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Antibiocompetent and Bacteriocidal Relevance of Ascorbic Acid on Coliforms Isolated from Feces of Apparently Healthy Students in Rivers Nigeria

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Abstract
In recent times, Ascobic acid has been discovered to posses antibiocidal potential against various bacterial strains that has been resistant which is in sharp contrast as it was previously regarded as potential antioxidant especially as it is thought to reduce oxygen radicals both in vivo and in vitro biochemical processes. This study investigates the anti-biocompetent and bacteriocidal relevance of ascorbic acid on Staphylococcus aureus, Escherichia coli, and Proteus mirabilis recovered from the apparently healthy student feces at Madonna University. Standard microbiological methods were applied for the isolation of fecal coliforms using sterile blood agar, mannitol salt agar and eosin methylene blue (EMB) agar plates. The agar diffusion disc technique was used to determine an ascorbic acid fortified antimicrobial susceptibility testing of test bacteria at various concentrations varying from 0.167g/ml to 1.0g/ml, while their mean values were compared using one-way analysis of variance. The results revealed that Proteus mirabilis were resistant to the various ascorbic acid concentrations, whereas S. aureus and E. coli were susceptible to various ascorbic acid concentrations. An ascorbic acid content (g/ml) changes/shift (at $p < 0.05$) the zones of inhibition (mm) of the isolates (E. coli and S. aureus) Such observed antibacterial potential suggests that ascorbic acid may be applied in the treatment of enteric or coliforms infections associated with E. coli and Staphylococcus aureus.

Keyword: Ascorbic acids; coliforms; *E.coli*; *Staphylococcus aureus*; bacteriocidal

INTRODUCTION
Ascobic acid is powerful antioxidant with potential relevance in reducing oxygen radicals (Daviese *et al*., 2005). However, in 1907, two Norwagian doctors discovered an additional potential in guinea pig animal model as a crucial disease-preventative food ingredient to prevent beriberi. These doctors eventually identified the dietary component as vitamin C (Daviese *et al*., 2005). It may be a naturally produced chemical substance while its impure

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form can make it look yellowish as it is a white solid in pure form (Lachapelle *et al*., 2010). It is water soluble and produces a slightly corrosive solution. Ascorbic acid is associated with the new term L-hexuronic acid taken from the Greek words a-, which means "no," and scorbutus, which is the Latin name for the condition known as scurvy. Many animals can make it because it is made from glucose, but people need it as part of their nutritional requirement (Lachapelle *et al*., 2010). Other primates, guinea pigs, teleost fish, bats, and birds are vertebrates that lack the capacity to make ascorbic acid, which they all need as a dietary micronutrient, or vitamin (Lachapelle *et al*., 2010). Although the compound has been produced in animals, its potential bioactive relevance has not been fully harnessed especially amongst enteric coliforms. Ascorbic acid is a crucial vitamin required for many metabolic processes in people, such as the regulation of free radicals, which is essential for the metabolism of bacterial cells (Igere *et al*., 2020; Levine, 2008).

It is noteworthy that as a primary agent of nosocomial and community-acquired infections, most coliforms such as *Staphylococcus aureus*, produces a wide range of human illnesses in all age groups, including infections of surgical wounds and epidermal skin disorders in newborn babies (Curran *et al*., 2004; Igere *et al*., 2021). When *S. aureus* infections are not adequately managed, they may spread to nearby tissues and cause a variety of health problems. The majority of *S. aureus* types are currently methicillin resistant, which is what is causing the rise in *S. aureus* infections (Holt *et al*., 2001). Although *S. aureus* is one of the most common sources of gastroenteritis arising from consumption of contaminated food globally with a 40% mortality rate, it is suggested that interpersonal hygiene and personal cleanliness must be given top priority while using regular ascorbic acid (Vitamin C) among other things (Levine, 2008; Holt *et al*., 2001).

