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ANTIBACTERIAL POTENCY OF CRUDE CITRUS JUICE ON BACTERIA ISOLATED FROM ZOBO DRINKS SOLD IN NNAMDI AZIKIWE UNIVERSITY, AWKA, NIGERIA.

***[1]Ezeadila Joachim O., [1]Umenonihu Chiamaka M., [1]Kyrian-Ogbonna Evelyn A. and [1]Udemezue Onyekachukwu I.**

[1]Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, P.M.B. 5025, Awka, Anambra State, Nigeria.

***Author for Correspondence: Ezeadila Joachim O.**
Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, P.M.B. 5025, Awka, Anambra State, Nigeria.

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ABSTRACT
Zobo drink is an indigenous non-alcoholic drink made from hot water Roselle calyces of *Hibiscus sabdariffa*. The greatest limitation for large-scale production of zobo drinks is the rapid deterioration of the drink. This study thus, focuses on the antibacterial activity of crude juices of some *Citrus* fruits on bacteria isolated from Zobo drink sold within Nnamdi Azikiwe University, Awka, Anambra state. A total of 10 samples were randomly collected and processed by culturing them on suitable media. Microorganisms isolated include *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterobacter* species, *Proteus mirabilis, Klebsiella pneumoniae* and *Salmonella* species. The antibacterial activities of the crude juices were determined using the disc diffusion method. Standard antibiotic discs were also used on the test organisms. The results obtained confirmed antibacterial activity of the crude juices of some of the Citrus fruits. The highest zone of inhibition (14mm) was observed with the crude juices of *Citrus aurantifolia* and *Citrus limonum* at 100% concentration on *Proteus mirabilis* while *Citrus paradisi* produced the lowest inhibition zone diameter (8mm) against *Escherichia coli* and *Pseudomonas aeruginosa*. All the isolates were resistant to the crude juices of *Citrus sinensis* while *Salmonella* species was resistant to the juices of all the *Citrus* fruits. This study revealed that crude *Citrus* juices (especially those of *Citrus limonum* and *Citrus aurantifolia*) have antibiotic potentials and their possible use to treat some infections caused by the isolated bacteria or as natural preservative to prolong the shelf- life of zobo drinks is recommended.

KEYWORDS: Citrus fruits, antibacterial potency, zobo drinks, pathogenic bacteria.

INTRODUCTION
Zobo drinks are aqueous extracts of calyx of roselle, *Hibiscus sabadariffa* an annual herb cultivated widely in Africa and India. Zobo is derived from zoborodo a local *Hausa* (Northern Nigeria) name for *Hibiscus sabadariffa* plant. The non-alcoholic drink or zobo is popular especially in Northern Nigeria and it is usually served chilled at various social gatherings.[1,2] The zobo drink is prepared by boiling the dry calyces of *Hibiscus sabadariffa* in water for about 10-15 minutes from which the pigment or flavor embedded is extracted. After extraction the filtrate may be taken hot as tea or allowed to cool and packaged in plastic sachet containers then taken as a refreshing drink when chilled.[1,3] Demand for zobo drinks is on the increase due to its low prices, nutritional and medicinal properties.[4,5] The greatest limitation for large-scale production of zobo drinks is the rapid deterioration of the drink due largely to unregulated nature of the trade. Poor hygienic practices as well as lack of running water, toilet, proper storage and waste disposal facilities at preparation and services point has resulted in poor unsanitary conditions exposing it to potential contaminants and increasing its public health risk.

The shell-life of zobo is approximately twenty-four hours following production if not refrigerated. The sharp sour taste of the raw extract is usually sweetened with sugar cane or granulated sugar, pineapple, orange or other fruits depending on choice. Microorganisms associated with the dried calyx and the processing for the production of zobo drinks and other factors may contribute to its spoilage.[3,6] Thus, as a result of microbial activities, the sweetness of Zobo drink does not last long.[4]

The search for new antimicrobial compounds is ongoing. Its importance cannot be overemphasized in an era of emerging resistant pathogenic organisms.[7] *Citrus* is one of the most important fruits and the juices are consumed majorly because of their nutritional value and special flavor. They are consumed mostly fresh and have been

used as herbal medicine, additives or food supplements as they are believed to possess bioactivities such as antioxidant, anti-inflammatory, anti-cancer and antimicrobial conferred on them by bioactive compounds such as phenolics, flavonoids, vitamins, and essential oils which they possess.[8] Also, the positive health benefits of juices have been ascribed in part to vitamin C (ascorbic acid), the major vitamin found in fruits and vegetables.[9,10,11]

The disadvantages of chemical preservative ranges from having adverse effect on humans to being too expensive for the local people that produce zobo drink. There is the need for alternative source of preservation that is natural, cheap or affordable, readily available and safe. The aim of this study therefore, is to investigate the effect of citrus juice on the bacteria isolated from zobo drinks sold in Nnamdi Azikiwe University (UNIZIK), Awka, as success means that it will form a veritable tool in the preservation of this treasured drink.

