ISSN: 0976-3031

Available Online at http://www.recentscientific.com

CODEN: IJRSFP (USA)

International Journal of Recent Scientific Research
Vol. 9, Issue, 2(J), pp. 24479-24484, February, 2018

International Journal of Recent Scientific Research

DOI: 10.24327/IJRSR

Research Article

PRODUCTION OF VIOLACEIN PIGMENT FROM *CHROMOBACTERIUM VIOLACEUM* AND ITS ANTIBACTERIAL ACTIVITY AND SYNERGISM ON *E.COLI* ISOLATED FROM UTI SAMPLES

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DOI: http://dx.doi.org/10.24327/ijrsr.2018.0902.1669

ARTICLE INFO

Article History:

Received 16th November, 2017
Received in revised form 27th December, 2017
Accepted 4th January, 2018
Published online 28th February, 2018

Key Words:

C.violecium – Uropathogens- Antibacterial susceptibility – violacein production - combinatory effect – MIC.

ABSTRACT

Chromobacterium violaceum a Gram-negative bacterium produces a spectacular pigment called violacein. Violacein is a quorum-sensing metabolite compound and is also an active antimicrobial, anticancer agent. Despite its efficiency as a potential drug, alone or in synergy with other active principles has not being completely demonstrated. With the advent of the multi-drug resistant uropathogenic strain *E.coli*, it becomes essential to find a new natural product(s) that could be effectively used as a therapeutic agent. This current research work is projected on the extraction of violacein from *C. violaceum* and its combinatory effect with commercial antibiotics against various pathogens. Violacein production was optimized and characterized by Gas -mass spectrometry and infrared spectroscopy. It was observed that violacein works synergistically with most commercial antibiotics and could be used as a drug in combination with other antimicrobial agents

INTRODUCTION

Urinary tract infection (UTI) is the second most common infectious presentation in community medical practices. Worldwide, it accounts for about 150 million people diagnosed with UTI each year, and grouped as uncomplicated or complicated. UTI is referred to bacteriuria, when bacterial counts are ≥105 cfu/ml, and occur in combination with clinical symptoms (Hedda *et al*., 2012). Uncomplicated UTIs are present in sexually active healthy female patients with structurally and functionally normal urinary tracts. Complicated UTIs are those that are associated with co-morbid conditions that prolong the need for treatment or increase the chances for therapeutic failure, and it could affect the kidneys, ureters, bladder or urethra in humans. Different factors such as female gender, sexual activity, menopause, diabetes, catheter use, and urinary tract obstruction provide the risks for increasing the possibilities of urinary tracts infection. Among many bacteria such as *Escherichia coli*, *Staphylococcus* sp., *Klebsiella* sp., *Enterobacter* sp., *Proteus* sp., and *Enterococcus* sp., *Chromobacterium violaceum* was also found to be one of the most important infectious agents responsible for UTI.

Chromobacterium violaceum is a saprophytic, Gram-negative, facultative anaerobic, oxidase positive, motile bacillus bacterium belonging to the *Rhizobiaceae* family. It was discovered first in water buffalo by Wooley in 1905 (Christopher *et al*., 2001). It is ubiquitously distributed in natural aquatic environments and soils of tropical and subtropical regions. Infections due to *C. violaceum* are of low virulence and causes occasional localized infection. Though human infections caused by *C. violaceum* are rare, the increasing incidence suggests this to be an emerging pathogen, Due to its rare infectious state in human, the awareness of the disease caused by *C. violaceum* are limited. The predominant portal entry of these bacilli is through minor skin trauma or through ingestion of contaminated soil and water. *C. violaceum* rarely infects humans causing skin lesions, sepsis, soft tissue infections, urinary tract infections, and diarrhea and liver abscesses that may be fatal. It may be responsible for pneumonia, gastrointestinal infection, localized cutaneous lesions, localized or metastatic abscesses, osteomyelitis, meningitis, peritonitis, brain abscess, endocarditis, hemophagocytic syndrome, respiratory distress syndrome, and fulminant sepsis. *C violaceum* can survive under diverse environmental conditions because it produces several proteins

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contributing for its tolerance to antimicrobial compounds, heavy metals temperature, and acid.

