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Methanolic extract of crude prodigiosin shows potential antibacterial activities *in vitro*

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Abstract
Prodigiosin is a natural pigment produced by *Serratia marcescens*. The colony of *Serratia marcescens* was grown in MacConkey agar, Nutrient Agar and Luria Bertani Agar. Excessive pigment was produced in Luria Bertani Agar when compared to MacConkey agar and Nutrient Agar. Antibacterial activity was evaluated using test organisms such as *E. coli* and *Staphylococcus aureus* by well diffusion method. The pigment was impregnated in the wells prepared on the surface of the test organism in different concentrations and incubated. The clear zone of inhibition formed around the well indicates antibacterial activities *in vitro*.

Keywords: *Serratia marcescens*; pigment production; prodigiosin; antibacterial activity *in vitro*

1. Introduction
The natural pigments are obtained from the two major sources plants and microorganisms. Phyto pigments have numerous drawbacks such as seasonal availability, Instability against light, heat, pH and low water solubility. Pigment production from microorganisms includes easy and fast production in culture medium and it is independent of weather conditions.
Prodigiosin is a secondary metabolite, a pyrrol dipyrromethane core structure mainly produced by *Serratia marcescens*. *Serratia marcescens* is gram negative bacilli belonging to the family Enterobacteriaceae which is motile and facultative anaerobe in nature. Prodigiosin has a tripyrrole in its structure belongs to the family prodiginines produced by Gamma proteobacteria eg. *Serratia marcescens* and some Actinomycetota eg. *Streptomyces coelicolor*. The production of prodigiosin is susceptible to temperature and is significantly inhibited at a temperature higher than 37 °C in *Serratia marcescens* and utilized to produce antimicrobial solution.

2. Aims and objectives
1. To extract the prodigiosin pigment from *Serratia marcescens*.
2. To demonstrate the antibacterial activity *in vitro* on gram positive and gram negative bacteria.

3. Materials and Methods
3.1 Collection of Sample
Soil samples (10 Nos.) were collected from various regions in Agartala city in April 2023. The soil samples were collected from a depth of 2 - 3 inches using a spatula and stored in clean polybags and transported to the lab. (Mahmood, 2007)

3.2 Isolation and Identification of *Serratia marcescens*
One gram of each sample was added to 10 ml of sterilized normal saline solution in test tubes and mixed thoroughly (Zbar, 2011). Serial dilutions for each sample were achieved, and then 100 µL aliquots from the appropriate dilution were spread on Nutrient Agar plates. After incubation at 30ºC for 18-24 hrs, red pigmented colonies were selected for further subculture on MacConkey Agar, Nutrient Agar and Luria Bertani Agar for pure isolation and identification. For identification of *Serratia marcescens*, several biochemical tests were done such as Catalase (+ve), Oxidase (-ve), NLF, Motility (+ve), Citrate Utilization (+ve), Urease (-ve). Apart from this, Microscan Walk Away 40 (SIEMENS) was used for confirmation.

3.3 Bacterial Inoculum Preparation
A standardized inoculum was prepared in nutrient broth by growing *Serratia marcescens* to the turbidity of the 0.5 McFarland standard.

Cultures adjusted to the 0.5 McFarland standard contain approximately 1.5 × 108 CFU/ml.

3.4 Koch's Plating Technique [4]
The inoculum was streaked on MacConkey Agar, Nutrient Agar and Luria Bertani Agar by 'Koch's Plating Technique' i.e. preparation of a well with a sterile plastic disposable loop, then a primary streak from the well, a secondary streak from the primary one and so on up to tertiary streaking ending in a tail end [4].
The petridishes were kept inside the incubator at 30 °C for 48 hours. After 48 hrs, the plates were observed to have yellow coloured colonies on MacConkey Agar and pinkish red coloured colonies on Nutrient Agar and Luria Bertani Agar.

Fig 1: *Serratiamarcescens* on Luria Bertani Agar

3.5 Prodigiosin production on MCA
These colonies from the MacConkey Agar were inoculated into LB broth and kept in CO_2 incubator in room temperature for 48 hrs. After 48 hrs, the prodigiosin pigment was observed.

3.5.1 Extraction of prodigiosin from MacConkey Agar
Serratia marcescens in MacConkey agar was centrifuged at 8000 g for 30 min. The Cell debris was formed in low concentration.