MATERIALS AND METHOD

Study Area

This study was carried out in Nnamdi Azikiwe University, Awka, Awka South Local Government Area, Anambra State, Nigeria between May-July 2016.

Sample Collection

Fresh sweet oranges (*Citrus sinensis*), grapefruit (*Citrus paradisi*), lime (*Citrus aurantifolia*) and lemon (*Citrus limonum*) were purchased from the local market (Eke-Awka) in Awka, Anambra state, Nigeria. The fruits were identified by a botanist Mrs. Aziagba B.O at the Department of Botany, Nnamdi Azikiwe University, Awka. Confirmation of taxonomic identity of fruits was achieved by comparison with voucher specimens kept at the herbarium of the Department of Botany.

Ten Zobo drinks were purchased randomly from different locations within Nnamdi Azikiwe University, Awka and were taken immediately to the Applied Microbiology and Brewing Laboratory for analysis. The Ten samples were bought as packed in plastic bottles and 20mls of each sample was transferred into universal sterile containers.

Source of Test Organisms

The test organisms (*Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella* species, *Klebsiella pneumoniae, Enterobacter* species and *Proteus mirabilis*) were isolated from the Zobo drink purchased randomly from different locations within Nnamdi Azikiwe University, Awka, Anambra State, Nigeria. A total of ten samples were collected and labeled appropriately. The samples were placed in nylon bags and transported to the Departmental laboratory of Applied Microbiology and Brewing, Nnamdi Azikiwe University, Awka for analysis.

Processing of the zobo samples

A total number of ten samples in universal sterile containers were diluted using serial dilution method as described by.[12] With the aid of pipette 0.1ml was aseptically placed on the surface of a well sterile dried Nutrient agar that was prepared and spread evenly using sterile bent glass rod. Each experiment was carried out in duplicates to get a mean standard value of the colony forming units (cfu /ml) on the plates. The inoculated plate was incubated at 37^0C for 24 hours.

Viable Count and Isolation of Pure Culture

After the period of incubation, the colonies on the nutrient agar plates were counted and recorded as colony forming units per millilitre (Cfu mL^{-1}). The plates were carefully examined and representative colony types were picked and sub cultured on nutrient agar using streaking techniques and incubated to obtain pure cultures, after which their morphology was recorded. The pure cultures were then stored on agar slants in Bijou bottles to preserve the isolates for further use.

Characterization and Identification of the Isolates

The identification of microorganisms was based on their cultural characteristics, Gram reaction, biochemical tests such as motility, citrate utilization, indole, methyl red, voges-proskauer (vp), coagulase, catalase, urease, starch hydrolysis, klinger's iron agar, sugar test as described by.[13] Growth on MacConkey agar was used to separate the lactose fermenter from non-lactose fermenter. Growth on eosin methylene blue agar (for the production of green metallic sheen), centrimide agar (for yellowish green pigmentation), mannitol salt agar (to determine if the organism will utilize mannitol and produce yellow colonies), salmonella and shigella agar to determine if the organism can produce transparent colorless colonies.

Extraction of Crude Extracts

The fresh oranges, grape, lime and lemon were washed with water and the surfaces disinfected with 70% alcohol. They were peeled aseptically with sterile knife, cut into halves and squeezed to get the crude extract. The extract was then filtered into a sterile conical flask using a sterile white handkerchief.

Sterility Check

To check for sterility, the Citrus extracts (0.1 ml) was inoculated into Mueller- Hinton agar using spread plate technique and incubated at 37ºC for 24 hours.