Other coliforms such as *Escherichia coli* are bacteria type that thrives in the bowels and stomach as Gram-negative facultative anaerobic, non-sporulating bacilli. Water, milk, food, bugs, and other animals are potential agents of spread of *E coli* (Daini *et al*., 2008). The cells have a cell volume of 0.6–0.7 micrometers, are about 2 micrometers length, and have a 0.5 micrometer width. *E. coli* grows most effectively in temperatures up to 49°C (Fotadar *et al*., 2005). *E. coli* has been linked to several frequent serious illnesses and conditions including pyelonephritis, septicemia, meningitis, endocarditis, urinary tract infections, and pandemic adult- and child-related diarrhea (Daini *et al*., 2008). It is majorly isolated from blood and implicated in both nosocomial and community acquired infections in people (Karlowsky *et al*., 2004).

Proteus is another member of coliforms which are gram-negative, actively motile, non-capsulated, rather pleomorphic coliform rod (Fawole and Ose, 2008). They are normal flora of the gut, but may also be found in the sewage, soil and vegetable with abundance in intestine of animals (Fawole and Ose, 2008). It is implicated in urinary tract infection and produce bacteremia, pneumonia and focal lesion in debilitated patients. One of the characteristic of Proteus species is to produce a putrefying odour as a result of production of acid and gas, which distinguish most species from the other group of entrobacteriaceae (Fawole and Ose, 2008).
The proliferation and antibiotic resistant nature of such coliform has necessitated the need for the use of alternative methods and the use of already known agents, as ascorbic acid promises a future. The study investigates the antibiocompetent and bactericidal relevance of ascorbic acid on few selected bacteria strains isolated from apparently healthy students from students' stools in Elele, Rivers State Nigeria.

MATERIALS AND METHODS

Study Area

The research was conducted in Elele, Rivers State, Nigeria which is situated in the Southern regions of Nigeria. The study area (Elele) is located at latitude 5°27-5° 31N and longitude 60 56-7 85E.

COLLECTION OF SAMPLES

A total of 24 samples (which is 0. 1% of total population) were collected from apparently healthy Madonna University students' stool using a ubiquitous sterile plastic receptacles. *Staphylococcus aureus, Escherichia coli,* and *Proteus mirabilis* were isolated from stool samples as test potential pathogens.

RESEARCH DESIGN

A once-off 24 total feces samples were taken once a week from both genders (boys and girls) at the University hostels. The samples are divided in four (4) samples per week for six (6) weeks.

METHODS OF IDENTIFICATION OF THE TEST ORGANISMS

a. Bacterial Culture

The samples were processed within three hours of collection by culturing onto blood agar, mannitol salt agar and eosin methylene blue (EMB) agar and incubating for 24 hours at 37 °C. Isolated b**acterial** were purified by subculturing colonies onto nutrient agar and storing pure colonies in freshly prepared agar slant.

b. Colonial Morphology and Gram Reaction

The colonial morphology of the test organisms were observed after inoculation at 37 ^{o}C and recorded as well as their Gram reaction, which was confirmed by the method of Gram staining.

Applying Gram staining technique, bacterial isolates were tested and the reports divided strains into Gram positive and Gram negative strains (Cheesbrough, 2005).

BIOCHEMICAL TEST

An array of various standard microbiological biochemical tests were conducted including motility, indole test, urease test, citrate utilization test, catalase test, sugar fermentation test (including: glucose, galactose, maltose, mannitol, fructose, and sucrose) and coagulase test were conducted and positive reports were recorded for various isolates (Igere *et al*., 2022a; Brooks *et al*., 2007; Cheesbrough, 2005).

PREPARATION OF THE LOCAL DISC

This was accomplished by using a perforator to pierce Whatman number one filter paper. The disc was then placed in a Petri dish and sanitized for 30 minutes in a hot air oven at Specific Gr**avity** temperature. Ascorbic acid in various concentrations was measured using an automated weighing scale (0.5, 1.0. l.5, 2.0, 2.5, 3.0g). Test containers containing the various ascorbic acid concentrations were sterilized in a heated air furnace at 160°C for 1 hour after being dissolved in 3mL of distilled water. The disc was placed in a Petri dish and submerged in an ascorbic acid solution before being fully desiccated in a heated air oven at a low temperature.

