

Original Research Article

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## Antimicrobial Susceptibility of Spoilage Bacteria of 'Atama' (*Heinsia crinata*) Soup

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### ABSTRACT

#### Keywords

'Atama' soup,  
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The study examined the sensitivity of spoilage bacteria to antibiotics and crude ethanol, methanol and aqueous extract of *Heinsia crinata* employed in the preparation of the 'atama' soup as well as its proximate composition using standard methods. The bacterial isolates were identified as *Bacillus tequilensis* strain ADIP3, *Bacillus cereus* strain SB2, *Bacillus thuringensis* strain EB-151, *Escherichia coli* strain sanji and *Pseudomonas aeruginosa* strain PG1. *Heinsia crinata* crude extracts had no inhibitory activity against all the isolates. The isolates however, showed varying cumulative percentage antibiotic resistance against gentamicin (80%), ofloxacin (40%), augmentin (100%), cefuroxime (100%), ceftazidime (100%), ampicillin (100%), nitrofurantoin (40%) and ciprofloxacin (40%). The proximate analysis shows the chemical composition such as moisture (63.92%), ash (1.04%), carbohydrate (7.96%), protein (5.71%), lipid (19.15%) and fibre (2.22%). The study has shown the inability of employing *H. crinata* in the shelf life extension of 'atama' soup.

### Introduction

'Atama' (*Heinsia crinata*) soup is a delicious palm nut soup, native to the Efik/Ibibio people of Southern Nigerian. It is very similar to 'banga' soup native to the Urhobo/Isoko people of Delta State; the major difference is the *Heinsia crinata* leaves which give it a distinctive taste and aroma. According to Inyang (2016) leaves of *Lasianthera africana* and *Heinsia crinita* are used for the preparation of indigenous traditional soups known respectively as "editan" and "atama" soups. Folk tales has it that many housewives use it to win back their husband's heart after a serious quarrel.

*Heinsia crinita* (Afz.) G. Taylor, also known as "Bush apple" is called "Atama,"

"Tonoposho" and 'Fumbwa' in Efik, Yoruba and Igala cultures in Nigeria, respectively belongs to the family *Rubiaceae* (Dalziel, 1937; Ebong *et al.*, 2014; Bob *et al.*, 2016).

Almost all parts of the plant can be utilized by humans for food or for medicinal purposes. Its fruits, leaves and flowers are edible. Its roots can be used as medicine (Eze *et al.*, 2014).

*Heinsia crinita* is also used as a component of various herbal portions in ethnomedicine in the Southwest of Nigeria against acute bacterial infections and for the treatment of infertility (Enyi-Idoh *et al.*, 2013).

Phytochemical investigations have shown this plant to contain some bioactive constituents with beneficial medicinal properties (Ebong *et al.*, 2014).

A comparative pharmacognostic and antimicrobial studies of leaves of *H. crinita* have been reported (Ajibesin *et al.*, 2003). Abo *et al.*, (2011) reported that extracts of *H. crinita* exhibited profound antibacterial activity against strains of *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Candida* species.

There is however, a dearth of information on the antimicrobial activity of *Heinsia crinita* on spoilage bacteria of 'atama' soup hence, the aim of this study is to evaluate its effects on possible spoilage bacteria with a view to establishing the possible role of this spice in enhancing the soup's shelf-life, the antibiotic sensitivity of the isolates and the determination of the soup's proximate composition.

## **Materials and Methods**

### **Sample collection**

The soup ingredients comprising atama (*Heinsia crinita*) leaves, stock fish, smoked or fresh fish, assorted meat, palm fruits, periwinkles, aidan fruit and condiments to taste were purchased at the Choba Junction market, Port Harcourt.

### **Preparation of 'atama' soup**

The soup was prepared using the customary method. Fresh palm fruits are boiled for 30 min, pounded in a mortar after which hot water is added and strain through a sieve to separate the oily extract from the pulp. Cut fresh meat seasoned with salt, pepper and maggi are steamed for 5 min on low heat before adding stock fish and a little water. It

is boiled till the meat is almost cooked. Cut 'atama' leaves are pounded till finely ground for about 10 min. The oily extract in a pot is boiled for about 5 min before adding the cooked meat, stock fish, washed smoked fish and other ingredients. It is allowed to boil for 10 min before adding the 'atama' leaves and allowed to boil for another 10 min without stirring. Salt is then added to taste and allowed to simmer for another 15 min or until slightly thick.