Preparation of Different Concentrations of the Crude Citrus Juice

Different concentrations were made by diluting the crude juice with sterile physiological saline. To prepare 1ml of 25% concentration of Citrus juice, 0.25ml juice was added to 0.75 ml of saline. To prepare 1ml of 50% and 75% concentration, 0.5ml and 0.75ml of Citrus juice was respectively added to 0.5ml and 0.25ml of saline. For 100% concentration, 1ml of the crude Citrus juice was used.[14]

Preparation of Discs for Sensitivity Testing

The Whatman no 1 filter paper was punched out to get the discs (6mm in diameter). The discs were transferred into a glass Petri dish well covered with a foil and sterilized in the autoclave at 121^0C for 15 minutes. The crude citrus juices of different concentrations (100%, 75%, 50%, and 25%) were aseptically transferred into well labeled different sterile Petri dishes. The discs were then transferred into the Petri dish containing the different concentration of the crude juice and were left in the Petri dish to absorb the juice for about 20 minutes. Later the discs were dried in the incubator for 2 hours at 35^0 C and kept for use.

Turbidity Standard for Inoculum Preparation

To standardize the inoculum density for the susceptibility test, barium sulphate turbidity standard, equivalent to 0.5 McFarland standards was used. One percent v/v solution of sulphuric acid was prepared by adding 1 ml of concentrated sulphuric acid to 99 ml of water and mixed well. A 1.175% w/v solution of barium chloride was prepared by dissolving 2.35g of dehydrated barium chloride in 200ml of distilled water. To make the turbidity standard, 0.5ml of the barium chloride solution was added to 1% 99.5ml sulphuric acid solution and mixed well. A portion (5ml) of the resultant turbid solution was transferred into sterile test tube and plugged with cotton wool. A 0.5ml of McFarland standard equals 1×10^8 colony forming unit per ml (CFU/ml). Using a sterile wire loop, discrete colonies each of 24 hours pure culture of the test organisms was emulsified in 5ml of peptone water in a test tube which was also plugged with cotton wool. The turbidity of the suspension was then matched with the turbidity of the standard.

Determination of the Antibacterial Activity of Crude Citrus Juices on the Isolated Organisms.

Antimicrobial sensitivity was determined using disc diffusion technique as demonstrated by.[15] Each test organism was seeded onto already prepared plates of Mueller Hinton agar. The discs impregnated with the crude citrus juice were then placed aseptically on the plates using sterile forceps. Conventional antibiotic discs (Ciprofloxacin, Gentamicin, Ceporex and Septrin) for the Gram positive bacteria and those (Amoxicillin, Augmentin, Chloramphenicol, Sparfloxacin, Septrin, Streptomycin, Gentamicin, Ceporex, Tarivid and erythromycin) for the Gram negative bacteria were also used for the purpose of comparison. The plates were incubated at room temperature for 24 hours after which the diameter of zones of inhibition were checked and measured in millimeter with a meter rule.

RESULTS AND DISCUSSION

In this study, disc diffusion method was used to evaluate the antibacterial potency of the crude juices of some citrus fruits against some bacteria isolated from 10 zobo samples (coded Z1-Z10) sold within Nnamdi Azikiwe University, Awka, Anambra State, Nigeria. Some conventional antibiotics were used for comparison. The total bacterial count of the zobo drinks is shown in table 1. It can be seen that all the zobo drinks sampled had varying levels of bacterial contamination ranging from bacterial counts of 5.0 x 104 CFU/ml to 1.9 x 106 CFU/ml. All the zobo drinks had the unacceptable total bacterial counts of >104 CFU/ml. This obviously implies that the zobo drinks have been extremely contaminated. Similar findings have been reported by.[3]

Table 1: Total bacterial count (cfu/ml) of the zobo samples.

Zobo Samples	Bacterial count (cfu/ml)
Z_1	1.6×10^5
Z_2	5.0×10^4
Z_3	3.0×10^5
Z_4	9.0×10^4
Z_5	1.7×10^6
Z_6	1.9×10^6
Z_7	1.1×10^6
Z_8	1.2×10^5
Z_9	2.0×10^5
Z_{10}	6.8×10^5

Seven (7) bacteria genera were isolated from the zobo samples. These include *Staphylococcus aureus*, *Escherichia coli*, *Proteus mirabilis*, *Enterobacter* species, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Salmonella* species. Table 2 shows the percentage (%) occurrence of bacteria in the zobo samples. The isolation of microorganisms such as *Escherichia coli* and *Enterobacter* species from the zobo samples is an indication of fecal contamination. This implies fecal materials would have been introduced into the zobo drinks through the use of contaminated water, contamination from unsanitary environment or even through the human handlers[12] since these microorganisms are known to inhabit the gastrointestinal tracts of humans. These microorganisms are causal agents of gastrointestinal diseases.