ANTIMICROBIAL ACTIVITY TEST

DISC DIFFUSION

This was done to ascertain the test **bacteria** susceptibility to ascorbic acid's antibacterial effects. The test bacteria suspension was made and stand**ardized** using the McFarland standard. A streak was formed on the nutrient agar using a swab stick, and using clean tweezers, the prepared local discs, which contained various concentrations of ascorbic acid, were impregnated on the corresponding nutrient agar with their corresponding b**acterial**. Three strains of each test bacterial were placed on nutrient agar dishes, which were then kept at 37 °C for 24 hours. Using a meter rule, the zones of inhibition of the different test b**acteria** and the ascorbic acid contentrations were noted (Igere *et al*., 2022b).

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

Test bacteria was prepared using a wire loop, a colony of the desire isolate *(Escherichia coli Staphylococcus aureus and Proteus mirabilis),* was taken and mixed unto normal saline. After which the test organism was standardized with McFarland standard to standardize the bacteria suspension. Using the double serial dilution, 2 mL of nutrient broth was introduced into ten test tubes and was labeled according to dilution factor of 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, 1/1024 and was sealed with cotton wool together with aluminum foil then autoclaved at 121°C for l5 minutes.

Half a gram of ascorbic acid was measured and dissolved into 3 mL of distilled water after the soup had been sterilized. One milliliter of ascorbic acid was pipette and introduced into the first test tube, mixed thoroughly, and then transferred into the second tube. This is followed in the same manner to the prepared test tubes or the last test tube is treated, and last content removed.

Each test container containing an bacteria was given a drop of one of the contents concentration. The entire setup was kept for 24 hours at ambient temperature. Turbidity was assessed in the test tube after 24 hours for bacteria growth (Chessbrough, 2005). The nutrient agar plate was swabbed with the contents of the test container using a swab stick, and it was then kept for 24 hours at 37°C. The dishes were then examined for inhibition after that, minimum inhibitory concentration (MIC), which was used in the antimicrobial test, was the lowest quantity at which it was possible to prevent any discernible bacterial growth.

DETERMINATION OF MINIMUM BACTERICIDAL CONCENTRATION (MBC)

Following the incubation time, the lowest bactericidal concentration was determined to be the ascorbic acid concentration with the greatest dilution factor that exhibited no apparent growth or turbidity. The agar diffusion technique was used to ascertain the minimum bacteriocidal concentration (MBC). MBC of a particular organism is measured and reported for the test tube with the dilution factor prior to the minimal inhibition.

McFarland Standard

A one percent sulphuric acid solution was made by mixing 1ml of pure sulphuric acid with 99ml of water. One percent solution of barium chloride was also made by dissolving 1 g of barium chloride in 99 mL of water and thoroughly mixing it. 99.6 mL of sulfuric acid and 0.4 mL of barium chloride were combined and thoroughly blended. The fluid was faintly cloudy (Cheesbrough, 2005).

STATISTICAL ANALYSIS

Collected data were analyzed with the statistical package for social science (SSPS) version 18.0 for Windows. To assess means, analysis of variance (ANOVA) was used, and values were deemed significant at $p < 0.05$. Post hoc multiple test for ANOVA was performed using the least significant difference (LSD). In addition, Pearson's correlation was used to determine the extent of the connection (Agwung- Fobellah, 2007).

