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ANTI MRSA AND ANTITUBERCULAR ACTIVITY OF PHENOXAZINONE CONTAINING MOLECULE FROM BORRA CAVES *STREPTOMYCES* SP. BCA1

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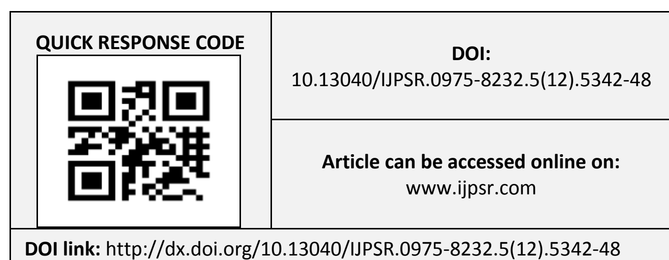
ABSTRACT: An attempt was made to study the anti MRSA and antitubercularactivity of *Streptomyces* sp. BCA1 isolated from Borra Caves soil. In agar plug method, strain BCA1 showed 12-17 mm inhibition against Gram positive and Gram negative bacteria, including MRSA. Crude pigment produced from strain BCA1 by agar surface fermentation showed 15 – 17 mm inhibition in disc diffusion method against bacterial pathogens. In luciferase reporter phage (LRP) assay the crude pigment showed more than 80% reduction against standard strain *M. tuberculosis* H37Rv, drug sensitive and multi drug resistant (MDR) clinical isolates of *M. tuberculosis.* Active pigment was separated by TLC and its activity was confirmed by bioassay guided fractionation. The MIC value of the purified pigment against bacterial pathogens ranged between 6.25 and 50µg/ml. MIC value for *M. tuberculosis* H37Rv and drug sensitive clinical isolate was found to be 6.25 µg/ ml while that of MDR *M. tuberculosis* isolate was 25 µg/ ml. The solubility of purified pigment in acid and alkali as well as the appearance of absorption peak at 440 nm in UV spectral analysis revealed that the active pigment contains the phenoxazinone chromophore. Strain BCA1 was found to produce pigment and exhibited bioactivity even when grown at a low nutrient concentration (1/10X) YEME agar. This study, for the first time, reported an antitubercular pigment from actinobacteria isolated from Indian Caves

INTRODUCTION: Need for cure from infectious diseases is highly warranted. Molecules discovered from natural resources yielded an impressive number of antibiotics to treat human diseases. Actinobacteria – group of gram positive filamentous bacteria – are the major microbial producer of bioactive secondary metabolites including antibiotics which are in the market today.



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Till date around 50% of microbial bioactive metabolites are isolated from less than 1% of the total culturable actinobacteria recovered from various ecosystems[1]. In recent decades, due to the increase in the reisolation of actinobacteria from known ecosystems, the rate of isolation of known metabolites from them is also increasing. Searching of rare/unexplored ecosystems may lead to the isolation of novel actinobacteria which in turn produce novel bioactive metabolites [2, 3, 4].

Caves are the nutrient, light intensity and temperature–limited, but humidity rich environments. They contain low levels of available organic carbon to support diverse microbial communities[5]. Caves can be considered as extreme

environments for life and provide ecological niches for highly specialized microorganisms. Microorganisms in caves are the biological habitant and remarkably contribute to cave ecology[6]. The practical applications of cave microbiology include the preservation of historical monuments and sculptures, through the identification of microbial species that can precipitate protective calcite coatings and the potential to produce bioactive substances [7].

It is known that actinobacteria are dominant among the heterotrophic bacterial population present in caves [8]. However, only limited works are carried out on actinobacteria from Indian caves in terms of biodiversity and bioprospecting. During the course of our microbial bioprospecting programme, actinobacterial cultures were isolated from Borra caves soil. This study reports the antibacterial and antitubercular activity of extracellular pigment produced by one of the actinobacterial strain BCA1 isolated from Borra cave soil.

MATERIALS AND METHODS:

Description of Borra caves: The Borra Caves are the second largest caves in the Indian subcontinent situated in the Araku Valley, approximately 90–95 km from Visakhapatnam ($18^0 5' N$; $83^0 3' E$), Andhra Pradesh. It lies in the forest area and consists of 14 villages inhabited by tribes in the Ananthagiri Mandal of Visakhapatnam district. It is also one of the important hill regions of the Eastern Ghats and is known not only for the diversity of its flora and fauna, but also for the richness of its minerals. The length of the cave is approximately 2 km; it is 12 m high and 1300 m asl. The bedrock is mainly limestone and the average temperature of the inner cave wall was approximately 16^0C. Mucus-like biofuels, which are thick orange microbial mats (2.5–3 cm thick) with patches of yellow biofilms extending 3 m from the aphotic deep cave orifice were seen floating on the spring waters [9].