Table 2: % Occurrence of bacteria in the zobo samples.

Isolates	Frequency	% Occurrence
Staphylococcus aureus	2	10.52%
Escherichia coli	5	26.32%
Proteus mirabilis	3	15.79%
Enterobacter species	1	5.26%
Pseudomonas aeruginosa	4	21.05%
Klebsiella pneumoniae	1	5.26%
Salmonella species	3	15.79%

The antibacterial activity of the crude juices (100%) of the different citrus fruits on the isolated microorganisms is presented in table 3. It is observed that at 100% concentration, the crude juice of *Citrus aurantifolia (*lime) and *Citrus limonum (*lemon) have the highest

antibacterial activity (with inhibition zone diameters of 14mm each) against *Proteus mirabilis.* Also, the inhibition zone diameter of *C. limonum* and *C. aurantifolia* was 12mm each against *Staphylococcus aureus* and *Klebsiella pneumoniae* respectively (Fig 1 & 2). Similar findings have been reported by some other researchers.[16,17]

In this study, all the microorganisms were resistant to the crude juice of *Citrus sinensis* at all concentrations (100%, 75%, 50% and 25%). This result is similar to that of[14] in which all the bacteria used (except *Vibrio choleriae*) where resistant to the different concentrations of juices of *Citrus sinensis*. The work of[16] also supports the findings of this study. However, the work of[18] disagrees with the findings of this study. This is because in their study, all the concentrations (100%, 75%, 50% and 25%) of juices of *Citrus sinensis* had activity against *Staphylococcus aureus*. *Salmonella* species was resistant to all the different concentrations of the crude juices of the four *Citrus* species used (Table 3). This contradicts the work of[14] and[19] in which they reported that crude juice of *Citrus limonum* (lemon) had antibacterial activity against *Salmonella* species. A possible reason for these differences could be the method of extraction of the citrus juices[20] and also, the species and even strains of *Salmonella* isolated in the present study could be different from theirs. The time of harvest and age of the Citrus fruits could also influence the antibacterial potency of the juices.[21]

Table 3: Antibacterial activity of the crude juices (100%) of the different *Citrus* species on the isolated microorganisms.

Microorganism	Inhibition zone diameter (mm)			
	C. aurantifolia	***C. limonum***	***C. paradisi***	***C. sinensis***
Staphylococcus aureus	10	12	-	-
Proteus mirabilis	14	14	10	-
Escherichia coli	10	10	8	-
Enterobacter species	10	10	-	-
Klebsiella pneumoniae	12	10	-	-
Salmonella species	-	-	-	-
Pseudomonas aeruginosa	10	10	8	-

Key.C.
= Citrus, - = No zone of inhibition

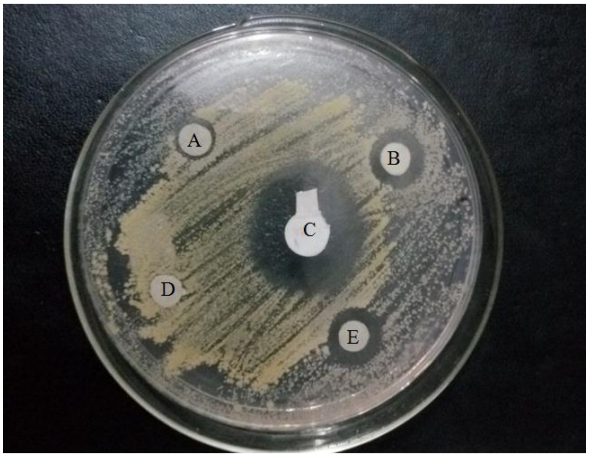

Fig 1: Zones of Inhibition by juice of *Citrus aurantifolia* against *Klebsiella pneumoniae.*

Key.
A= 50% Concentration, **B=** 100% Concentration, **C** = Control (Amoxicillin)
D= 25% Concentration, **E**= 75% Concentration