3.6 Prodigiosin production on Luria Bertani Agar
The plate was placed at room temperature for 48 hrs. After 48 hrs, the plate was observed to have pinkish red coloured colonies. LB broth was taken and pinkish red coloured colonies were inoculated into the LB broth and kept in room temperature for 48 hrs. After 48 hrs, the prodigiosin pigment was produced in the broth.

3.6.1 Extraction of prodigiosin from Luria Bertani Agar
Serratia marcescens in Luria Bertani broth was centrifuged at 8000 g for 30 min. The supernatant was discarded and the cell pellets were collected. The acidified methanol was added with the extracted cell pellet and was centrifuged at 5000 g for 15 min. The supernatant was collected in a tube and placed in a bacteriological incubator at 60 °C for 48 hrs to obtain a crude pigment.

Fig 2: Acidic Methanolic Extraction of Prodigiosin

4. Estimation of prodigiosin from Luria Bertani Agar
The cell absorbance of the bacterial culture was measured at 620 nm. For the pigment absorbance, the broth was mixed with methanol, subjected to centrifugation. The supernatant was used for the measurement of absorbance at 534 nm. The pigment prodigiosin was estimated by the formula [5].

$$\text{Prodigiosin (U/C)} = \frac{[OD_P - (1.381 \text{ X } OD_C) \text{ X } 1000}{OD_C}$$

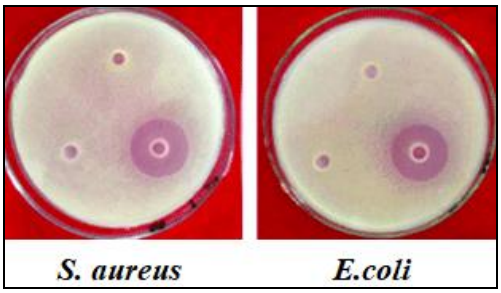
$$\text{Prodigiosin (U/C)} = \frac{[OD_{534} - (1.381 \text{ X } OD_{620}) \text{ X } 1000}{OD_{620}}$$

[OD_P = pigment absorbance; OD_C = bacterial cell absorbance; 1.381 = constant]

The pigment prodigiosin was estimated by the following formula and it was found to be 36.7 units/cell.

5. Antibacterial Activities *in vitro*
One gram negative (*Escherichia coli*) and one gram positive (*Staphylococcus aureus*) bacteria were used for the evaluation of antibacterial activities *in vitro*.
The antibacterial activity of the pigment was carried out using agar well diffusion method separately. The test bacteria such as *Escherichia coli* and *Staphylococcus aureus* were inoculated on the MHA plates. 5 µL, 10 µL, 15 µL and 20 µL and 25 µL of methanolic extracts of this pigment was impregnated in the wells prepared on the surface of test bacteria and incubated overnight at 37 °C [5].

6. Results
The clear zones of inhibition of varying diameters were observed with different volumes of the extracted pigment used after incubating the plates at 37 °C for 48 hrs [5].



7. Discussion

The results of extraction of prodigiosin from *Serratia marcescens* and demonstration of antibacterial properties on two bacterial isolates, obtained in this study, imply that the growth rate of the prodigiosin pigment is higher in LB Agar when compared with MacConkey Agar.

Incubation of MacConkey Agar plates under increased CO_2 has been observed to reduce growth of microorganism. The pigment from MacConkey Agar was obtained in lower concentration. The antibacterial activity of the pigment from LB Agar was estimated and checked by well diffusion method on *E. coli* and *Serratia marcescens* in different concentrations. In *E. coli* the antibacterial activity started at a concentration of 15 µL and above, whereas, the inhibition activity started fro 5 µL, in case of *S. aureus*. This study showed that the pigment prodigiosin can be considered as a possible alternative source of antibacterial solution *in vitro.*

