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PHARMACEUTICAL POTENTIAL OF FLEXIRUBIN PIGMENT FROM CHRYSEOBACTERIUM

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ABSTRACT: The study was carried out to isolate and characterize pigment producing bacteria from rhizosphere soil. In our study, isolation and characterization of bacteria that produced pigments were performed using serial dilution, spread plating method followed by gram staining and various other biochemical tests (Oxidase, Catalase and IMViC tests). Our study concluded that bacteria isolated from rhizosphere soil are capable of producing pigment which is flexirubin (light orange in colour) and the bacterium belongs to the genus Chryseobacterium. After extracting the pigment, antimicrobial activity was performed, and it showed that the pigment has antimicrobial activity against both bacteria (E. coli and B. sphaericus) and fungi (Candida albicans). At 100µg/ml concentration, the pigment showed highest antioxidant activity with an absorbance of 0.116AU. The anti-inflammatory activity of the pigment was found to be highest at 1200µg/ml with an absorbance of 0.128AU. It is found that the pigment has the ability to increase SPF property of commercially available sunscreen by 0.713%. Thus different potentials of the pigment can be applicable in various industrial (pharmaceutical, textile, food and cosmetic) purposes.

INTRODUCTION: The desire for pigments as a natural color in food, medications, cosmetic, textile, and printing dye industries has prompted a burgeoning interest in natural pigments due to growing awareness of the danger of synthetic colors. Natural pigments are increasingly being used in the creation of food, colors, cosmetics, and pharmaceutical products. Because they are nontoxic, non-carcinogenic, and biodegradable by nature, natural colorants and dyes originating from flora and wildlife are thought to be safe ¹. Despite having low market, they are regarded for their safe use as a natural alternative to synthetic food dyes.



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Therefore, it is crucial to investigate the potential of various natural sources of food-grade pigments ². Microbial pigments are among the natural pigments that could provide acceptable substitutes for manmade colors. Microbial pigments are considered natural, quickly developing, do not present seasonal production issues, and exhibit great productivity makes them a possible replacement for other color additives derived from vegetables and fruits ³.

Natural pigments are reasonably priced, Environment -friendly, compostable - recycling is not a problem and they are in harmony with nature ⁴. It is very important to investigate the potential of microbial pigment, to use it in various industries. The antimicrobial activity of the pigment is tested by antibacterial and antifungal assay ⁵. Both gram positive and gram-negative bacteria are susceptible to antimicrobial effects of bacterial pigment ⁶. The antioxidant activity was determined using ferric

reducing antioxidant power assay simply means the reduction of ferric ion to ferrous ion 7. The growing interest in anti-inflammatory foods (green tea, oily fishes, blueberries, etc.) accelerated the research on naturally occurring pigments with inflammatory properties. Examples of natural pigments with anti-inflammatory characteristics are anthocyanin, curcumin, quercetin and pigments from monascus, etc 8. Bacterial pigments also show photoprotective activity and can be used in skin protection creams, soaps, lotions etc. Several bacteria that block UV light also have antibacterial and antioxidant properties ⁹. Here is the study about extraction of pigment, estimation antioxidant, anti-inflammatory antimicrobial, activity and SPF of the pigment produced by bacteria isolated from rhizosphere soil.

Review of Literature: The soil seen attached to the root part of the plant is called rhizosphere soil which is abundant in microorganism populations ¹⁰. Pigments are vibrant secondary metabolites created by bacteria ¹¹. The pigmented bacteria are a huge field of interest because of their Chemical use, effectiveness, and specific qualities, such as antifungal. anti-cancer. and immunological suppression, which have been reported so far in various research ¹⁰. One example for pigment producing bacteria are Chryseobacterium. The carotenoid pigment produced by Chryseobacterium species is called flexirubin and these species can be isolated from rhizosphere soil ¹¹. Natural pigments are reasonably priced, have pharmacological impacts and are composed of Environment-friendly materials³. Bacteria can be a good alternative source for the creation of synthetic pigments due to their short life cycles and accessibility of genetic alteration methods ⁹. The antibacterial activity of bacterial pigments was more efficient against gram negative pathogenic strains than gram positive ¹⁰. The antioxidant assay results indicated IC₅₀ which is defined as the concentration of antioxidants needed to reduce 50% of the starting concentration of ferric thiocyanate ¹². The anti-inflammatory effect of the pigment was increased with an increase in concentration ¹³. A lot of people use sunscreen to shield themselves from dangerous UV radiation. Many are switching to natural sunscreen creams because of the negative side effects of synthetic photo-protective products, such as acne, itching, burning, skin redness etc. Researchers also concentrate on the identification of innovative and reasonably priced natural sunscreen agents due to the growing awareness and demand for natural sunscreens ¹⁴.