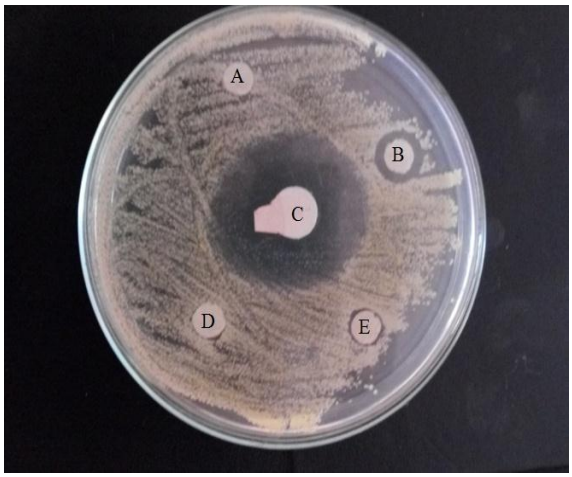

Fig 2: Zones of Inhibition by juice of *Citrus limonum* against *Klebsiella pneumoniae.*

Key
A= 25% Concentration, **B=** 100% Concentration, **C** = Control (Chloramphenicol)
D= 50% Concentration, **E**= 75% Concentration

In table 4, is presented the antibacterial activity of the conventional antibiotics on the isolated microorganisms. From this study, it can be seen that septrin showed the highest antibacterial activity followed by ciprofloxacin with inhibition zone diameter of 48mm and 40mm against *Staphylococcus aureus* respectively. Similar observations have been made by[22] in which

ciprofloxacin was the second most effective antibiotics used against *Staphylococcus aureus.*

In this study, all the conventional antibiotics used showed much higher zones of inhibition than the juices of the *Citrus* fruits. This doesn't agree with the findings of a similar research carried by[16] in which the antibacterial activities of juices of *Citrus limonum* and *Citrus aurantifolia* against the test organisms (especially *Staphylococcus aureus*) compared favourably with those of the conventional antibiotics such as septrin, gentamicin and ciprofloxacin. The time of harvest and age of the *Citrus* fruits, as well as the strains of microorganisms[21,23] could have accounted for these differences.

Table 4: Antibacterial Activity of the Conventional Antibiotics on the Bacterial Isolates.

Microorganisms	Inhibition zone diameter (mm)										
	CPX	SXT	CN	AU	CEP	OFX	E	AM	CH	SP	S
S. aureus	40	48	26	*	30	*	*	*	*	*	*
Escherichia coli	*	20	*	18	*	22	*	*	*	*	*
Proteus mirabiis	*	*	20	*	30	*	31	*	*	*	*
Enterobacter spp	20	*	*	*	18	*	*	*	16	*	*
P. aeruginosa	30	18	18	*	*	*	*	*	30	*	*
K. pneumoniae	*	*	*	*	*	*	*	26	22	28	30
Salmonella spp	32	24	-	*	*	30	*	*	*	*	*

Key
*SXT= Septrin, CH= Chloramphenicol, SP = Sparfloxacin, CPX = Ciprofloxacin, AM = Amoxicillin, Au = Augmentin, CN = Gentamicin, OFX = Tarivid, S = Streptomycin, E = Erythromycin, CEP = Ceporex, * = Not used against the test organism, - = Resistant*

CONCLUSION

In the present study, all the zobo drinks sampled had varying levels of bacterial contamination ranging from bacterial counts of 5.0 x 104 CFU/ml to 1.9 x 106 CFU/ml. All the zobo drinks had the unacceptable total bacterial counts of >104 CFU/ml. This obviously implies that the zobo drinks have been extremely contaminated. Consumption of these zobo drinks is thus a potential health risk.

The crude juices of *Citrus aurantifolia (*lime) and *Citrus limonum (*lemon) at 100% concentration had the highest antibacterial activity (with inhibition zone diameters of 14mm each) against *Proteus mirabilis*. Septrin showed the highest antibacterial activity followed by ciprofloxacin with inhibition zone diameter of 48mm and 40mm against *Staphylococcus aureus* respectively. In this study, all the microorganisms were resistant to the crude juice of *Citrus sinensis* at all concentrations (100%, 75%, 50% and 25%) while *Salmonella* species was resistant to all the different concentrations of the crude juices of the four *Citrus* species used.

Though none of the crude juices of the *Citrus* fruits used compared favorably with any of the antibiotics used, the marked antibacterial activity of crude juices of *Citrus aurantifolia (*lime) and *Citrus limonum (*lemon) especially against *Proteus mirabilis* reveal great potential antibacterial activity of these juices against these microorganisms. Thus, further research to extract the active ingredients in these Citrus juices is recommended. Also, a standard procedure for preparation, packaging and handling of zobo drinks should be provided by bodies such as NAFDAC (National Agency for Food and Drug Administration and Control) with a view to reducing the level of contamination of the zobo drinks.

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