Characterization of strain BCA1: The actinobacterial strain BCA 1 was isolated from soil samples collected from Borra caves using starch casein agar (SCA) medium which produced small powdery colonies with diffusible pigment. The viability of strain BCA1 was maintained on YEME (ISP2) agar slants as well as in 30% glycerol broth[10].Microscopic characteristics were studied by adopting slide culture technique on glycerol asparagine agar medium. Mycelia and spore chain morphology was recorded using bright field microscope at 400 magnifications (Olympus microscope).

Spore structure and spore surface were recorded under scanning electron microscope (JEOL model JSM5600LV).Media and procedures used for determination of cultural, carbon and nitrogen source utilization were those described by Shirling and Gottileb[11]. Effect of pH, temperature, NaCl concentration and anaerobic condition was studied using modified ISP 2 medium. Lipase, protease and amylase activity were determined by adopting the agar plate method [10].

***In-vitro* screening for antimicrobial activity:** Spores of strain BCA1 was inoculated onto YEME agar plates (20 ml/plate) and incubated at 28^0C for 10 days. After scraping the mycelial growth, 5 mm diameter agar plugs were taken and placed over Muller Hinton Agar (MHA) plates seeded with test pathogens such as *Staphylococcus aureus*MTCC96, *Bacillus subtilis* MTCC441, methicillin resistant *S. aureus*, Enterotoxigenic *Escherichia coli*, *Vibrio cholerae*, *Vibrio mimicus*, and *Pseudomonas aeruginosa*. Agar plug prepared from uninoculated YEME agar was tested as medium control. All the plates were incubated at 37 ^0C for 24 hours. A zone of inhibition was expressed in millimeter in diameter[12].

Production of bioactive metabolites and antimicrobial activity: Production of extracellular pigment from strain BCA1wascarriedout by agar surface fermentation using YEME agar After 10 days of incubation at 28^0C the soluble pigment secreted into the agar medium was extracted in a crude form by solid-liquid extraction method using ethyl acetate at 1:2 ratio for 24 hours. The solvent was concentrated using rotary evaporator and the resulted crude pigment was completely dry[12].

Testing for antibacterial and antitubercular activity: Antibacterial activity of crude pigment was tested by disc diffusion method at 200 µg/disc concentration[13].Crude pigment impregnated sterile filter paper disc was placed over the MHA plates inoculated with test bacterial pathogens. Zone of

inhibition was measured after 24 hours of incubation at 37^0C.

Antitubercular activity of crude pigment was studied against standard laboratory strain *Mycobacterium tuberculosis* H37Rv, SHRE (streptomycin, isoniazid, rifampicin and ethambutol) sensitive and multi drug resistant (MDR) (resistant to rifampicin and isoniazid) clinical isolates of *M. tuberculosis* by adopting LRP assay[3]. Two mg per ml of working concentration of crude pigment was prepared using dimethyl sulfoxide (DMSO) (10 %) and filtered using 0.45 µ filters.

High titer of mycobacteriophage phAETRC202 was prepared using *M. smegmatis* mc^2155 in Middle brook 7H9 complete medium[14].About 350 µl of G7H9 broth supplemented with 10 % albumin dextrose complex and 0.5 % glycerol was taken in cryo vials and added with 50 µl of crude pigment in order to get the final concentration of 200 µg/ml. Hundred µl of *M. tuberculosis* cell suspension was added to all the vials. The above procedure was followed for all the three *M. tuberculosis* isolates used. DMSO (1 %) was also included in the assay as solvent control. All the vials were incubated at 37 ^{0}C for 72 hours. After incubation, 50 µl of high titerpage phAETRC202 and 40 µl of 0.1 M $CaCl_2$ solution were added into the test and control vials. All the vials were incubated at 37 ^{0}C for 4 hours. After incubation, 100 µl from each vial was transferred to luminometer cuvette. About 100 µl of D-Luciferin was added and the relative light unit (RLU) was measured in a luminometer (Monolight 2010).