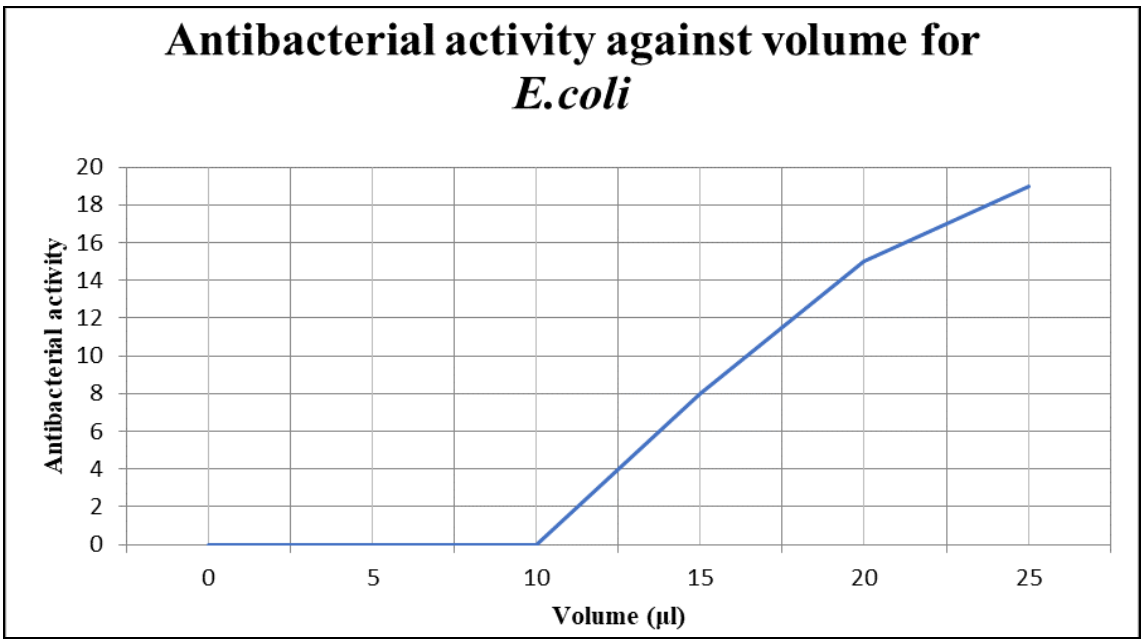


Fig 3: Graph showing Antibacterial Activity against *E. coli*

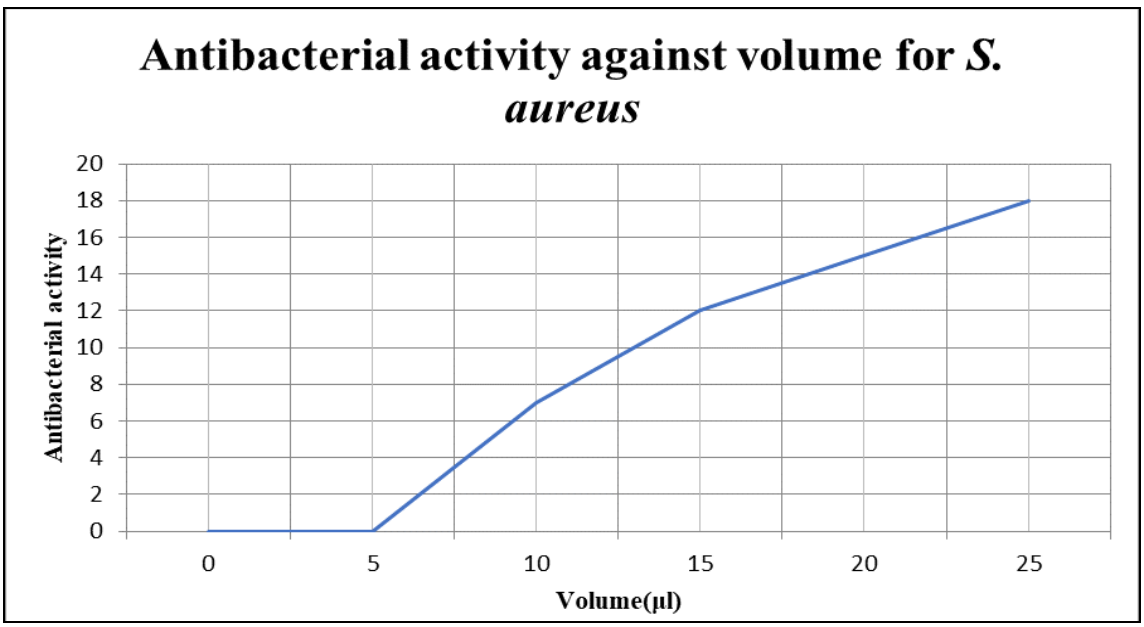


Fig 4: Graph showing Antibacterial Activity against *S. aureus*

8. Conclusion

The pigment prodigiosin extracted from the gram negative bacteria *Serratia marcescens* showed *in vitro* susceptibility against the gram negative and gram positive bacteria with more inhibitory activity against gram positive ones, but further research works are required for in depth study before its application for the human being.

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10. Conflict of Interest
Not available

11. Financial Support
Not available

12. References
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