Usually it grows readily on different bacteriological culture media including nutrient agar (NA), blood agar (BA), chocolate agar (CA), and Muller-Hinton agar (MHA) at 35 to 37°C by developing a distinctive smooth low convex colonies with a dark violet metallic sheen in the typical pigmented strain. It is indole negative, non lactose fermenter on TSI media, utilized citrate, reduced nitrate, fermented mannitol and decarboxylated arginine. All these biochemical characters and pigment production were identical for *C. violaceum*. The pigmented strains produce a violet non-diffusible pigment known as violacein, which is soluble in ethanol and insoluble in water and chloroform.

Violacein is a versatile pigment from *C. violaceum* that exhibits several biological activities and has currently gained an increasing importance in industrial sectors of medicine, cosmetics, and textiles (Gozde and Zerrin, 2015). It possesses antioxidant property, anti-leishimanial, anti-viral, anti-tumoral and anti-mycobacterial tuberculosis activities (Ponte, 1992; Leon *et al*., 2001). In most of violacein-producing bacterial strains isolated from nature, this bisindole is a secondary metabolite that is associated with biofilm production (Seong Yeol *et al*., 2015). Many of these molecules are biologically active, and some have toxic properties against competing species. In addition, many secondary metabolites have been found to have pharmacological properties which are of interest to clinical use. Violacein has strong antibacterial effects which makes it a promising candidate antibiotic extraction. Concomitantly violacein has also been shown to have antibacterial properties particularly towards Gram-positive bacterial strains (Seong Yeo *et al*., 2015). Also, the antiprotozoan properties of violacein could be exploited to treat diseases in humans, such as malaria and leishmaniasis (Leon *et al*., 2001). Moreover, when administered in combination with other antibiotics, the impact is more effective in fighting bacteria than the use of antibiotics alone (Subramaniam *et al*., 2014). The most studied clinical use of violacein is its potentiality as a cancer therapeutic (De Carvalho *et al*., 2014). Of late, it has been demonstrated that the purple pigment has antibacterial properties against methicillin resistant *Staphylococcus aureus*, proposing a possible binding to the bacterial DNA as a mechanism of action (Huang *et al*., 2012). Violacein are generally not involved in pathogenicity and therefore, isolating new violacein producing strains or other molecules with antimicrobial activity could contribute to the diversification in the application of natural products produced by bacteria.

The present study unwraps the potentiality of violacein for commercial activity against human pathogenic bacteria obtained from clinical samples.

MATERIALS AND METHODS

Sample Collection and Isolation

A total of 372 urine samples for microbiological examinations were received from patients who was professionally diagnosed with UTI at different hospitals and sample collection centers of Kanyakumari District, Tamilnadu, India. Samples were spread plated on nutrient agar and incubated at 37 ºC for 24 h. After incubation, bacterial isolates that expressing shades of dark violet colored colonies were isolated. Since dark violet colored colonies were considered as rare pathogens, they were sub-cultured in nutrient agar slants for further investigations. The isolates were identified using a battery of biochemical characteristics as described in Bergey's manual of systematic bacteriology (1985).

Antibiotic Susceptibility Pattern of C. violaceum

Antimicrobial susceptibility test for *C. violaceum* and all pathogenic isolates were performed by Kirby-Bauer Disk diffusion (Bauer *et al*., 1966) and the breakpoints used were as per the recommendations of CLSI (Clinical and Laboratory Standard Institute) on guidelines for susceptibility testing. Antibiotic disks contained Amikacin (30mcg), Amoxycilin+culvalinic acid (30mcg), Ampicillin (30mcg), Azithromycin (15mcg), Aztreonam (30mcg), Cefepime(30mcg), Ceftriaxone (30mcg), Cefotaxime (30mcg), Ceftazidime (30mcg), Cefuroxime (30mcg), Cefoxitin (30mcg), Chloramphenicol (30mcg), Ciprofloxacin (5mcg), Cotrimoxazole (25mcg), Gentamycin (10mcg) Imipenem (10mcg), Meropenem (10mcg), Piperacili+Tozobactum (30mcg), Tobramycin (10mcg) and Tetracycline (30mcg) were used. Following incubation, the zones of inhibition around the antibiotic disks were noted.

Optimization of the C. violaceum isolates for violacein production

Optimization study with the isolates of *C violaceum* was done to identify the expression of violacein production in relation to media composition, media type and temperature. The ability of the isolates in violacein pigment production was assessed by using various concentrations of Nutrient Broth with increasing concentration of media from 1% to 5%.