Percentage RLU reduction = Control RLU – Test RLU / Control RLU X 100

RLU reduction by 50 % or more of the crude pigment when compared to control was considered as having antitubercular activity.

Bioactive metabolite production, purification and bioautography: Strain BCA1 was cultivated on 100 YEME agar plates (20 ml/plate) by agar surface fermentation for 10 days to get large quantity of pigment. The crude pigment was purified by thin layer chromatography using silica gel coated ready-made TLC sheets. Chromatogram was run using different organic solvents such as methanol, ethyl acetate, chloroform, n-hexane in various proportions. The antibacterial activity of the separated pigment was confirmed by adopting bioautography method using *B. subtilis* MTCC441 as test organism. After confirming the activity, active yellow pigment from the crude extract was separated in large quantity by preparative TLC using the optimized solvent system[13].

MIC of purified pigment against bacterial pathogens was determined by broth dilution method using Muller Hinton Broth whereas MIC against *M. tuberculosis* isolates was determined by LRP assay using 7H9 broth. Purified pigment was tested at concentration ranged between 100µg and <1 µg/ml.

Solubility and UV-Vis spectral analysis: To determine the solubility, one mg of purified pigment was mixed with each 10 ml of concentrated H2So4, 1 N NaOH and solvents such as methanol, chloroform, ethyl acetate and n-hexane. All the tubes were allowed to stand for 10 minutes and observed for solubility and colour change, if any. Ultraviolet (UV) spectrum of the purified pigment was determined using Shimadzu UV-1700 series. One milligram of sample was dissolved in 10ml of methanol and the spectra were recorded at a wavelength of 190 – 900nm.

Effect of nutrient concentration on bioactive pigment production: To determine the effect of nutrient concentration on the pigment production and the growth, spores of strain BCA1 was inoculated onto different nutrient concentrations (2X, 1X, 1/2X, 1/4X and 1/10X) of YEME agar and incubated at 28^0C. Growth and pigment production was observed for every 24 hours. After 10 days of incubation, crude pigment secreted in the agar medium was extraction using ethyl acetate and concentrated using eppendorf concentrator. The crude pigment was tested against *S. aureus* MTCC96, *B. subtilis* MTCC441 and *V. cholerae* at 200 µg/disc concentration.

RESULTS:

Characterization of actinobacterial strain BCA1: Strain BCA1 was showed the presence of aerial mycelium with spiral arrangement and substrate mycelium with no fragmentation and any

other special structures like sporangium (**Figure 1**). Under SEM observation, about 10-20 spores per chain of aerial mycelium with smooth surface were observed. Strain BCA1 showed good growth on ISP2, ISP3, ISP and ISP6 medium, pH 7-9 and temperature 30-40^{0}C. It utilized sugars such as glucose, fructose, mannitol and xylose as a carbon source (**Table 1**). Based on the studied phenotypic characteristics, the actinobacterial strain BCA1 was identified as species of the genus Streptomyces.

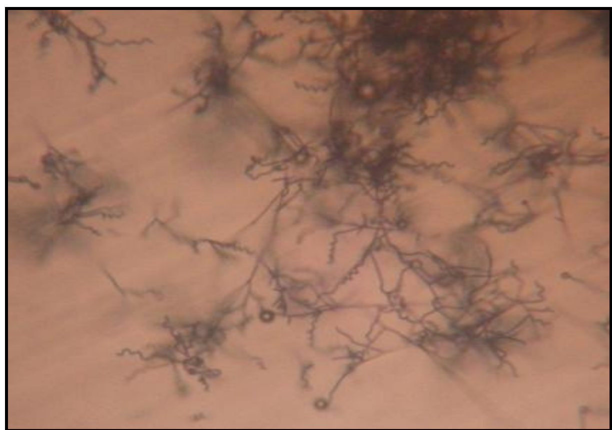

FIG. 1: MICROMORPHOLOGY OF STRAIN BCA1 UNDER BRIGHTFIELD MICROSCOPE AT 400 MAGNIFICATIONS

TABLE 1: PHENOTYPIC CHARACTERISTICS OF *STREPTOMYCES SP* BCA1

Characteristics	Result
Micromorphology	
Aerial mycelium	Present
Substrate mycelium	Present
Spore chain morphology	Spiral (S)
Spore surface	Smooth
Cultural characteristics	
Colony consistency	Powdery
Aerial mass colour	White
Reverseside pigment	Absent
Soluble pigment	Yellow
Growth on different ISP media	
ISP1	Moderate
ISP2	Good
ISP3	Good
ISP4	Good
ISP5	Moderate
ISP6	Good
ISP7	Absent
Growth at pH	7-9
Growth at temperature	30-40^{0}C
Utilized carbon sources	Glucose, fructose, mannitol, xylose

