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Evaluation of Antimicrobial Activity of Prodigiosin Produced from *Serratia marcescens* against Some Pathogenic Bacteria.

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Abstract

In this work, the activities of prodigiosin produced by *Serratia marcescens*isolated from soil samples were evaluated against some pathogenic microorganisms. Spread plate method was used for its isolation on MacConkey agar while MacConkey broth was used for fermentation and production of prodigiosin. The production of prodigiosin was confirmed by its antimicrobial activity against *Streptococcus pyogenes, Klebsiellapneumoniae, Staphylococcus aureus*and *Pseudomonas aeruginosa.*The various dilution concentrations of the crude extracts were obtained by diluting the corresponding decimal percentage millilitre of the supernatant from the production broth into 10 ml peptone water already inoculated with the corresponding test organism. The Minimum inhibitory concentrations of prodigiosin against test organisms were determined.The highest zone of inhibition (22.33 mm) was observed with *Staphylococcus aureus*followed by*Streptococcus pyogenes*(20.67 mm), *Klebsiella pneumoniae*(16.67mm) and*Pseudomonas aeruginosa* (13.67 mm) in that order. At 70, 65, 50 and 45 % concentrations, prodigiosin was inhibitory to *Staphylococcus aureus*but not at 30 %. Prodigiosin was inhibitory to *Klebsiella pneumoniae*only at 70 % and 65 % concentrations while inhibitory to *Streptococcus pyogenes* at 70, 65 and 50 % concentrations. Prodigiosin was inhibitory to *Pseudomonas aeruginosa* at 70 % and 65 % concentrations only. Since prodigiosin produced marked inhibitory effects on these pathogenic organisms, it can be used to treat diseases caused by these organisms.

Keywords: Antimicrobial, Prodigiosin, pigment, metabolite, Pathogenic, *Serretia marcescens*.

1. Introduction

Microorganisms are found in every sphere and biome of life and if these organisms are left to keep proliferating without control measures, they would one day outgrow the human population. Any compound that inhibits or kills microorganism is said to have an antimicrobial activity. Antibiotics are produced by only a few genera of microorganisms (Anita, 2015). These antibiotics are an example of secondary metabolites and are produced at the stationary and death phases of growth.

Prodigiosin is a red pigment produced by many strains of the bacterium *Serratia marcescens*and other Gram negative gamma proteobacteria, collectively called prodiginines (Pankaj, Deepali, Isha, and Sheetal, 2015). *Serretia*species are opportunistic Gram negative bacteria classified in the group Klebsielleae and the large family Enterobacteriaceae (Huayda, 2015). *Serretia*species are wide spread in the environment but are not a common component of the human faecal flora (Darah Teh, Jain and Sheh-Hong, 2014). The chemical structure of prodigiosin has been elucidiated (Hejazi and Falkiner, 2012). *Serratia*are capable of surviving in diverse environment including water, soil and the digestive tract of various animals. *S. Marcescens*has a predilection for growth on starchy foodstuff where the pigmented colonies are easily mistaken for drops of blood (*Eleanor*, *Nurhafizah* and Fahrul , 2012).

*S. marcescens*can be identified in the laboratory by the use of methyl red test, of which they are negative. Their negation is due to their ability to produce 2, 3-butanediol and ethane. *S. marcescens*is positive to Voges-Proskaeur which shows their ability to convert pyruvate to acetoin (Anthony, Chandana, Senthilkumar and Narendra, 2011). In this research work, *Serratiamarcescens*was isolated from soil samples, cultured, used to produce prodigiosin and the antimicrobial effect of the produced prodigiosin was evaluated on some pathogenic microorganisms in a view to assess its efficacy as an alternative therapeutic agent.

2. Materials and Methods

2.1 Collection of Samples

Soil samples were collected within the environs of Daughters' of Mary Mercy Hospital Ahiaeke in Umuahia, Abia State, Nigeria. The soil samples were put in a plastic sterile zip lock bag and taken to the laboratory for analysis.

2.2 Collection of Test Organisms

The test organisms used were *Staphylococcus aureus, Klebsiellapneumoniae, Streptococcus pyogenes*and *Pseudomonas aeruginosa.* These organisms were collected from Standard isolates from previous student research works. Biochemical and confirmatory tests were run on isolates to authenticate their identities.

2.3 Biochemical Tests Run on Test Organisms

2.3.1 Catalase Test

This test is used to demonstrate the presence of the enzyme catalase. This enzyme breaks down hydrogen peroxide to water and oxygen gas. The oxygen gas escapes as bubbles. An overnight bacterial broth culture was picked using an applicator stick. The stick was then dipped into 3ml of freshly prepared hydrogen peroxide reagent in a test tube. The presence of gas indicated a positive result (Cheesbrough, 2006).

