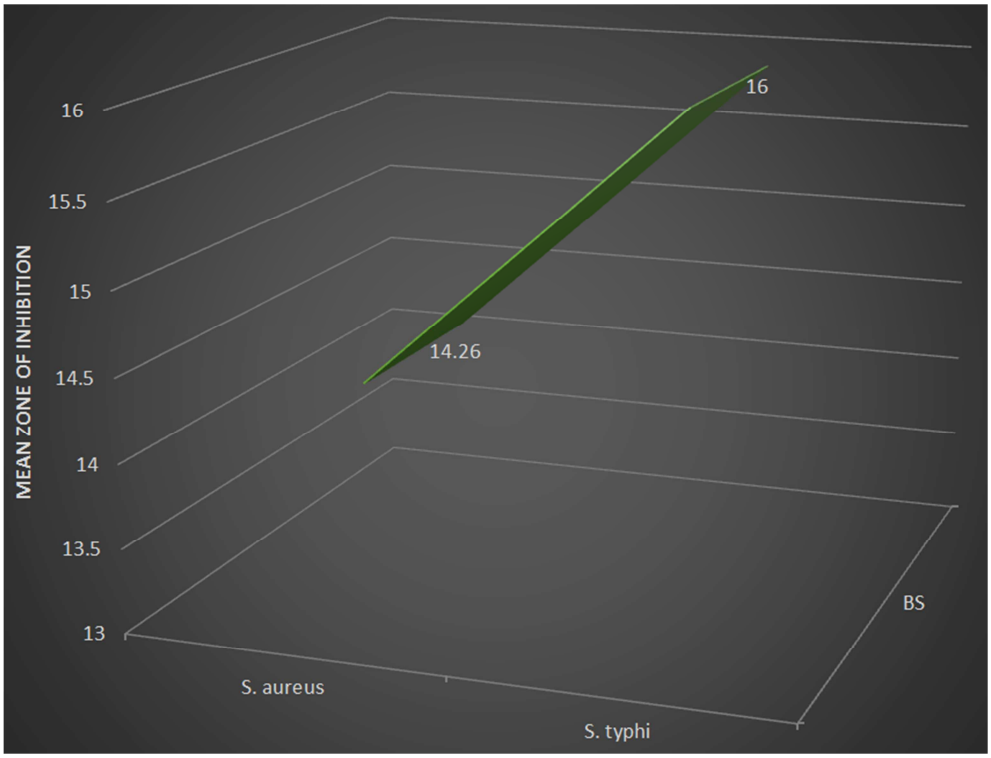

Figure 1. *Comparative Mean Zone of Inhibition (mm) of Black soup on S. aureus and S. typhi.*

Minimum Inhibitory Concentration (MIC) and minimum bactericidal concentration Black Soup against *Staphylococcus aureus* and *Salmonella typhi.*

The MIC of black soup against both bacterial isolates was 250mg/ml. There was growth on the plates for MBC.

Figure 2. *BLAST and Electrograph sequence of S. aureus* respectively.

4. Discussion

4.1. Isolation of Selected Bacterial Isolates

MSA has high salt concentration that only permit the cultivation of *S. aureus* and mannitol sugar which is utilized by the *S. aureus* and its' indicated by yellowish colouration in the presence of phenol red as a dye. On the other hand, *Salmonella typhi* selectively shown black colour on SSA media because of its ability to produce H_2S after the utilization of glucose in the medium.

The tentative bacterial isolates of *S. aureus* and *Salmonella typhi* were subjected to array of biochemical. Colonies from MSA are Gram positive cocci in cluster, catalase, coagulase and mannitol sugar positive *S. aureus*. The isolate was Gram positive because of its thick peptidoglycan to retain primary stain, its ability to breakdown hydrogen peroxide into gas and water, and the capacity to agglutinate serum, potential to

ferment mannitol sugar respectively. After genomic extraction and PCR, the bands for *S. aureus* was 800kbp.

Salmonella isolates prepared for API web gave 040450 number combination. The number was search on API web which confirmed the Salmonella specie to be 99% *Salmonella typhi*.

4.2. Antibacterial Activity of the Black Soup Against the Bacterial Isolate

The result of this study showed that the cooked black soup inhibited the growth of both the Gram positive *S. aureus* and the Gram negative *Salmonella typhi*. Therefore the antibacterial activity of the extracts was broad spectrum activity. This is because of the presence of bioactive compound present according to previous research done by [1].

The cooked black soup showed less antibacterial sensitivity when compared to the ciprotab. This may be due to the process of preparation of the soup which required heating which may have denatured some bioactive compound and presence of palm oil which reduces the function of saponins and possible flavonoid [1, 9, 10].

The Zone of Inhibition was lesser in Gram positive *Staphyloccocus aureus* than *Salmonella typhi.* This should be attributed to the fact that Salmonella has somatic layer, lipopolysaccharide and thin peptidoglycan while *S. aureus* has thick peptidoglycan on their cell wall hence penetration of these bioactive compounds maybe seemingly tedious in the Gram positive *S. aureus* [11-14].

4.3. Minimum Inhibitory Concentration and MBC

However, the MIC for the black soup against both *S. aureus* and *S. typhi* is the same at 250mg/ml. There was growth on the MBC plates that was subcultured. This clearly indicates that black soup used for this study is bacteriostatic not bactericidal, except if the concentration is increased to 1g/ml.