***In-vitro* screening for antibacterial activity:** Strain BCA1 showed broad spectrum activity against both gram positive bacteria including methicillin resistant *S. aureus*and Gram negative bacterial pathogens with 11 -15 mm zone of inhibition except *P. aeruginosa* for which no activity was exhibited (**Figure 2**).

Preparation of crude pigment, its antibacterial and antitubercular activity: Strain BCA1 started to produce extracellular yellow pigment on ISP2 agar medium from the second day of incubation and it reached maximum coloration on the fifth day of incubation. Maximum of 45 mg of crude pigment was extracted using ethyl acetate from 100 ml of ISP2 agar medium. In the disc diffusion method, the crude pigment inhibited all the test pathogens, except *P. Aeruginosa* with 15-17 mm zone of inhibition (**Figure 2).**

In general, most of the discovered antibiotics are found to be active against gram positive bacteria when compared to gram negative bacteria (Berdy, 2005). In the present study the yellow pigment produced by the *Streptomyces* sp BCA1 showed good activity against gram negative bacterial pathogens.

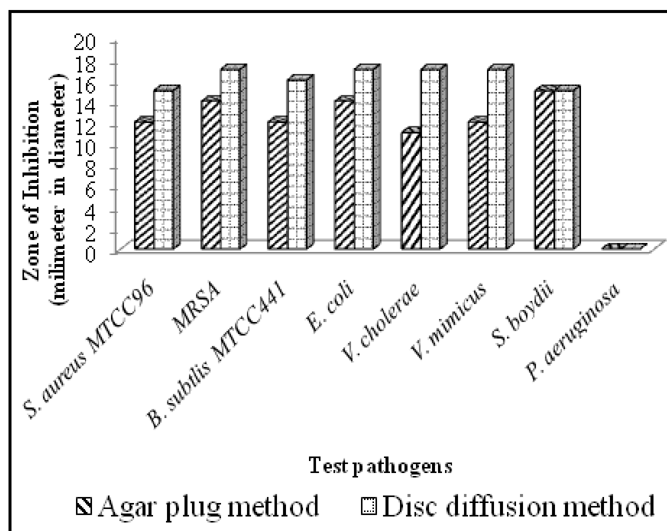

FIG. 2: ANTIBACTERIAL ACTIVITY OF *STREPTOMYCES SP* BCA1 AND ITS CRUDE PIGMENT

In LRP assay crude pigment showed >80% inhibition against the standard, drug sensitive and multi drug resistant clinical *M. tuberculosis* strains at 200µg/ml concentration **(Table 2).**

TABLE 2: ANTITUBERCULAR ACTIVITY OF THE CRUDE PIGMENT PRODUCED FROM *STREPTOMYCES SP* BCA1

Test pathogens	% Reduction in Relative Light Unit
M. tuberculosis H37Rv	85.14
M. tuberculosis (SHRE sensitive)	80.45
M. tuberculosis (multi drug resistant)	81.39

Production, purification and bioautography: Strain BCA1 was produced about 520 mg of crude pigment per 1000 ml of YEME agar medium. Separation of the crude pigment resulted in 4 separated spots with Rf value 0.7, 0.5, 0.4 and 0.2 when chloroform: methanol used in 9:1 ratio as the solvent system. In bioautography, the separated spot with Rf value 0.7 was yellow in color and it produced clear zone of inhibition around *B. subtilis* MTCC441. The preparative TLC based separation yielded about 50 mg of purified pigment/ gram of crude extract.

Determination of MIC: The MIC of purified pigment against various bacterial pathogens was given in **Figure 3.** *B. subtilis* MTCC441 was inhibited at the lowest of 6.25µg/ml concentration. MIC against *M. tuberculosis* H37Rv was found to be 6.25µg/ml whereas the drug sensitive and MDR *M. tuberculosis* isolates were inhibited at 25µg/ml concentration.