Objectives: The objective of the current study is to isolate and characterize the pigment producing bacteria from rhizosphere soil and to determine the antimicrobial, antioxidant, anti-inflammatory and Sun Protecting Factor (SPF) of the pigment produced by the bacteria.

MATERIALS & METHODS: All the experiments required for the study was done in Bioroot Exploration India Pvt Ltd, Thiruvananthapuram, Kerala, in the year 2023. The required chemicals were bought from different companies like Himedia, Kanton laboratories, NICE, SRL etc.

Sample Collection: The rhizosphere soil was collected from the garden area of Bioroot in 2023. The soil sample was serially diluted and spread plate was done for last test tubes of serial dilution on nutrient agar (NA) plate. After 48 hrs of incubation at 37°C, the colonies with different pigment production were selected and pure culture was obtained on nutrient agar plate. The colony with light orange colored pigment was further studied for identification of bacteria.

Gram Staining: To a drop of sterile water placed on a clean slide, a single colony was spread to make a thin smear, air dried and heat fixed over a gentle flame. The smear was flooded with primary stain crystal violet, kept for 1 minute (min) and washed off. Then Gram's iodine was added to the slide, after 1 min, rinsed the slide with water. Then immediately Gram's decolourizer was added drop by drop. Again after 5 seconds (sec) the slide was rinsed with water. Finally, the slide was flooded with counter stain safranin for 30 sec. Washed off the stain, air dried the slide and observed under microscope.

Biochemical Characterization:

Catalase Test: One drop of hydrogen peroxide (H_2O_2) was placed on a clean glass slide and a loop full of bacterial culture was mixed with it. The formation of bubbles indicated positive result for catalase test.

Oxidase Test: To determine the ability of the bacteria to release cytochrome oxidase enzyme, oxidase disc test was performed. A loopful of subcultured colony from NA plate was spotted onto the oxidase disc placed on petri plate. A color change to deep blue, recorded within 10 secs, indicated a positive result.

Indole Test: To a test tube containing 4 ml distilled water (DW), tryptone (0.04g) and sodium chloride (NaCl) (0.02g) was added and autoclaved at 121°C, 15lbs for 15 mins. A loop full of culture was then inoculated into the mixture and incubated at 37°C for 24hrs in incubator. Later Kovac's indole reagent was added, and the presence of pink colored ring indicated the formation of indole.

Methyl Red (MR) Test: To a test tube, containing 4 ml DW, MR-VP medium (0.06g) was added, mixed well and autoclaved at 121°C, 15lbs for 15 mins. After 24hrs of incubation, methyl red indicator was added and the formation of red color indicated positive result.

Voges Proskaeur (VP) Test: To a test tube, containing 4 ml DW, MR-VP medium (0.06g) was added, mixed well and autoclaved at 121°C, 15lbs for 15 mins. Barritt's reagent A followed by Barritt's reagent B were added to the test tube and the formation of red colour indicated positive result.

Citrate Test: To a test tube containing 4 ml DW, Simmon's citrate agar (0.1g) was added and autoclaved at 121°C, 15lbs for 15 mins. The test tube was then inoculated with the sample and incubated at 37°C for 24hrs in the incubator. The result was observed after 24hrs of incubation and the presence of deep blue coloration indicated positive result.

Extraction of Pigment: The culture was inoculated in an agar plate and then the colonies were taken using an inoculation loop and mixed in methanol for methanol extraction and centrifuged at 4000 revolutions per minute (rpm) for 15 mins and kept for 24 hrs for methanol to get evaporated and the crude pigment obtained was used for further analysis.