RESULTS

MICROBIOLOGICAL ANALYSIS

Table 1 shows the percentage occurrence of bacteria found in the stools of male and female students of Madonna University Elele Campus, Rivers State. The strains are *Escherichia coli, Staphylococcus aureus* and *Proteus mirabilis* with percentage occurrence of 12 (50%), 8 (33%) and 4 (17%) respectively among male students. Whereas female students reports showed *Escherichia coli, Staphylococcus aureus* and *Proteus mirabilis* with percentage occurrence of 12 (50%), 7(29%) and 5 (21%) respectively. Table 2 shows the mean values for ascorbic acid zones of inhibition (mm) for the various isolates (*Escherichia. coil* and *Staphylococcus aureus)* subjected to various concentrations (in g/ml) of ascorbic acid. Amongst the *Staphylococcus aureus* strains, the inhibitory effect exerted by the ascorbic acid is highest at the concentration 1.0 g/mL with zone of inhibition of 39.00mm and is least at the concentration 0.167 g/mL with zone of inhibition of 18.00mm. Also amongst *Escherichia coli,* the inhibitory effect exerted by the ascorbic acid is highest at the concentration 1.0 g/mL with zone of inhibition of 40.33mm and is least at the concentration 0.167 g/mL with zone of inhibition of 13.00mm. There was a significant difference ($p < 0.05$) in the ascorbic acid zones of inhibition (mm) in the isolates (*Escherichia coli* and *Staphylococcus aureus*) as the quantity of ascorbic acid (g/mL) increases/changes. There was also a positive significant association ($p < 0.05$) between ascorbic acid zones of suppression (mm) and ascorbic acid concentrations. Figure 1 shows the mean values for ascorbic acid zones of inhibition (mm) amongst isolates *(Escherichia coli* and *Staphylococcus aureus)* subjected to various concentration (g/mL) of ascorbic acid.

Figure 2 depicts the mean values of ascorbic acid's minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) on chosen strains (*Escherichia coli* and *Staphylococcus aureus*). The mean values of the MIC and MBC of ascorbic acid in *Staphylococcus aureus* strains are 0.012 g/mL and 0.025 g/mL, respectively. The mean values of the MIC and MBC of ascorbic acid on the chosen strains are 0.012 g/mL and 0.023 g/mL, respectively, among *Escherichia coli.*

Table 1: Percentage occurrence of bacteria found in stools of male and female students of Madonna University Elele, River State

Sample	*Staphylococcus aureus*	*Escherichia coli,*	*Proteus mirabilis*	*Total number of isolates*	*% Occurrence*
Male	8 (33)	12 (50)	4 (17)	24	100
Female	7 (29)	12 (50)	5 (21)	24	100
Total number of isolates	15	24	9	48	

Table 2: Comparison of mean values for ascorbic acid zones of inhibition among bacterial isolates subjected to various concentrations of ascorbic acid

Isolate/ Concentration	0.167 g/mL	0.333 g/mL	0.5 g/mL	0.667 g/mL	0.833 g/mL	1.0 g/mL	P-value
***S. aureus* (mm)**	18.00±1.00	20.33±0.58	23.67±01.15	27.33±7.57	33.67±5.13	39.00±6.00	P < 0.05
***E. coli* (mm)**	13.00±1.00	13.33±1.15	19.67±1.52	26.00±2.00	34.67±0.58	40.33±2.31	P < 0.05