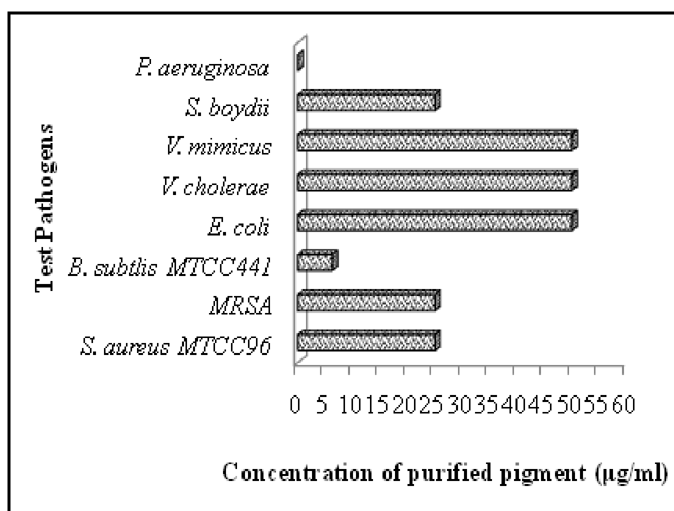

FIG. 3: MINIMUM INHIBITORY CONCENTRATION OF PURIFIED PIGMENT AGAINST BACTERIAL PATHOGENS

Reactions of purified pigment and UV-vis spectral characteristics: The yellow colour of the purified pigment was changed into cherry red upon the addition of conc. H2So4 while the yellow color was turned colorless upon the addition of 1N NaOH. The pigment was found soluble in methanol, chloroform, ethyl acetate and DMSO, but not in n-Hexane. The pigment showed the broad absorption band at 442nm under UV spectral analysis **(Figure 4)**

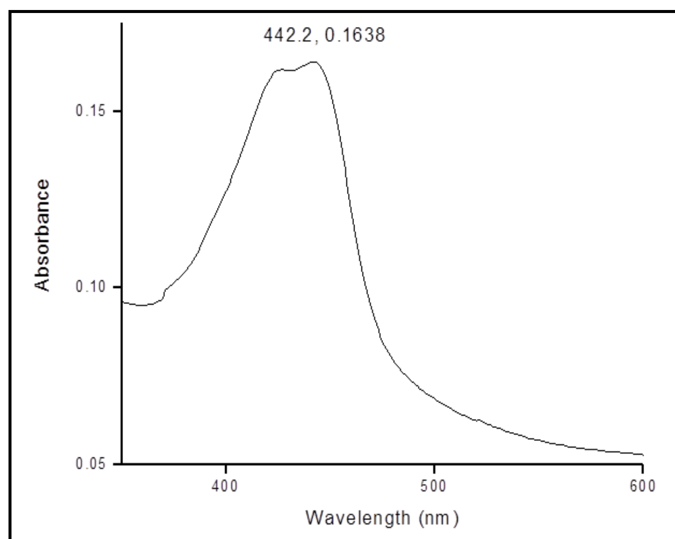

FIG. 4: UV SPECTRUM OF PIGMENT ISOLATED FROM *STREPTOMYCES* SP BCA1

Effect of nutrient concentration on growth and bioactivity of strain BCA1: The sporulation and pigment production was rapidly in decreased concentration of YEME agar. Maximum pigment production and bioactivity was on 1X, 1/2X and 1/4X concentrations of YEME agar. Large amount of vegetative mycelium was observed in 2X YEME agar medium **(Figure 5)**.