2.3.2 Voges-Proskaeur Test

Voges-Proskaeur (VP) test is a test used to detect acetoin in a bacterial broth culture. A bacteria broth of the inoculum was prepared in a test tube containing Voges-Proskaeur broth and alpha-naphthol was added followed by potassium hydroxide. A cherry red colour indicates a positive result, while a yellow-brown colour indicates a negative result (Cheesbrough, 2006).

2.3.3 Citrate Test

The citrate test differentiates bacteria according to their ability to use citrate as the sole source of carbon and energy. In this study, the bacteria isolate were inoculated on a medium containing sodium citrate. These colonies were picked up from the sub culture of the pure bacteria isolate using a straight wire and inoculated into slope of Simmons citrate agar and incubated overnight at 37^{o}C. A medium change in colour from green to bright blue indicates citrate utilization (Cheesbrough, 2006).

2.4 Preparation of Media

MacConkey agar, MacConkey broth, Peptone water, Mueller-Hinton agar and Centrimide agar were prepared following manufacturer's instructions and dispensed into the corresponding vessels i.e. MacCartney bottles for the broth and Petri dishes for the agar.

2.5 Isolation of *Serratiamarcescens*

After serial dilution of the soil sample, 1ml each of the 3rd and 4th tubes were plated out unto the gelled surface of MacConkey agar, using spread plate technique. The plates were incubated at 37^{O}C for 24 hours.

2.6 Confirmatory Tests Run on the Isolates to Authenticate their Identity as *Serretiamarcescens*.

2.6.1 Casein Hydrolysis

This test is primary based on the ability of an organism to utilize casein. Casein is a protein present in milk. So the ability of an organism to hydrolyse casein invariably means that the organism can grow in a medium incorporated with milk as its sole nitrogen source (Joanne, 2013). The hydrolization of casein by any organism allows the organism to produce extra-cellular metalloproteinase which aids in extracellular interactions.

2.6.2 Tryptophan Degradation

The test organism was cultured in a medium which contains tryptophan. Indole production was detected by Kovac's reagent which contains 4 (p)-dimethylaminobenzaldehyde. This reacted with the indole to produce a red coloured compound (Cheesbrough, 2006).

2.6.3 Citrate Degradation

Citrate is a ready carbohydrate and can be degraded by some microorganisms as their carbon source. *Serratiamarcescens* is an example of such microorganism and can readily

breakdown citrate into carbon for utilization in the generation of energy. This test is carried out using Simmons agar (Cheesbrough, 2006).

2.6.4 Methyl-Red

This test is used to determine if an organism is a mixed fermenter or not. A test tube containing MR-VP Broth with was inoculated with a pure culture of the test organism and incubated at 35°C for 4days and 5 drops of the methyl red indicator solution was added to the tube.A positive reaction is indicated, if the colour of the medium changes to red within a few minutes (Cheesbrough, 2006).

2.7 Presumptive Test for the Production of Prodigiosin

A 24 h old broth culture each of *Staphylococcus aureus, Klebsiellapneumoniae, Streptococcus pyogenes* and *Pseudomonas aeruginosa* was obtained. The organisms were streaked on the surface of gelled Mueller-Hinton agar plates. A sterile wire loop was then used to collect the colonies of *Serratiamarcescens.* The loop was then used to streak a straight line on the plates. The plates were then incubated at 37^{O}C for 24 hours. After the incubation of the plates, the presence of clearance along the line of *Serratiamarcescens* inoculation shows the presence of an inhibitory substance which inhibits the growth of the test organisms (Lorian, 1991).

2.8 Production of Prodigiosin.

After isolation of *Serratiamarcescens* using MacConkey agar, the inoculum was inoculated into MacConkey broth and incubated at 37^{O}C for 24 hours. After incubation, the broth was centrifuged to separate the supernatant from the residue. The supernatant was used for the antimicrobial assay, since it contained the prodigiosin.

2.9 Antimicrobial Assay for the Production of Prodigiosin (Agar Well Diffusion Method)

After centrifugation, an equal volume of ethanol was added to the supernatant to extract the prodigiosin and was then tested for antimicrobial activity using test organism on Mueller-Hinton agar plate sand incubated at 37^{O}C for 24 hours. After incubation, the plates were checked for the presence or absence of clearance and the corresponding zones of clearance, if present, were measured using a millimetre ruler. The agar well diffusion was done in triplicates (NCCLS, 1998).

2.9.1 Minimum Bactericidal and Inhibitory Concentration Determination

This test is done to determine at what minimum concentration, a given antibiotic is still effective. In this testing, after the production of the prodigiosin, the supernatant from the fermentative broth was separated out.