### **Proximate analysis**

The moisture, crude protein, crude fibre, crude fat, carbohydrate and total ash contents of the soup was analysed using the method described by Association of Official Analytical Chemists' (AOAC, 1990).

### **Isolation procedure**

From an overnight deteriorating soup after 24h, 10 ml was aseptically transferred to 90ml sterile peptone water and homogenized. After a ten-fold serial dilution, 0.1ml was spread plated on Nutrient agar plates and incubated at room temperature ( $29\pm 2^{\circ}\text{C}$ ) for 24 h. Distinct colonies were purified in fresh Nutrient agar and stored in slants for further analysis.

### **Identification of bacterial isolates**

The isolates were identified using standard conventional (Gram staining, catalase, indole, motility, citrate, Methyl red, Voges Proskauer, oxidase, starch hydrolysis,  $\text{H}_2\text{S}$  production, sugar utilization) and molecular methods (Polymerase Chain Reaction and sequencing).

### **DNA extraction**

Extraction was done using a ZR fungal/bacterial DNA mini prep extraction kit supplied by Inqaba South Africa. A heavy

growth of the pure culture of the isolates was suspended in 200 microlitre of isotonic buffer into a ZR Bashing Bead Lysis tubes, 750 microlitre of lysis solution was added to the tube. The tubes were secured in a bead beater fitted with a 2ml tube holder assembly and processed at maximum speed for 5 min. The ZR bashing bead lysis tubes were centrifuged at 10,000xg for 1 minute.

Four hundred (400) microlitres of supernatant was transferred to a Zymo-Spin IV spin Filter (orange top) in a collection tube and centrifuged at 7000 xg for 1 min. One thousand two hundred (1200) microlitres of fungal/bacterial DNA binding buffer was added to the filtrate in the collection tubes bringing the final volume to 1600 microlitre, 800 microlitre was then transferred to a Zymo-Spin IIC column in a collection tube and centrifuged at 10,000xg for 1 min, the flow through was discarded from the collection tube. The remaining volume was transferred to the same Zymo-spin and spun. Two hundred (200) microlitre of the DNA Pre-Wash buffer was added to the Zymo-spin IIC in a new collection tube and spun at 10,000xg for 1 min followed by the addition of 500 microlitre of fungal/bacterial DNA Wash Buffer and centrifuged at 10,000xg for 1 min.

The Zymo-spin IIC column was transferred to a clean 1.5 microlitre centrifuge tube, 100 microlitre of DNA elution buffer was added to the column matrix and centrifuged at 10,000xg microlitre for 30 s to elude the DNA. The ultra pure DNA was then stored at -20°C for other downstream reaction.

### **Amplification of 16S rRNA**

The 16S rRNA regions of the rRNA genes of the isolates were amplified using the 27F (AGAGTTTGATCMTGGCTCAG): and 1492R (CGGTTACCTTGTTACGACTT):

primers on an ABI 9700 Applied Bio-systems thermal cycler at a final volume of 50 microlitres for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95°C for 5 minutes; denaturation, 95°C for 30 seconds; 2666annealing, 52°C for 30 seconds; extension, 72°C for 30 seconds for 35 cycles and final extension, 72°C for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 15 minutes and visualized on a UV transilluminator.

### **Sequencing of 16S rRNA**

The amplified 16S products were sequenced on a 3500 genetic analyzer using the Bigdye-Termination technique by Inqaba South Africa.

### **Phylogenetic analysis**

The sequences were edited using the bioinformatics algorithm Bioedit, similar sequences were downloaded from the National Biotechnology Information Center (NCBI) data base using BlastN, these sequences were aligned using ClustalX. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0 (Saitou and Nei 1987).

The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor, 1969) and are in the units of the number of base substitutions per site.