Analysis of Anti Microbial Activity of Pigment: The method here followed was Agar well diffusion method, for this 8 number of autoclaved petriplates were taken and 100 ml of NB medium was prepared and 40 ml of potato dextrose agar (PDA) medium was prepared and swabs were also made and were autoclaved at 121°C, 15lbs for 15 mins. The media was poured onto sterile petriplates and kept for solidification. The colonies taken for antimicrobial activity test were 2 bacteria which were (Escherchia coli) E. coli and Bacillus sphaericus (B. sphaericus) and 1 fungi namely Candida albicans (C. albicans) and they were inoculated in 2 test tubes containing 5 ml NB media each for both bacteria and 1 test tube containing 5 ml PDA media for fungi and were incubated at 37°C for 24 - 48 hrs. After the incubation, agar well diffusion method was performed. The microbes were swabbed from 48 hrs grown broth and spread on respective plates. Three wells were made on each plate, one well for positive control - gentamicin and one well for negative control which - DW and last well for the sample (extracted pigment). Then kept for incubation at 37°C for 24 - 48 hrs. Formation of zone of inhibition around the sample showed that the pigment has antimicrobial activity.

Determination of Antioxidant Activity of the Pigment:

Frap Assay: FRAP was the simplest method that was used to detect the antioxidant property of the sample. The extracted pigment was dissolved in 2.5ml sodium phosphate buffer (PBS) with pH 6.6 in different concentrations such as 25µg/ml, $50\mu g/ml$, $75\mu g/ml$, $100\mu g/ml$. 2.5ml freshly prepared 1% of potassium ferric cyanide solution was added to the mix and the solution was thoroughly mixed well and was kept in water bath for 20 mins at 50°C. After the incubation period the solution was treated with trichloroacetic acid (TCA) and shaken well. After that 0.5ml of 0.1% Ferric chloride solution was added and incubated at RT for 10 mins. The absorbance of the sample was measured at 700nm using a spectrophotometer. Ascorbic acid was used as positive control.

Determination of Anti-Inflammatory Activity of the Pigment:

Protien Denaturation Assay: Anti-inflammatory activity was determined using Protein Denaturation Assay by measuring the absorbance of the sample. 1 ml of 1% Bovine Serum Albumin (BSA) was

added along with PBS into different concentrations of the extracted pigment ($800\mu g/ml$, $1000\mu g/ml$, $1200~\mu g/ml$). Later it was incubated at room temperature (RT) for 15 mins and then kept in water bath for 5 min at 70° C. Later the turbidity was measured using a spectrophotometer at 660 nm. Diclofenac was used as positive control. The percentage (%) inhibition of denaturation was calculated using the following equation.

% inhibition of denaturation = $(1-(Vt/Vc)) \times 100$

Where Vt = Absorbance of test sample, Vc= Absorbance of control.

Estimation of Photoprotective Activity of the Pigment: The ability of the bacterial pigment to increase the Sun Protection Factor (SPF) of sunscreens was measured using the following method. 0.1g of the commercial sunscreen was mixed with 10ml of ethanol (70%) and 10% (v/v) of the crude bacterial pigment and stirred for 5 mins at 500 rpm using a magnetic stirrer.

This was then filtered using a muslin cloth and the initial few drops were discarded. Absorbance of each of these solutions was then measured in the UV range (290 to 320 nm) at 5-nm intervals using ethanol as blank. Commercial sunscreen of SPF30 without any added pigment was taken as control. The SPF was calculated using the following equation:

SPFspectrophotometric = CF $\times \sum 320290 \text{ EE } (\lambda) \times I (\lambda) \times \text{Abs}$

Where, CF - correction factor (10), EE-erythemal effect spectrum, I-solarintensity spectrum, Abs (l) - absorbance of the test solution. The values of (EE x I) are constants and were obtained from literature and given below in **Table 1.**

TABLE 1: NORMALIZED VALUES OF $EE \times I$ AT DIFFERENT WAVELENGTHS

Wavelength (nm)	EE×I (Normalized
290	0.015
295	0.0817
300	0.2874
305	0.3278
310	0.1864
315	0.0839
320	0.018

RESULTS & DISCUSSION:

Serial Dilution, Spread Plating & Subculturing: 1g rhizosphere soil sample was weighed and transferred into the first test tube (10⁻¹) of a series of 8 test tubes and mixed well. Later 1 ml from first test tube was transferred into the second test tube (10⁻²) and mixed well. The same was repeated till 10⁻⁸ and 1 ml was discarded. 100 microliters (µL) from three different dilutions (10⁻⁶ and 10⁻⁸) were spread onto NA plates (spread plating). The plates were then kept at room temperature (RT) for 48 hours (hrs). After 48 hrs, the culture plates were observed. Bacterial colonies of different morphology were picked and streaked onto NA using an inoculation loop. The plates were then kept in an incubator at 37°C for 24 hrs (subculturing).