P < 0.05 Significant

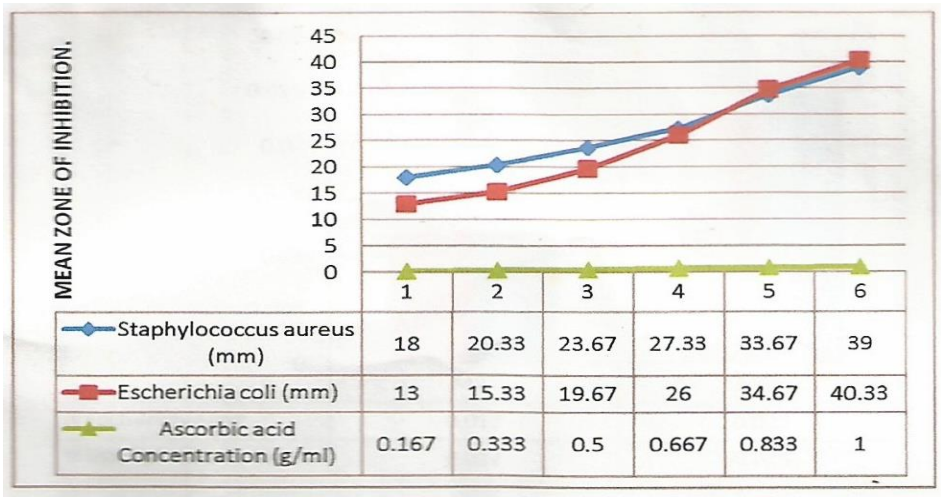

Figure 1: Mean values for ascorbic and zones of inhibition (mm) among bacterial isolates subjected to various concentration (g/mL) of ascorbic acid.

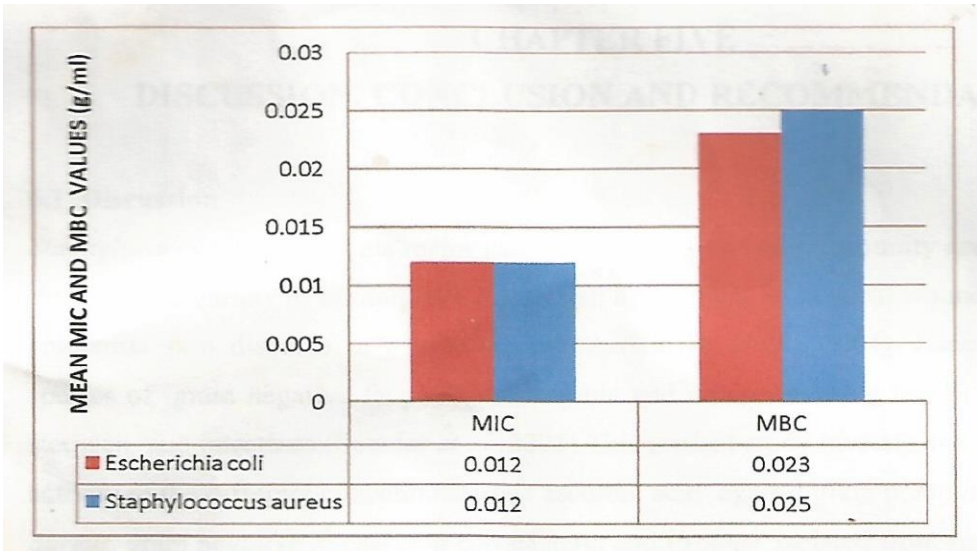

Figure 2: Mean values for minimum inhibition concentration and Minimum bacteriocidal concentration of Ascorbic acid on the selected isolates