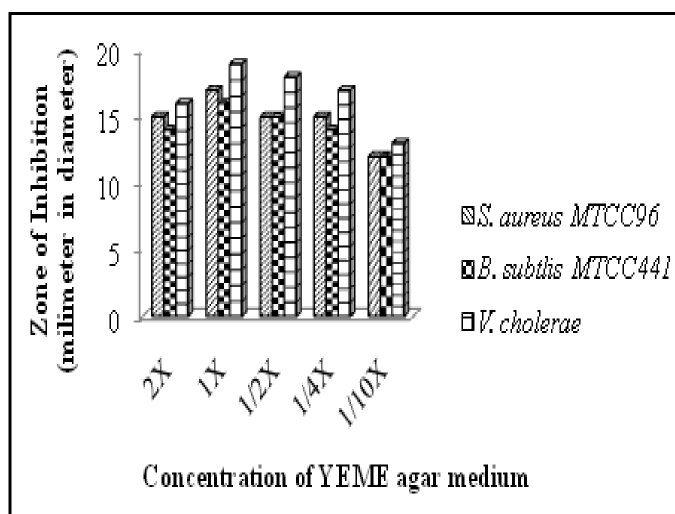

FIG. 5: EFFECT OF NUTRIENT CONCENTRATION ON ANTIBACTERIAL ACTIVITY OF STRAIN BCA1

DISCUSSIONS: Knowledge of cave microbial diversity is very limited, despite the fact that caves are found abundantly throughout the world. Cave habitats are of interest as the main focus for a new pool of microbial diversity and as a major source of microorganisms that produce antimicrobial agents[15]. Though more than 1500 caves are known to exist in India, no detailed and systematic microbiological explorations from most of these caves have been carried out[5]. Borra caves are one among the many unexplored caves in India in terms of actinobacterial bioprospecting.

The earliest microbiological studies of cave and karst habitats, predominately based on the examination of sediment and water by using microscopy or enrichment and isolation culture-based approaches, identified that microbes were prevalent but not as diverse as in surface habitats like soil [16,17]. Cave microorganisms in nutrient rich or limited environments (high and low energy caves) are metabolically versatile acquiring energy from cave compounds, gases and by oxidizing metals from rocks[18]. During our actinobacterial isolation program, strain BCA1 was isolated as one of the only eight actinobacterial colonies recovered from Borra cave soil using the starch casein agar medium. More number of novel actinobacterial colonies may be recovered from Borra cave soil using different isolation media and pretreatment methods as described by Hayakawa [19].

Generally, caves are low in nutrients, temperature and light intensity but have high humidity [6]. These factors might encourage the competition which enhances the production of substances such as antibiotics and hydrolytic enzymes that inhibit the growth of other microorganisms. Thus caves are potentially valuable places for discovering microorganisms with the potential to produce bioactive substances. The crude pigment prepared from the strain BCA1 showed promising activity against the bacterial pathogens including methicillin resistant *S. aureus* and MDR *M. tuberculosis*.

During the screening for novel bioactive metabolites, actinobacterial isolates are often encountered which show antibiotic activity on agar (Primary screening) but not in liquid culture (Secondary screening) [20].In the present study also, strain BCA1 produced the soluble yellow pigment only in solid medium but not in submerged liquid culture. In a study by Shomura *et al* .[21] the antibiotic production of *Streptomyces halstedii,* which showed activity against gram negative bacteria only in agar dishes, was well correlated with its mycelial morphology. The vegetative mycelium was filamentous in antibiotic producing agar cultures, but fragmented in nonproducing submerged cultures.

In this study, maintaining submerged cells filamentous by using diluted media, production of antibiotic in the submerged fermentations was accomplished. In our early study, *Streptomyces* sp D25 also produced the yellow pigment only in solid culture but not in liquid media with different concentrations. Medium consistency and filamentous mycelial structure did not have significant effect on bioactive pigment production by the strain D25 even while using different nutrient concentrations [22].Hence further optimization of nutrient components and concentrations are needed to overcome this problem. In addition, strain BCA1 rapidly produced the bioactive yellow pigment even in 1/4X YEME agar. This observation clearly supported the low nutrient origin of the strain BCA1.

The bioassay guided fractionation, acid base reaction and spectral analysis results in the tentative identification of active compound as phenoxazinone containing molecule. Phenoxazinones are reported to range from yellow to orange in colour and exhibit antibacterial, antifungal, anticancer and phytotoxic activities [23, 24, 25]. The chromophore is the part of complex natural products like actinomycin, auratin, cryptomycin and chandrananimycins and responsible for their colour [25]. These coloured antibiotics show antibacterial and anticancer activities. However, their antitubercular activity was not explored. But the phenoxazone chromophore containing yellow pigment isolated from strain BCA1 showed promising activity against both MRSA and MDR *M. tuberculosis* evidencing that the functional groups present in the BCA1 are responsible for its activity. In addition, this may be first report on antitubercular pigment from actinobacteria isolated from Indian Caves.

CONCLUSION: Findings of the present study conclude that Borra Cave is a potential source for actinobacterial bioprospecting. But extensive ecology-guided investigation is needed for the maximum recovery of the culturable actinobacterial population. Actinobacterial strain BCA1 isolated in this study will be a potential source for bioactive pigments against MRSA and MDR *M. tuberculosis*.

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