FIG. 2:- GRAM STAINING OF PIGMENT

TABLE 2: BIOCHEMICAL CHARACTERIZATION OF PIGMENT PRODUCING BACTERIA

Table	Observation	Inferences
Oxidase test	Purple colour	Positive
Catalase test	Bubbles formed	Positive
Indole test	Yellow ring	Negative
MR test	Red colour	Positive
VP test	Yellow colour	Negative
Citrate test	Green colour	Negative

Extraction of Pigment: The pigment was extracted by methanol extraction method, and showed orange in color assumed as flexirubin pigment which was produced by *Chryseobacterium*.



FIG. 3: EXTRACTED PIGMENTS

Analysis of Anti Microbial Activity of Pigment: By agar well diffusion method, the pigment showed better antibacterial activity against *E. coli* than *B. sphaericus* and antifungal activity against fungi *C. albicans*. The amount of antimicrobial activity is determined according to the diameter of zone of inhibition observed.

This showed that the pigment has better antibacterial and anti-fungal activity. The zone of inhibition was high against *E. coli* and fungi *C. albicans*, whereas the zone was less against *B. sphaericus*. The positive control (PC) used was gentamicin, negative control (NC) used was distilled water.

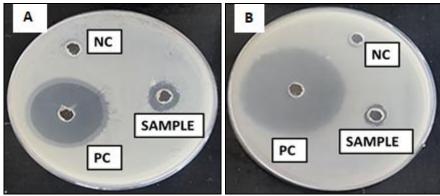


FIG. 4: ANTI MICROBIAL ACTIVITY OF THE PIGMENT AGAINST (A) E. COLI AND (B) B. SPHAERICUS

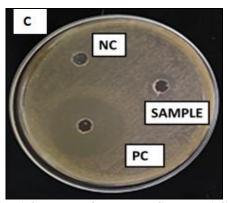


FIG. 5: ANTI-MICROBIAL ACTIVITY OF THE PIGMENT AGAINST (C) C. ALBICANS

TABLE 3: ANTI-MICROBIAL ACTIVITY OF THE PIGMENT AGAINST E. COLI, B. SPHAERICUSAND C. ALBICANS

ALDICANS			
Samples	Zone of inhibition (mn)		
_	EE. coli	B. spearicus	C. albicans
Gentamycin (PC)	33mm	42mm	35mm
Distilled water (NC)	0mm	0mm	0mm
Sample (pigment)	14.5mm	10.5mm	14mm

Determination of Antioxidant Activity of the Pigment Using Frap Method: The antioxidant activity denoted by absorbance value kept on increasing from different concentrations 25µg/ml,

 $50\mu g/ml$, $75\mu g/ml$, $100\mu g/ml$ and showed that the pigment has antioxidant activity and is comparatively high at $100\mu g/ml$ concentration as shown in **Table 4** and **Graph 1**.

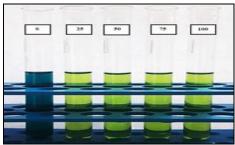
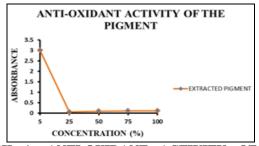


FIG. 6: ANTI-OXIDANT ACTIVITY OF THE PIGMENT AT DIFFERENT CONCENTRATIONS (25, 50, 75, 100 $\mu g/ml$) OF THE SAMPLE AND THE STANDARD (S)

TABLE 4: ANTI-OXIDANT ACTIVITY OF THE PIGMENT AT DIFFERENT CONCENTRATIONS

Concentration (µg/ml)	Absorbance (700nm)
Standard	3
25	0.060
50	0.098
75	0.112
100	0.116



GRAPH 1: ANTI-OXIDANT ACTIVITY OF THE PIGMENT AT DIFFERENT CONCENTRATIONS (25, 50, 75, 100 µg/ml) AND STANDARD (S)

Determination of Anti-Inflammatory Activity of Pigment:

Protein Denaturation Assav: The Anti-Inflammatory activity denoted by % inhibition of denaturation kept increasing from on concentrations $800 \mu g/ml$, $1000 \mu g/ml$ and 1200µg/ml respectively and showed that the pigment has anti-inflammatory activity and is high at 1200µg/ml as shown in **Table 5** and **Graph 2**.