The media were autoclaved and inoculated with 10 ml of bacterial suspension previously cultured in nutrient broth for 24 h. Another trial included the production of the pigment in a modified cotton carpet technique (Rettori & Duran, 1998). Here sterilized cotton wool was placed into pre-sterilized Petri plates to a thickness of 1 cm. Furthermore, 5ml from 0.625, 1.25 and 25% of LB broths pre-cultured with *C. violaceum* for 24 h were taken and spread onto the cotton bed and incubated at 37ºC. Subsequent trials were conducted with different media for violacein production. Trials were done using Bennett's agar, MacConkey agar, brain heart infusion agar, tryptone glucose yeast extract agar, Muller Hinton agar, nutrient agar and tryptic soy broth. The effect of temperature on the production of violacein was observed by incubating the inoculated flasks at 4, 25, 30, 32 and 35ºC. Observation of pigment production for all the trials were made during 12 to 48 hr period and results noted.

Extraction and Characterization of Violacein Pigment

After incubation, the appearance of violet pigment and turbidity in broth indicated the optimal growth of *C. violaceum* and it was centrifuged at 10,000 rpm for10 min at 4ºC. The supernatant was discarded and the pellets were mixed with 2ml of phosphate buffered saline (PBS) and centrifuged at 10 000 rpm for 10 min at 4ºC. Then to the pellet was added with 1 ml

of ethanol and kept for 1 h. After 1 hr, the solution was again centrifuged at 10 000 rpm for 10 min at 4ºC. The solvent phase was removed and dried in an oven at 80◦C. It was then kept in a desiccator and later its structure was confirmed using spectroscopic methods (1H NMR and MS) (Anju *et al*., 2015).

GC-MS Analysis of Violacein Pigment

The extracted pigment *C. violaceum* was characterized by Gas Chromatography-Mass Spectrometry (LC-MS) on Thermo GC - Trace Ultra Version 5.0, Thermo MS DSQ II system equipped with an column of DB 35 - MS capillary standard non - polar column with dimension of 30 Mts length, 0.25 mm ID and film 0.25 µm film thickness.. Temperature was programmed from 70-260 ºC at a rate of 6ºC/min. Sample injection volume 1µL Helium was used as a carrier gas with 1.0mL/min flow rate. The peaks obtained in the GC-MS results were analyzed to identify the components of the purple pigment violacein (Nakata *et al*., 1979).

FT- IR Spectroscopic Analysis of Violacein Pigment

The FTIR spectra for the violacein pigment were recorded using the instrument in the range of 400-4000 cm^{-1}.

Determination of Minimum Inhibitory Concentrations of Violacein

The MIC for violacein and antibiotics were performed using the micro dilution assay as described in Clinical and Laboratory Standards Institute (2015) with minor modifications. The medium used was LB broth supplemented with 2% glucose. The test pathogen was prepared in a 0.85% saline solution to obtain a final concentration ranging from 3×10^3 to 6×10^3 CFU/ml.

Minimal Inhibition Concentration (MIC)

The minimum inhibitory concentration (MIC) was determined for all the antimicrobial agents and combinations (violacein + antibiotics). The resazurin micro titer assay with modifications (Farida *et al*., 2006) was employed. The violacein pigment and its combination with the selected some commercial antibiotics were diluted with nutrient broth (1:1) into a series of seven sets of three test tubes for the Uropathogenic *E.coli* pathogens (Sharma *et al*., 2010). An aliquot of 1ml of the bacterial suspension ($1x10^6$) of *E.coli* was inoculated into each tube. The control tubes were inoculated with same quantity of sterile distilled water and 75% ethanol. All tubes were incubated at 37^{o}C for 24hrs. The lowest concentration that did not permit any visible growth when compared with the control was considered as the minimum inhibitory concentration. The content of tubes that showed no visible growth were cultured on nutrient agar and incubated at 37^{o}C for 24 hrs. The minimum bactericidal concentration was considered as the lowest concentration that could not produce even a single bacterial colony.

The fractional inhibitory concentration index (FICI) was found as the sum of the FICs of each of the drugs. FIC could be defined as the MIC of each drug used in combination divided by the MIC of the drug when used alone (Shankar *et al*., 2014). The interaction was defined as synergistic if the FIC index was less than or equal to 0.5; additive if the FIC index was greater than 0.5 and less than or equal 1.0; indifferent if the FIC index was greater than 1.0 and less than or equal to 2.0 and antagonistic if the FIC index was greater than 2.0.

