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ANTIBACTERIAL ASSESSMENT OF VANILLIC ACID AGAINST *ESCHERICHIA COLI*

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ABSTRACT

Phenolic compounds have been widely researched for their antibacterial activity throughout the years. Vanillic acid's antibacterial activity against Escherichia coli was investigated using a broth microdilution technique in 5ml sterile tubes. To get the minimum inhibitory concentration, the inocula was treated with vanillic acid in increasing quantities ranging from 150 g/ml to 2000 µg/ml in the tubes. The antibacterial activity of the phenolic compound was further investigated using time kill susceptibility. The lowest inhibitory concentration found was 900 g/ml. During the time kill susceptibility evaluation, a substantial reduction in viable bacteria cells was also detected. This study's findings support the antibacterial activity of phenolic compounds and the antibacterial potential of vanillic acid. Further extensive research on vanillic acid and other phenolic compounds is recommended to provide more insight on the mechanism of antibacterial activity. Adoption of these naturally occurring antibacterial compounds for treatment alternatives for infectious diseases caused by pathogenic bacteria may provide a solution to the pharmaceutical industry's multi-resistance conundrum.

Keywords: Vanillic, Antibacterial, Phenolic, MIC

INTRODUCTION

Antibiotic resistance in pathogenic bacteria species has prompted the search for plant-derived antimicrobial agents (Tanase et al. 2018). Vanillic acid, one of the major phenolic derivatives, can be found in a wide variety of edible plants and fruits (Folami et al. 2020).It is a benzoic acid derivative that is used in the food industry as a flavoring agent, preservative, and food additive(Sharma et al., 2020). Vanillic acid is a phenolic molecule that is an oxidized vanillin molecule. It is derived from a variety of cereals, including whole grains, herbs, fruits, green tea, juices, beers, and wines(Sharma et al., 2020). It has antimicrobial and pharmacological properties such as antioxidant, anti-inflammatory, immunostimulant, neuroprotective, hepatoprotective, cardioprotective, and antiapoptotic properties(Satpute et al. 2019; Sharma et al. 2020).

Escherichia coli is a Gram-negative, rod-shaped bacterium that belongs to the Enterobacteriaceae family and the Gammaproteobacteria class (Tenaillon et al. 2016). It is a medically important human and animal pathogen that causes severe diseases and significant economic loss (Roberta et al. 2020).Although it is found in feces of homeothermic animals because the gastrointestinal tract is its natural habitat, it is one of the world's leading causes of bloody-liquid diarrhea, which can lead to conditions such as anemia and colitis (Roberta et al. 2020).
One of the major concerns with microbial therapeutics is the persistent increase in pathogen resistance to antimicrobials (Oloyede et al. 2017). This resistance could be attributed to a variety of physiological and biochemical activities (Oloyede et al. 2017). However, research into the antibacterial potentials of phenolic compounds has yielded promising results in terms of offering therapeutic

alternatives against pathogens that cause infectious disease (Ajiboye et al. 2017; Oloyede et al. 2017; Folami et al. 2020). The purpose of this study was to determine the minimum inhibitory concentration of vanillic acid against *Escherichia coli.*

MATERIALS AND METHODS

Antibacterial Agent

Vanillic acid was obtained from AK Scientific Inc. in San Francisco, California, USA. The investigation into the minimum inhibitory concentration of vanillic acid against *Escherichia coli* included the use of various concentrations such as 150 µg/ml, 300 µg/ml, 450 µg/ml, 600 µg/ml, 750 µg/ml, 900 µg/ml, 1500 µg/ml and 2000 µg/ml. Ciprofloxacin was used as a reference antibiotic in the study at concentrations of 5 µg/ml and 10 µg/ml.

Test Organism

Escherichia coli was obtained from the Department of Medical Laboratory, Health Services Directorate, Olabisi Onabanjo University, Ago Iwoye, and grown at 37°C in Luria-Bertani Broth.

Media Preparation and Sterilization

Glassware was washed, rinsed, and sterilized in 70% ethanol. The workbench's surface was also disinfected with ethanol.Luria Bertani broth, MacConkey Agar, and Peptone Water were the media used in this study. They were measured per the manufacturer's instructions and sterilized for 15 minutes at 121°C.

MINIMUM INHIBITORY CONCENTRATION

The minimum inhibitory concentrations (MICs) of Vanillic acid were determined using a modified method described by Balouiri et al. (2016). In this study, a broth microdilution method with 5ml sterile tubes was used. To achieve the 0.5 McFarland standard, the inoculum was first incubated in peptone water at 37°C for two hours. The inoculum was mixed with vanillic acid in increasing concentrations ranging from 150 µg/ml to 2000 µg/mlin the tubes.As a control antibiotic, ciprofloxacin concentrations of 5µg/ml and 10µg/ml were used. Tubes containing 0.04% Dimethyl Sulfoxide (DMSO) with inoculum and plain broth without inoculum were used as positive and negative controls, respectively. The tubes were then incubated at 37°C for 24 hours. Based on the lack of turbidity in the sterile tubes, the MIC was expressed as the lowest concentration that inhibited growth. The turbidity of each tube was determined by comparing its clarity to that of the negative control.

vv

Minimum Bactericidal Concentration

The contents of the turbidity-free tubes were centrifuged and washed aseptically with normal saline to remove the vanillic acid and ciprofloxacin, then diluted with normal saline and sub-cultured on Muller Hilton agar using the pour plate method. The plates were examined after 24 hours of incubation at 37°C. The minimum bactericidal concentration was determined to be the concentration that completely inhibits colony formation (Ajiboye et al. 2017).