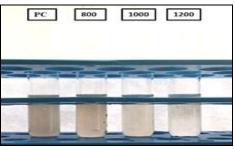
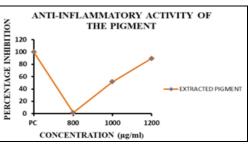


FIG. 7: ANTI-INFLAMMATORY ACTIVITY OF THE PIGMENT AT DIFFERENT CONCENTRATIONS. (800 μ G/ML, 1000 μ g/ml, 1200 μ g/ml) AND POSITIVE CONTROL (PC)

TABLE 5: ANTI-INFLAMMATORY ACTIVITY OF THE PIGMENT AT DIFFERENT CONCENTRATIONS

Concentration (µg/ml)	Absorbance (nm)	Percentage inhibition (%)
PC	1.211	100
800	1.191	1.7
1000	0.585	54.7
1200	0.128	89.5



GRAPH 2: ANTI-INFLAMMATORY ACTIVITY OF THE PIGMENT AT DIFFERENT CONCENTRATIONS. (800μg/ml, 1000μg/ml, 1200μg/ml) AND POSITIVE CONTROL (PC)

Estimation of Photoprotective Activity of the Pigment: The result was calculated in terms of SPF value for the sample and was found to be 35.2352, whereas for the control it was found to be 34.5218. The pigment showed SPF activity with an increase of 0.713% Compared to commercially available sunscreen. The absorbance obtained was as follows:

TABLE 6: ABSORBANCE VALUE OF CONTROL, PIGMENT AND EEXI AT DIFFERENT WAVELENGTHS

Wavelength (nm)	EE×I	Absorbance control	Absorbance of sample
290	0.015	3.470	3.535
295	0.0817	3.466	3.536
300	0.2874	3.458	3.529
305	0.3278	3.454	3.524
310	0.1864	3.447	3.521
315	0.0839	3.423	3.498
320	0.018	3.400	3.467

DISCUSSION: The study was conducted to isolate and identify pigment producing bacteria from

rhizosphere soil and to perform various assays of the pigment. Serial dilution and spread plating were

done to isolate the bacterial colony with pigment production. Light Orange colored colonies were isolated and subcultured, gram staining and biochemical tests were performed and confirmed the colony as *Chryseobacterium* sps. The bacterial isolate was found to be gram-negative, rod-shaped bacteria and the biochemical tests (Catalase, Oxidase and IMViC tests) showed positive results for Catalase, Oxidase, MR & negative results for Indole, citrate tests and VP tests. In 2003 the presence of *Chryseobacterium* in soil was confirmed using gram staining and biochemical tests with similar results 15. The pigment was extracted using methanol and was identified as flexirubin. In 2022 ³ and in 2020 ¹⁶ researchers performed the isolation of pigment producing bacteria from different soil sources earlier.

The antimicrobial activity performed by agar well diffusion method showed that the extracted pigment had antimicrobial activity against E. coli, B. sphaericus & C. albicans. The pigment showed high antimicrobial activity against E. coli with a zone of inhibition as 14.5mm. Previously in 2019 17 the antimicrobial activity of the pigment isolated from soil bacteria was also performed. FRAP method was used to estimate the antioxidant activity of the pigment and was found to be increasing with increasing concentration, the antioxidant activity was high at 100ug/ml concentration with an absorbance value of 0.116AU. Previously the antioxidant activity of the Chryseobacterium pigment from spp performed in 2021 18.

The anti-inflammatory activity of the pigment was determined by protein denaturation assay. The pigment had high anti-inflammatory activity at $1200\mu g/ml$ conc. with a percentage inhibition of 89.5%. Determination of anti-inflammatory activity of the pigment from different bacteria was previously done in 2018^{-19} . The SPF activity of the pigment was determined using ethanol dilution method and the pigment has the potential to increase the SPF activity of commercially available sunscreen by 0.713% which is similar to the work performed in 2017^{-20} .

CONCLUSION: Experiments were carried out to isolate and identify pigment producing bacteria from rhizosphere soil using serial dilution and

spread plating. By gram staining and biochemical tests the bacteria and the pigment was identified as *Chryseobacterium* and flexirubin respectively. The pigment was extracted using methanol as solvent. The pigment showed antimicrobial activity against both bacteria and fungi. The pigment possesses both antioxidant and anti-inflammatory activities. The pigment also has the potential to enhance SPF of commercially available sunscreens.

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