### **Preparation of crude extract**

The methanol and ethanol crude extracts of *Heinsia crinita* were prepared according to the method of Akujobi *et al.*, (2004). The spice crude extracts were diluted with 30% dimethylsulphoxide (DMSO) to obtain 250mg/ml (0.5g in 2ml), 200mg/ml (0.5g in 2.5ml), 150mg/ml (0.3g in 2ml), 100mg/ml (0.5g in 5ml) and 50mg/ml (0.25g in 5ml). Aqueous crude extract was diluted in sterile deionised water at same ratio.

### **Antibacterial activity of crude *Heinsia crinita* extracts**

Agar diffusion method was employed. From an overnight broth culture of the various bacterial isolated in nutrient broth, a  $1 \times 10^8$  cell/ml McFarland standard was prepared (by first centrifuging the overnight broth at 4,000 rpm for 10min and supernatant decanted). Sterile deionized water (2ml) was then added vortexed and centrifuged again at 4,000rpm for 10min. The resulting pellets were transferred to a physiological saline while comparing with McFarland standards) and 0.1ml aseptically transferred to sterile Petri dishes before adding 20ml molten Mueller Hinton agar cooled to 50°C. The content was thoroughly mixed and then allowed to solidify. Five wells (5.0mm) were made in each plate using a cup borer and 0.2ml of the spice concentrations of the methanol, ethanol and aqueous extracts aseptically transferred into each well using a pipette. Plates were allowed to stand for pre-diffusion for 1h before incubation at  $29 \pm 2^\circ\text{C}$  for 24h. Average zones of inhibition were calculated.

### **Antibiotic sensitive testing**

Antibiotic sensitivity patterns of all the isolates confirmed biochemically and molecularly were performed by standard disk diffusion method on Mueller-Hinton agar (Titan, Biotech Ltd, Indian) following the procedures recommended by NCCLS (1997).

Eight commonly used antibiotics ( $\mu\text{g}/\text{disc}$ ) viz. gentamycin (GEN) 10, ofloxacin (OFL) 5, augumentin (AUG) 30, cefuroxin (CRX) 30, ceftazidime (CAZ) 30, ampicillin (AMP) 10, nitrofurantoin (NIT) 300 and ciprofloxacin (CPR) 5 (Abtek<sup>R</sup>), were tested. From an overnight culture in brain heart infusion broth, a  $10^8$  cell/ml (0.5 MacFarland turbidity standards) bacterial culture was prepared in sterile saline, from which 0.1ml was inoculated onto Mueller Hinton agar, after which antibiotic discs were carefully and aseptically placed on the surface of the agar. The plates were incubated at 37°C for 24h. Zones of inhibition were measured in millimeter.

### **Determination of spoilage time**

Fifty milliliter of freshly prepared soup were aseptically dispensed into pre-sterilized bowl, and 0.1ml of  $1 \times 10^8$  cell/ml (0.5) McFarland standard of individual and a mixed bacteria culture was added to each bowl and observed at 15min interval for spoilage signs at room temperature ( $29 \pm 2^\circ\text{C}$ ).

## **Results and Discussion**

### **Bacterial present in ‘atama’ soup**

The bacteria isolated from freshly spoilt ‘atama’ soup were *Bacillus tequilensis* strain ADIP3, *Bacillus cereus* strain SB2, *Bacillus thuringensis* strain EB-151, *Escherichia coli* strain sanji and *Pseudomonas aeruginosa* strain PG1 (Figure 1). There are no reports of the microbiology of ‘atama’ soup in available literature. However, *Bacillus* and *Pseudomonas* species are well known spoilage bacteria of a wide range of foods (Owolabi and Ichoku, 2014).

### **Antibacterial activity of ‘atama’ leaves**

The result of the antibacterial activity of crude aqueous, ethanol and methanol extract reveals

an inability of all the extracts (50 to 250mg/ml) to inhibit any of the bacteria implicated in the spoilage of ‘atama’ soup. Abo *et al.*, (2011) have however, reported the inhibitory potentials of methanol and hexane extracts of *H. crinata* against *S. aureus* and *E. coli* up to concentration of 50 mg/ml.