Time Kill Susceptibility of Bacteria Isolates

Absorbance reading

The bacteria isolate was grown overnight in Luria Bertani broth, then centrifuged and resuspended in 20 ml fresh LB broth (OD_{600} = 0.1) and grown aerobically at 37°C in 25ml sterile bottles. At mid log phase (OD_{600} = 0.5), vanillic acid (4 x MIC) was added and incubated for 3 hours at 37°C. Then, for the next two hours, a sample of treated culture was taken every 30 minutes to determine absorbance readings with a spectrophotometer (Ajiboye et al. 2017)

Colony Counting

Each sample of treated culture was centrifuged every 30 minutes for 2 hours to collect cells as pellets. The cells were then washed aseptically to remove the vanillic acid and ciprofloxacin, diluted with normal saline, and subcultured on Muller Hilton agar using the pour plate method. Colonies were counted after 24 hours of incubation at 37°C. (Ajiboye et al. 2017).

Data Analysis

Microsoft Excel was used to enter all of the data. To adequately express results, tables, graphs, and charts were used.

RESULTS

Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) used in this study was defined as the lowest concentration of vanillic acid that completely inhibits *Escherichia coli* growth after 24 hours of incubation at 37°C. Growth was inhibited in bacteria broth cultures containing concentrations of g/ml to µg/ml, and 2000 µg/ml vanillic acid. Growth was also inhibited in the study's broth culture containing ciprofloxacin concentrations. The MIC was determined to be g/ml to µg/ml vanillic acid concentration because it was the lowest concentration that inhibited bacteria growth. (Table 1).

Table 1: Minimum Inhibitory concentration of *Escherichia coli* with vanillic acid

Ciprofloxacin		Vanillic acid								DMSO	Plain Broth
Conc (µg/ml)	5	10	150	300	450	600	750	*900	1500	2000		
Observation	-	-	+	–	+	+	+	-	-	-	+	-

Key: *- MIC

Minimum inhibitory concentration was observed in broth culture containing 900 µg/ml of vanillic acid.

Minimum Bactericidal Concentration

The study's minimum bactericidal concentration (MBC) was defined as the lowest concentration among the growth inhibiting concentrations that produced no viable colonies. The broth culture containing concentrations inhibiting the growth of *Escherichia coli* was incubated overnight using the pour plate method to determine the minimum bactericidal concentration. Following incubation, viable colonies of *Escherichia* coli were found on all incubated plates, indicating that none of the concentrations completely killed the bacteria and thus no MBC was obtained.

Time Kill Susceptibility

Absorbance Readings

When compared to untreated cells, there was a significant decrease in viable bacteria cells treated with vanillic acid during the time kill susceptibility assessment. The values of the absorbance readings obtained at the 30-minute interval decreased as time increased (Figure 1). These readings were also comparable to those of the ciprofloxacin-treated cells, which served as the study's reference antibiotics.

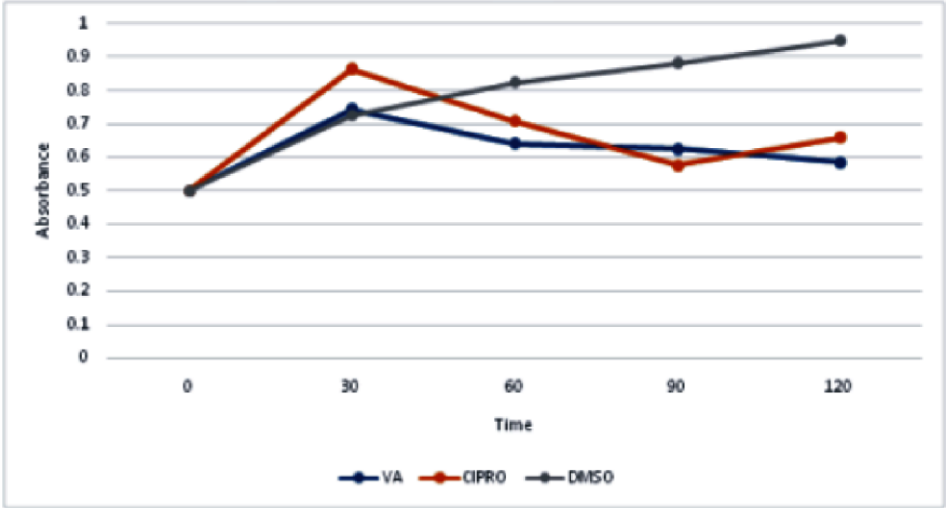

Figure1: Time kill susceptibility - Absorbance readings of *Escherichia coli*

Key: VA – Vanillic acid, CIPRO – Ciprofloxacin, DMSO - Dimethylsulfoxide

There was a decrease in the absorbance readings of cells treated with vanillic acid and ciprofloxacin at the end time intervals.