5. Conclusion

Antibacterial activity, MIC and MBC of Black Soup against *Staphylococcus aureus* and *Salmonella typhi*. The black soup showed sensitivity against the bacterial isolates and according to this research, the soup is bacteriostatic except the concentration of the soup is increased to proof that it can be bactericidal. This research validate the paradigm that the soup has medicinal value. Therefore, the soup has dual property of being nutritional and medical.

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Appendix

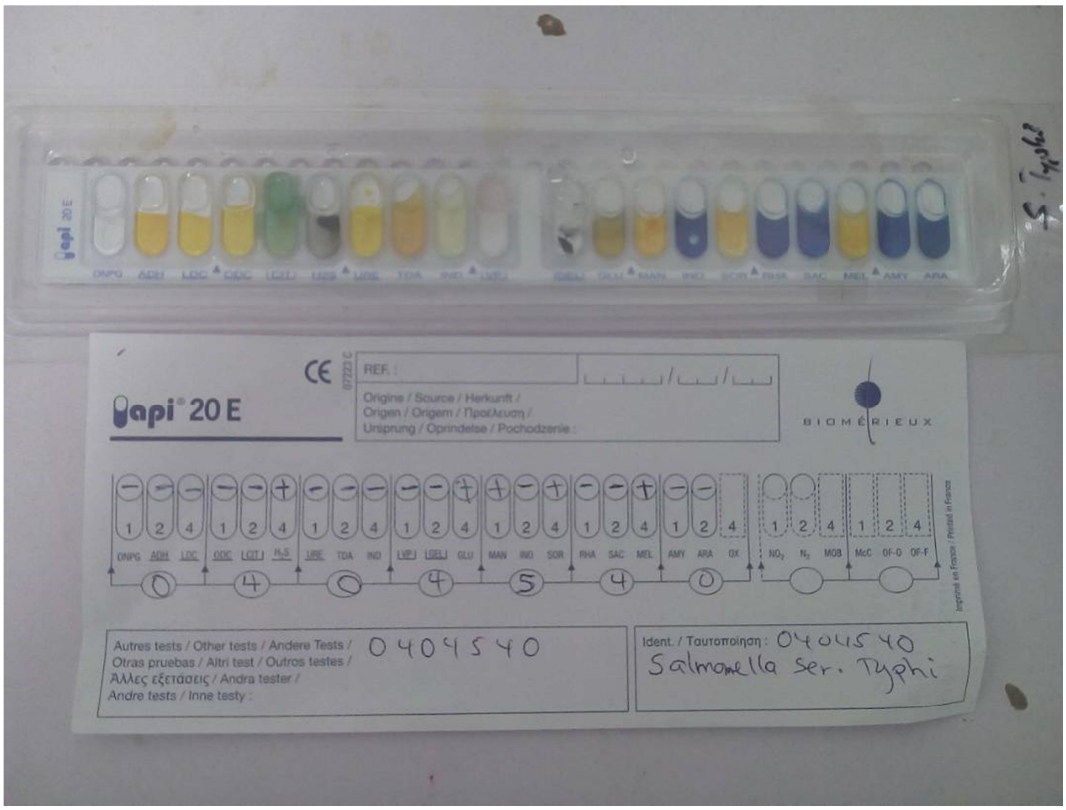

Figure A1. *API serokit for Enterobacteria.*

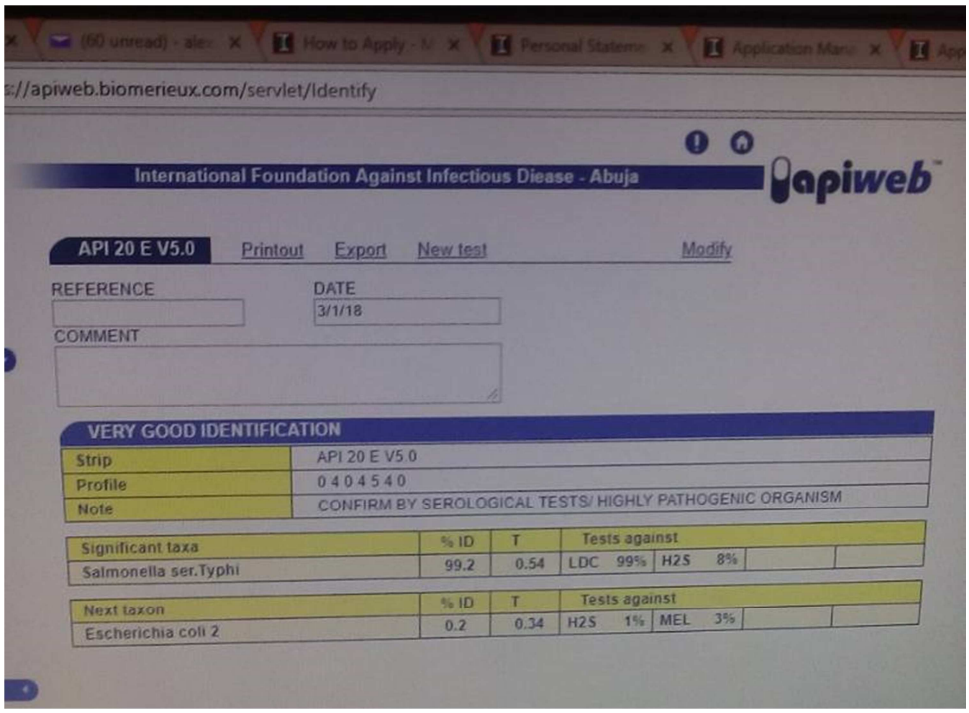

Figure A2. *API web with 99.2% identification accuracy.*

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