**Antibiotic susceptibility of isolated bacteria**

The spoilage bacterial showed varying antibiotic resistance against gentamicin (80%), ofloxacin (40%), augmentin (100%), cefuroxin (100%), ceftazidime (100%), ampicillin (100%), nitrofuratoin (40%) and ciprofloxacin (40%) (Table 1). The 100%

susceptibility of *Bacillus* species to ciprofloxacin and ofloxacin in this study is in agreement with previous reports of 100% susceptibility of *Bacillus* species isolated from different food sources (Andrews and Wise 2002; Whong and Kwaga, 2007; Dikbas, 2010; Agwa *et al.*, 2012; Ogidi and Oyetayo, 2013). A number of authors have reported a 0 to 100% resistance of *Bacillus* species to gentamicin, comparable to the findings of this study (Balakrishnan *et al.*, 2003; Dikbas, 2010; Ogidi and Oyetayo, 2013; Owolabi and Ichoku, 2014). *Pseudomonas aeruginosa* strain PG1 isolated from ‘atama’ soup was resistant to all the antibiotics employed in this study.

**Table.1** Distribution of resistance against common antibiotics

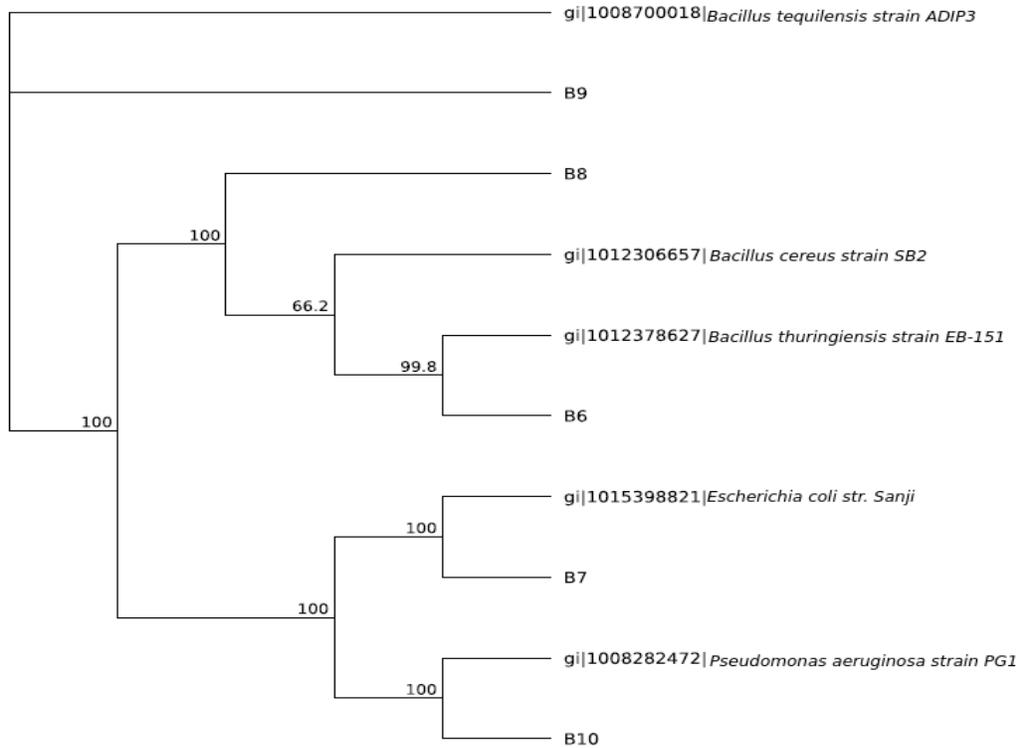
Antibiotics	Conc (µg/ml)	Susceptibility						% cumulative resistance
		BT ADIPS	BC SB2	BT EB- 151	EC Sanji	PA PG1		
AUG	30	R	R	R	R	R	100	
GEN	10	R	S	S	R	R	80	
OFL	5	S	S	S	R	R	40	
NIT	300	S	R	S	S	R	40	
CAZ	30	R	R	R	R	R	100	
CRX	30	R	R	R	R	R	100	
CPR	5	S	S	S	R	R	40	
AMP	10	R	R	R	R	R	100	

BT ADIPS= *Bacillus tequilensis* strain ADIP3, BC SB2=*Bacillus cereus* strain SB2, BT EB-151=*Bacillus thuringensis* strain EB-151, EC Sanji=*Escherichia coli* strain sanji and PA PG1=*Pseudomonas aeruginosa* strain PG1