Colony Counting

Parallel observations were also made during the assessment of viable bacteria colonies: the number of bacteria colonies incubated after treatment with vanillic acid and ciprofloxacin decreased as treatment time increased, whereas the number of bacteria colonies in untreated cells (cells treated with DMSO) increased (Figure 2).

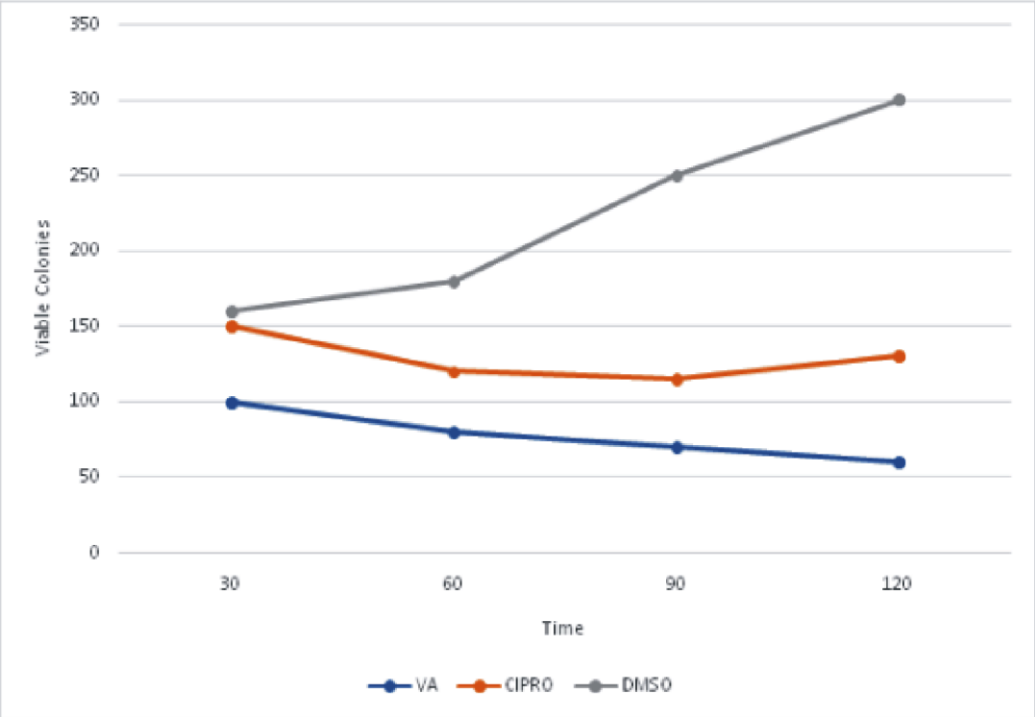

Figure 2: Time Kill Susceptibility- Number of viable colonies for *Escherichia coli*
Key: VA – Vanillic acid, CIPRO – Ciprofloxacin, DMSO - Dimethylsulfoxide

There was a decrease in the number of viable colonies treated with vanillic acid and ciprofloxacin at the end time intervals

DISCUSSION

Over the years, phenolic compounds' antibacterial action has been intensively researched and are considered to be the most active metabolites (Sharma et al., 2020). The essential feature of all phenols is the presence of one or more hydroxyl groups connected to the benzene ring, and vanillic acid is an example of a phenolic compound's benzoic acid derivative (Kim et al., 2010). According to Bernal-Mercado et al. (2018) and Sharma et al. (2020), vanillic acid has antibacterial activity at concentrations ranging from g/ml to µg/ml. Nonetheless, the least inhibitory concentration achieved in this investigation was 900 g/ml to µg/ml, which was somewhat higher than the minimum inhibitory concentrations reported in previous studies by Bernal-Mercado et al (2018) and Ibrahim et al (2020), which were 312.5 g/ml and 740 µg/ml, respectively. The discrepancy might be attributed to the study design and the surrounding environment.

The reduction in the number of colonies generated in bacteria cells treated with vanillic acid seen during the time kill susceptibility assessment suggests that the phytochemical progressively kills bacteria cells over time. Bacterial mortality caused by phenolic acids is thought to be caused by the formation of reactive oxygen species, which causes oxidative stress and, as a result, inhibits membrane function (Ajiboye et al. 2017). The observation made in this study corroborates previous studies that assessed the activity of phenolic compounds against variety of bacteria isolates (Ajiboye, et al. 2016; 2017; Oloyede et al. 2017). Vanillic

acid, along with other phenolic compounds, may offer a solution to the multi-resistance problem, however its mechanism of action in various microorganisms must be thoroughly investigated.

CONCLUSION

Vanillic acid was shown to have substantial antibacterial action against *Escherichia coli* in this investigation. This highlights vanillic acid's potential as a promising antibacterial agent for treating illnesses caused by bacterial infection, as well as its usage in the food sector as preservatives and surface disinfectants against pathogenic bacteria.

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