FIC index (FICI) = (MIC of violacein in combination (with antibiotic)/MIC of violacein alone) +MIC of antibiotic in combination (with violacein) / MIC of antibiotic alone)

RESULTS AND DISCUSSION

Of the total 372 urine samples collected in this study, 155 (42%) were found to be positive for UTI isolates. Among these, 46 (30%) samples were from males and 109 (70%) were from female patients. The age of patients ranged from 1 year to 90 years, with the mean age being 45.5 years. The demographic characteristics of the patients are depicted in Table 1.

Table 1 Frequency of urine positive culture with respect to age and sex distribution of patients suspected with UTI

S.No.	Age Groups (Years)	Sex	Number of Samples (n)	No. of Positive Cultures (n)	Pattern of Positive Cultures(n)	
					Monomicrobial	Polymicrobial
1.	1-30	Male	39	9	9	-
		Female	101	52	52	-
2.	31-60	Male	70	22	22	-
		Female	117	39	39	-
3.	61-90	Male	18	15	15	-
		Female	27	18	18	-
Mean Age: 45.5		Total:	372	155 (41.66%)	155	-

A total prevalence of the uropathogens was 155 (41.66%). Among these, 109 (70.32%) were isolated from females and 46 (29.67%) were from males. Comparatively, the highest isolation rate was observed in the age group between 1 to 60 years of age (Table 1).

Table 2 Prevalence of microbial isolates from urine samples

S. No.	Particulars	Total No. of Isolates (155)	Organisms Isolated	Total Pathogens Isolated (%)
1.	Gram negative bacteria	127 (81.9%)	*Escherichia coli*	64.56
2.			*Pseudomonas aeruginosa*	13.3
3.			*Klebsiella pneumoniae*	4.51
4.			*Enterobacter* sp.	6.2
5.			*Enterococcus* sp.	4.7
6.			*Proteus vulgaris*	3.9
7			*Chromobacterium violecium*	1.5
8.	Gram positive bacteria	16 (10.3%)	*Staphylococcus aureus*	50
9.			*Streptococcus* sp.	50
10.	Fungi	12 (7.7%)	*Candida* sp.	7.7

The 155 isolates were dignified as Gram negative (81.9%), Gram positive bacteria (10.3%) and fungi (7.7%) which are reported in Table 2. Of these Gram negative bacterial isolates, 7 genera were identified and the predominant was *E.coli* (64.56%), followed by *P.aeruginosa* (13.3%), *K. pneumoniae* (4.51 %), *Enterobacter* sp. (6.2%), *Enterococcus* sp. (4.7%) and *P. vulgaris* (3.9%), *C. violecium* (1.2%), Staphylococcus *aureus* (5.1%) and *Streptococcus* sp. (5.1%) constituted the Gram positive bacterial isolates. The fungi *Candida* sp. had 7.7% of incidence.

Due to rare infections caused in human by *C. violaceum*, the awareness of the diseases caused by them are limited (Chattopadhyay *et al*., 2002). Though human infections with *C. Violaceum* are rare, it can cause human infections like

septicemia, liver abscess, lung abscess, skin lesions, dental infections, urinary tract infections and diarrhoea (Harapriya *et al*., 2013). Thus, quick diagnosis, accurate bacterial identification, and specific treatment are very important, because *C. violaceum* may cause serious infection in healthy and young people (Ching-Huei Yang, 2011).

Informations on antimicrobial susceptibility patterns of *C. violaceum* are very limited owing to the rarity of isolation from clinical specimens. *In vitro* antibiotic susceptibility of the rare bacterial isolates *C. violecium* (Table 3) from positive urine cultures exhibited resistance to different commercial antibiotics such as Ampicillin (71.0±0.30), Imipenem (66.03±0.55), Ceftazidime (56.9±0.26), Cefoxitin (49.7±0.26), Cefuroxime (47.7±0.26), Amoxycillin + Clavulanic acid (42.43±0.40), Ceftriaxone (41.43±0.30), Ampicillin + Sulbactam (32.9±0.45) and Cefazolin (28.03±0.45) each. The clinical manifestations of *C. violaceum* infections are protean. It has been associated with pneumonia, gastrointestinal tract infections, urinary tract infections, localised cutaneous lesions, localised or metastatic abscesses, osteomyelitis, meningitis, peritonitis, brain abscess, endocarditis, hemophagocytic syndrome, respiratory distress syndrome, and fulminant sepsis (Vishnu *et al*., 2016).