10 ml each of peptone water was obtained and put into MacCartney bottles. For each test organism, dilution percentages of 70, 60, 50, 30 and 20 % were made. This was done by adding the corresponding mil of the supernatant into the 10 ml of peptone water.. A wire loop

of the respective test organism was inoculated into the MacCartney bottle and then incubated (NCCLS, 1998).

After the incubation for 24 hours at 37^0C, the tubes were checked for the presence or absence of turbidity which is an indication of bacteriostatic activity. Those test tubes where turbidity were not observed were subjected to Minimum Bacteriocidal Concentration testing by adding 0.1ml of the broth culture unto Mueller-Hinton Agar plate which has already been inoculated with the test organisms. A clear zone of inhibition indicates bacteriocidal activity (NCCLS, 1998).

3. Results

Table 1 shows the morphological and biochemical test used to confirm the isolates as *Serratiamarcescens.* All the isolates were gram positive cocci, hydrolysed casein, degraded tryptophan and citrate and methyl red positive.

Table 1: Morphological and Biochemical Test Used to Confirm the Isolates as *Serratiamarcescens.*

S/N	Gram Reaction	Casein Hydrolysis	Tryptophan and Citrate	Methyl red Degradation
Isolate 1	Negative Rods	Positive	Positive	Positive
Isolate 2	Negative Rods	Positive	Positive	Positive
Isolate 3	Negative Rods	Positive	Positive	Positive
Isolate 4	Negative Rods	Positive	Positive	Positive
Isolate 5	Negative Rods	Positive	Positive	Positive
Isolate 6	Negative Rods	Positive	Positive	Positive
Isolate 7	Negative Rods	Positive	Positive	Positive
Isolate 8	Negative Rods	Positive	Positive	Positive
Isolate 9	Negative Rods	Positive	Positive	Positive
Isolate 10	Negative Rods	Positive	Positive	Positive

The zone of inhibition diameter produced on *Staphylococcus aureus, Klebsiellapneumoniae, Streptococcus pyogenes* and *Pseudomonas aeruginosa* by prodigiosin is as shown in Table 2. The highest zone of inhibition was observed with *Staphylococcus aureus* with a mean inhibition diameter of 22.33 mm followed by *Streptococcus pyogenes* with a mean inhibition diameter of 20.67 mm. This was closely followed by *Klebsiellapneumoniae,* with a mean inhibition diameter of 16.67 mm. The least was *Pseudomonas aeruginosa* with a mean inhibition diameter of 13.67 mm.

Table 2: The Zone Inhibition Diameter Produced on *Staphylococcus aureus, Klebsiellapneumoniae, Streptococcus pyogenes* and *Pseudomonas aeruginosa*by prodigiosin.

Test organism	Plate 1 (mm)	Plate 2 (mm)	Plate 3 (mm)	Mean diameter inhibition (mm)
Staphylococcus aureus	20	25	22	22.33
Klebsiella pneumonia	15	18	17	16.67
Streptococcus pyogenes	22	21	19	20.67
Pseudomonas aeruginosa	12	15	14	13.67

Table 3 shows the minimum bactericidal and inhibitory concentration of prodigiosin against *Staphylococcus aureus.* At 70, 65, 50 and 45 % concentrations, prodigiosin was inhibitory to *Staphylococcus aureus.* At 30 % concentration, prodigiosin was not inhibitory against *Staphylococcus aureus.* The minimum bactericidal concentration was 45 %.

Table 3: Minimum Bactericidal and Inhibitory Concentration of Prodigiosin against *Staphylococcus aureus.*

Test Organism	Percentage Dilution	Appearance of Broth	MBC
Staphylococcus aureus	70	Not turbid	
	65	Not turbid	
	50	Not turbid	
	45	Not turbid	45
	30	Turbid	

The minimum bactericidal and inhibitory concentration of prodigiosin against *Klebsiella pneumoniae* is shown in table 4. At 65 and 70 % concentrations, it was inhibitory to *Klebsiella pneumoniae* but was not inhibitory at 30, 45 and 50 % concentrations. The minimum bactericidal concentration was 65 %. **Table 4: Minimum Bactericidal and Inhibitory Concentration of Prodigiosin against *Klebsiellapneumoniae.***

Test Organism	Percentage Dilution	Appearance of Broth	MBC
Klebsiellapneumoniae	70	Not turbid	
	65	Not turbid	65
	50	Turbid	
	45	Turbid	
	30	Turbid	

Table 5 shows the minimum bactericidal and inhibitory concentration of prodigiosin against *Streptococcus pyogenes.* At concentrations of 70, 65 and 50 %, it was inhibitory to

Streptococcus pyogenes but was not inhibitory at 45 and 30 %. The minimum bactericidal concentration was 50 %.