**Table.2** Spoilage time of ‘atama’ soup by individual and mixed bacteria culture

Bacteria	Spoilage time (Hrs)
<i>Bacillus tequilensis</i> strain ADIP3	15.30
<i>Bacillus cereus</i> strain SB2	15.30
<i>Bacillus thuringensis</i> strain EB-151	15.30
<i>Escherichia coli</i> strain sanji	15.45
<i>Pseudomonas aeruginosa</i> strain PG1	15.00
Mixed culture	15.00
Control	17.00

**Fig.1** The phylogenic tree of bacteria isolated from ‘atama’ soup



This is in agreement with 100% resistance of *Pseudomonas* species isolated from food sources to augmentin, nitrofuratoin, gentamicin, ampicillin and ofloxacin (Oluyeye *et al.*, 2009; Adeshina *et al.*, 2012; Ogidi and Oyetayo, 2013; Ghalem *et al.*, 2014).

The *Escherichia coli* strain sanji isolated in this study was only susceptible to nitrofuratoin. A number of authors have reported varying resistance of *E. coli* isolated from food and other sources to against augmentin (13.3 to 100%), ofloxain (0 to 39.3%), nitrofurantoin (26.17 to 80.0%), gentamicin (0 to 91.37%), ampicillin (22.2 to 100.0%), ciprofloxacin (4.07 to 82.0%) and ceftazidime ((0.0 to 94.02%) (Akond *et al.*, 2009; Oluyeye *et al.*, 2009; Adeshina *et al.*, 2012; Ogidi and Oyetayo, 2013; Poonia *et al.*, 2014; Rasheed *et al.*, 2014; Tiamiyu *et al.*, 2015; Boss *et al.*, 2016; Omobije *et al.*, 2016; Sudda *et al.*, 2016).

#### Proximate composition of 'atama' soup

The proximate analysis shows the chemical composition such as moisture (63.92%), ash (1.04%), carbohydrate (7.96%), protein (5.71%), lipid (19.15%) and fibre (2.22%). The finding of this study is comparable to report of moisture (66.56%), ash (3.23%), carbohydrate (7.79%), protein (5.99%), lipid (8.89%) and fibre (7.53%) by Kolawale and Obueh. (2012) and moisture (76.04%), ash (1.70%), carbohydrate (12.77%), protein (4.03%), lipid (5.00%) and fibre (0.48%) by Okeke *et al.* (2009) of 'banga' soup, another major palm nut soup in Nigeria..

#### Spoilage of soup by isolated bacteria

The result of the potentials of individual and a mixed bacteria culture to cause spoilage of 'atama' soup is presented in Table 2. The average spoilage time for all seeded soups ranged from 15.00 to 15.45 h compared to the control samples' average spoilage time of 17.00 h.

There is a dearth of information on the

spoilage microorganisms of Nigeria soups, however, a number of Authors have reported *P. aeruginosa*, and *Bacillus species* as major food spoilage bacteria of number of foods (Jansen and Aschehoug, 1951; Hanlin, 1998; Dogan and Boor, 2003; Nwagu *et al.*, 2010; Arslan *et al.*, 2011; Owolabi and Ichoku, 2014; Setlow, 2014; Rawat, 2015; Scatamburio *et al.*, 2015).

In conclusion, the findings of this study have revealed the proximate composition, presence of notable spoilage bacteria, namely: *Bacillus tequilensis* strain ADIP3, *Bacillus cereus* strain SB2, *Bacillus thuringiensis* strain EB-151, *Escherichia coli* strain sanji and *Pseudomonas aeruginosa* strain PG1 and their antimicrobial resistance to the spice, *Heinsia crinata* (a spice used in the preparation of 'atama' soup) and common antibiotics. The result obtained revealed available nutrients in 'atama' soup which makes it an ideal delicacy for human nutritional needs. The inability of *H. crinata* to inhibit the isolated bacteria, particularly the food spoilage bacteria is also established and a pointer to the fact that the soups' shelf life cannot be extended by the spice. The resistance of these isolates to common antibiotics followed established pattern portending great danger.

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