Table 3 Multiple antimicrobial resistance patterns of Microbial isolates

S.No.	Commercial Antibiotics Used(mcg)	Resistance Pattern of *C.violecium* (n= 2)
1	Ampicillin (10 mcg)	71.0±0.30
2	Imipenem (10 mcg)	66.03±0.55
3	Ceftazidime (30 mcg)	56.9±0.26
4	Cefoxitin (30 mcg)	49.7±0.26
5	Cefuroxime (30 mcg)	47.7±0.26
6	Amoxycillin + Clavulanic acid (30 mcg)	42.43±0.40
7	Ceftriaxone (30 mcg)	41.43±0.30
8	Ampicillin + Sulbactam (10 mcg)	32.9±0.45
9.	Cefazolin (30 mcg)	28.03±0.45

The growth potential of *C. violecium* was measured at 560 nm from 0 to 30 hours and its optimum rate of violacein pigment production was between 12 to 16 hours. When different media were used, nutrient medium was found to be good for violacein production.

The properties of violacein pigment from *C. violecium* were analyzed using GC-MS and FTIR. The results of GC- MS indicated different peaks representing various compounds in the pigment. The spectrum of GCMS revealed the stretching bands which developed at 22.54 and 34.62 due to C- O bonds (Fig.1).

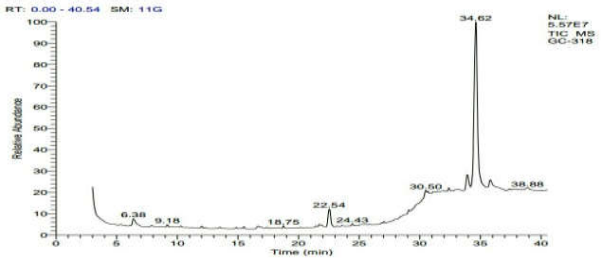

Figure 1 GCMS Analysis of Violacein Pigment

The result of FTIR spectra of the pigment exhibited the common functional groups of various chemical compounds such as aliphatic amines, ketones, aldehydes and phenolic compounds (Fig. 20. The infrared spectroscopy (IR) band at 1644.60 cm_1 corresponded to the carbonyl (C¼O) stretching frequency and a broad peak at 3362.35 cm_1 can be attributed to the NH group on the indole nucleus. All the FTIR data were in concurrence with the literature of Shankar *et al*. (2014).

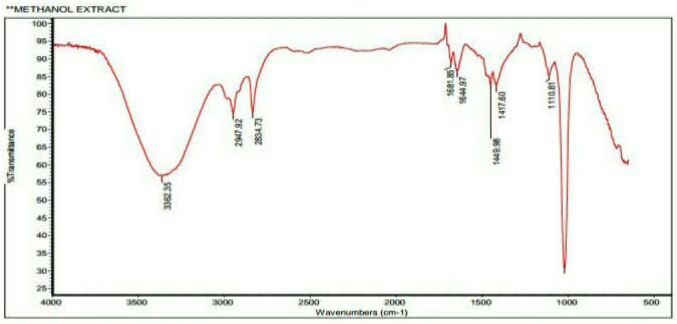

Figure 2 FTIR Analysis of Violacein Pigment

Violacein has strong antibacterial effect and this makes it a promising candidate as an antibiotic. Moreover, when administered in combination with other antibiotics, the impact is more effective in fighting bacteria than the use of antibiotics alone (The MIC of violacein and its combination with other commercial antibiotics against the Uropathogenic *E.coli* are summarized in Table 4. The application of violacein and its mixture with some selected commercial antibiotics (1:1 ratio) have shown synergistic, additive and antagonistic effects on *E.coli* (Table 4).

The fractional inhibitory concentration index (FICI) was found as the sum of the FICs of each of the drugs. FIC can be defined as the MIC of each antibiotics used in combination divided by the MIC of the antibiotics when used alone (Shankar *et al*., 2014). The interaction was defined as synergistic if the FIC index value was less than or equal to 0.5; additive if the FIC index was greater than 0.5 and less than or equal 1.0; indifferent if the FIC index was greater than 1.0 and less than or equal to 2.0 and antagonistic if the FIC index was greater than 2.0.