Table 5: Minimum Bactericidal and Inhibitory Concentration of Prodigiosin against *Streptococcus pyogenes.*

Test Organism	Percentage Dilution	Appearance of Broth	MBC
Streptococcus pyogenes	70	Not turbid	
	65	Not turbid	
	50	Not turbid	50
	45	Turbid	
	30	Turbid	

Table 6 shows the minimum bactericidal and inhibitory concentration of prodigiosin against *Pseudomonas aeruginosa.* At 70 and 65 % concentration, it was inhibitory to *Pseudomonas aeruginosa* and showed no inhibitory activity at 50, 45 and 30 % concentration. The minimum bactericidal concentration was 65 %.

Table 6: The Minimum Bactericidal and Inhibitory Concentration of Prodigiosin against *Klebsiellapneumoniae.*

Test Organism	Percentage Dilution	Appearance of Broth	MBC
Pseudomonas aeruginosa	70	Not turbid	
	65	Not turbid	65
	50	Turbid	
	45	Turbid	
	30	Turbid	

4. Discussion

Natural based antimicrobial compounds have enormous therapeutic potential as they can serve the function without any side effect that are often associated with synthetic types (Mohammadi, Anijad & Mohammadi, 2011).

Serratiamarcescens were isolated from soil samples using standard microbiological techniques. The isolates were confirmed to be *Serratiamarcescens* by their positive results

to gram staining, casein hydroxylation, tryptophan and casein degradation and their negative result to methyl red staining. This is in accordance with what was reported in Prescott *et al.,* (2013).

Prodigiosin is a peptide antibiotics produced as a red pigmentation by *Serratiamarcescens.* The antimicrobial activity exhibited by prodigiosin was in concordance with Chandni *et al.,* (2012) which said that *Serratiamarcescens* produced such metabolites at their secondary stage of growth curve.

The efficacy of prodigiosin was tested against four pathogenic microorganisms. It displayed a good inhibitory activity as evident by the production of large zone of inhibition against the test organisms. In a similar work done by Bhathimi *et al.,* (2013), it was found that prodigiosin has an inhibitory property over most pathogenic microorganisms of public health importance such as *Staphylococcus aureus, Klebsiellapneumoniae, Streptococcus pyogenes*and *Pseudomonas aeruginosa.* Prodigiosin exhibited this antimicrobial property due to the presence of a pyrrolylpyrromethane ring skeleton that affects the bacterial ribosomal components and the chitin components of fungi cell wall.

The antibacterial activity revealed a greater inhibition towards Gram positives than Gram negetives. Similar findings were observed by Ramani, Nair and Krithika (2014) and Lapenda *et al*., (2015) which reported that Gram negative bacteria had higher resistance to the prodigiosin. This is probably due the presence of lipopolysaccharides in cell wall of Gram negative bacteria. Lipopolysaccharides of cell walls can prevent influx of active substances into cytopasmic membrane of these bacteria (McKeegan, Borges-Walmsley & Walmsley, 2002)

For an antibiotic to be completely regarded as effective, its minimum inhibitory concentration needs be known (Hugo & Russel, 2004). The minimum inhibitory concentration of the produced prodigiosin was determined using dilution ratio percentages of 70, 65, 50, 45 and 30 %. Most of the test organisms were inhibited even at low percentage concentration of 30%. *Pseudomonas aeruginosa* was strongly a negation to this assertion due to the low inhibition diameters observed with it. This may be due to what was reported by Chuang (2013) that *Pseudomonas aeruginosa* are more resistant to permeation by germicides and most inhibitory compounds due to the nature of their gram negative cell walls.

The result of this work shows that with the increase in the concentration of prodigiosin (30, 45, 50, 65 and 70 %), there was an increase in the antibacterial activity of prodigiosin which is exactly correlating with the result of Ramani, Nair and Krithika (2014) and Khanafaeri, Assadi and Fakhr (2006).

5.0 Conclusion

Serratiamarcescens isolated from soil samples produced a diffusible red pigmentation in the production medium. This diffusible pigment was found to be prodigiosin. Prodigiosin was observed to be antimicrobial in nature when tested on *Staphylococcus aureus, Streptococcus pyogenes, Klebsiella pneumonia* and *Pseudomonas aeruginosa.* The minimum inhibitory concentration percentage of prodigiosin on *Staphylococcus aureus*is was 45 %. Similarly, the percentage minimum inhibitory concentration of prodigiosin on

*Klebsiellapneumoniae*and *Pseudomonas aeruginosa*is 65 % and lastly for *Streptococcus pyogenes*is was 50 %. Since prodigiosin has a notable inhibitory activity on these pathogenic microorganisms, it can therefore, be effectively used to treat disease conditions arising from invasion by these pathogenic microorganisms.

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