DISCUSSION

Most nosocomial and community acquired illnesses have been implicated with *Staphylococcus aureus*, which also causes infections in surgical incisions and epidermal skin disorders in newborn babies (Curran *et al*., 2004). The stomach and intestinal mucosa are home for diverse Gram-negative, facultatively anaerobic, and non-sportulating bacteria called *Escherichia coli* (Fotadar *et al*., 2005). This study examined the antibiocompetent and antibacteriocidal action of various ascorbic acid concentrations against stool-isolated Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* bacteria. The study revealed the presence of organisms in stool specimens as shown in Table 1. The observed strains are *Escherichia coli, Staphylococcus aureus* and *Proteus mirabilis* with percentage occurrence of 12 (50%), 8 (33%) and 4 (17%) respectively among male students. Whereas female students reports showed *Escherichia coli, Staphylococcus aureus* and *Proteus mirabilis* with percentage occurrence of 12 (50%), 7(29%) and 5 (21%) respectively. The study observed that *Escherichia coli* was most predominant/prevalent than *Staphylococcus aureus* while *Proteus mirabilis* was least prevalent. The presence of *Escherichia coli* in stool indicates its prevalence as gut flora in the lower intestine (Feng *et al*., 2004). The antibiocompetent/antibacteriocidal potential of ascorbic acid's demonstrated differing zones of inhibition against *Staphylococcus aureus* and *Escherichia coli*, however such effect was not observed among *Proteus mirabilis*. It is important to note that ascorbic acid exhibit a variety of effect including its functions as a biological hydrogen transporter for redox enzyme systems in cell metabolism (Alibi *et al*., 2004). It also help in food preservative to prevent oxidative rancidity in rich greasy foods and to prevent discoloration of conserved fruits and vegetables (Hancock and Speert 2000). The sensitivity pattern of the selected isolates to ascorbic acid, the minimum inhibitory concentration (MIC), and the minimum bacteriocidal concentration (MBC) of ascorbic acid on different selected/detected strains was significant ($p < 0.05$) (Table 2, fig 2). Such observation of ascorbic acid in this study is similar to the findings of Naresh *et al*. (2002), who reported that ascorbic acid was bacteriocidal for many types of bacteria, including *Bacillus pertussis* and *Mycobacterium tuberculosis*, resulting in inhibition of their growth in ascorbic acid-containing medium. In a related study, it was observed that ascorbic acid extracted from cranberry, amla, and lemon fruits pose antibacterial effect on bacterial strains (Hancock and Speert 2000). Their study reported antibacterial inhibitory effect on *Streptococcus species* (with zones of inhibition of 23mm) and *Staphylococcus saprophyticus* (with zones of inhibition of 23mm), *E. coli* (with zones of inhibition of 21mm), *Klebsiella pneumonia* (with zones of inhibition of 18mm) and *Pseudomonas aeruginosa* (with zones of inhibition ranging from 23mm - 16mm). The bacteria isolates exposed to vitamin C showed significant growth inhibition at the MICs, also viability experiments revealed that vitamin C had bactericidal activities at doses eightfold or higher than the MICs. In addition, growth inhibition and viability loss were also observed amongst some *Escherichia coli* strains as reported by some investigators (Can and Frei, 2005). Furthermore, ascorbic acid was found to have a mean minimum bacteriocidal concentration (MBC) of 0.023 g/mL against *Escherichia coli* isolates and 0.025 g/mL against *Staphylococcus aureus* isolates, which may be consistently applied in an *in vivo* treatment of enteric diseases cases. This is compatible with the findings of Dr. Klenner who first used vitamin C aggressively to treat illness. Large doses of vitamin C were used by Klenner to treat polio, measles, mumps, chicken pox, and other diseases (Thomas et al., 2009). Other investigators also reported that Ascorbic acid may act as an antimicrobial modulator (Gunics *et al*., 2000; Chakrabarty *et al*., 2002; Kristiansen and Amaral, 2002; Rajyaguru and Muszynski, 2003;). One consistent observation during the study was that ascorbic acid exhibited no antibacterial properties on *Proteus mirabilis*. This may be associated with the swamy nature of the strain in the presence of ascorbic acids. It may also be as a result of the minor adjustments made to the test organism's diffusion or microbe growth rates.

CONCLUSION

This study has demonstrated that *Proteus mirabilis* was unaffected by ascorbic acid's bacteriocidal effects whereas significant effect were observed among *Staphylococcus aureus* and *Escherichia coli* isolated from feces. This indicates that ascorbic acid may be used in combination with the majority of antibiotics, which may be advantageous in increasing drug sensitivity to the majority of bacteria. The functions of ascorbic acid reported above makes it abundantly obvious that it plays a part in cellular processes although, its biological action is yet to be completely described, it possess important features for human wellbeing. It is therefore necessary to conduct more study on how ascorbic acid is metabolized. Additionally, adequate policy must be developed on the suggested daily amounts for each nation on the globe. There is also need to create/evaluate research-based strategies for usage and/or management during specific illnesses, effectiveness, safe intake in various illnesses etc. Further studies should be conducted on Proteus to ascertain ascorbic acid antimicrobial activity as well as its minimum inhibitory concentration and minimum bactericidal concentration using other dilution factors with additional bacteria isolates (including both Gram positive and Gram negative strains).

Conflict of Interest

This study has no conflict of interest.

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