The MIC of violacein and other combinatory are enlisted in Table 4. The results observed underlines violacein to be active synergistically with many of the tested antibiotics (Table 4). In most combinations, the drug efficacy was either synergistic or additive. The FICI was found for each of the drug combinations based on the formula like MIC of violacein in combination /MIC of violacein alone] + [MIC of antibiotics in combination / MIC of antibiotics alone]. The combination of Violacein and Aztreonam was synergistic with a FICI of violacein against uropathogens *E.coli*, followed by Cefuroxime (0.41), Meropenem (0. 18) and Ampicillin/ Sulbactum (0.28). The Ceftazidime and Violacein mixture expressed significant additive effects with *E.coli* followed by Cefazolin, Tobramycin, Cefepime and Amikacin.

Furthermore, repeated or prolonged treatment with antibiotics is likely to contribute to the problems of antimicrobial resistance. Effective alternatives to antibiotics have the potential to improve public health as it offers an opportunity for patients to self manage the prevention of recurrent UTIs, which may improve their quality of life. The current study clearly establishes that violacein acts synergistically with most

common commercial antibiotics increasing the drug efficacy. It should also be noted that pigment of *C.violecium* is not a marker of pathogenicity (Diaz Perez, *et al.,* 2007).

No antagonistic effects were observed when violacein was tested in combination with commercial antimicrobial compounds. Therefore, in addition to the potential application of violacein as an antibiotic, this compound could be used further in combined therapy against multidrug-resistant isolates (Luciana Lacorte *et al*., 2011)

Table 4 Minimum inhibitory concentration (MIC) of violacein and its combinatory with commercial antibiotics

S.No.	Agents Used (mcg)	Uropathogenic *E.coli*			
		I	M	F	R
1.	Violacein	28.7	-	-	-
2.	Ceftazidime	20.1	11.9	1.0	A
3.	Aztreonam	3.9	1.7	0.48	S
4.	Cefuroxime	12.3	4.2	0.41	S
5.	Cefazolin	14.1	6.5	0.66	A
6.	Tobramycin	11.7	3.6	0.52	A
7.	Meropenem	3.7	1.9	0.18	S
8.	Ampicillin/ Sulbactum	4.1	3.7	0.28	S
9.	Cefepime	15	7.2	0.77	A
10.	Amikacin	12.7	8.6	0.9	A

I: individual, M: Mixture, F: fractional inhibitory concentration index (FICI) and R: results. Observations: S: synergistic, A: additive

Intraperitoneal doses of violacein up to 1 mg kg1 did not cause toxicity in blood, kidneys or liver of mice. The *in vivo* use of violacein as a therapeutic compound with low side effects as reported by Luciana Lacorte *et al*. (2011) Therefore, the use of violacein as an antibiotic compound is a reasonable prospect, and further studies relating to the in vivo antibacterial activity of violacein and its accumulation in tissues of food-producing animals could well reinforce their therapeutic potential of this drug against the uropathogenic isolates of *E.coli*.

CONCLUSION

Bacterially produced violacein, which is a secondary metabolite, has been found to possess antibacterial properties and more so when used synergistically with commercial antibiotics. The mode of activity of violacein needs to be studied in depth especially emphasizing the exact mode of action, its derivatives and bioactive component reactions to orbital levels

Acknowledgements

The author would like to thank Mrs.R.Vigila Christy, M.Sc., M.Phil., (Microbiologist), Mrs. S. Lalitha Srinivasan M.Sc., M.Phil. (Microbiologist), Mrs. P. Jagatha M.Sc., M.Phil. (Quality Manager), Prof. Dr. C. Ratnam Nadar, Ph.D., Prof. Dr. S. Ramesh, Ph.D., Mrs. A. Padma, B.Sc. MLT (Lab Technician) and other technicians of our institution for their help in the manuscript preparation, data collection and sampling

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How to cite this article:

Devi Priya *et al.*2018, Production of Violacein Pigment From Chromobacterium Violaceum And Its Antibacterial Activity And Synergism on E.Coli Isolated From Uti Samples. *Int J Recent Sci Res.* 9(2), pp. 24479-24484.
DOI: http://dx.doi.org/10.24327/ijrsr.2018.0902.1669

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