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Antimicrobial Activity of C- Phycocyanin from *Arthrospira Platensis* Isolated From Extreme Haloalkaline Environment Of Lonar Lake.

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Abstract: *The isolated Arthrospira (Spirulina) platensis from Lonar Crater Lake is rich in Cphycocyanin content. C- PC was extracted by implementing the Freeze Thaw method. The extracted C-PC concentration was found to be 0.0348 mg/ml. The crude C-PC was analyzed for the antimicrobial potency against some pathogenic bacteria and fungi by different standard microbiological assay methods. The antibacterial activity of C-PC was found to be maximum against S. aureus and Shigella spp. exhibiting 10 mm and 09 mm of diameter of zone of inhibition by disc diffusion method and 17 mm and 16 mm by agar well diffusion technique. Among the bacterial pathogens all showed sensitivity towards the C-PC resulted to show maximum relative percent inhibition of C-PC as high as up to 94.44% against S.aureus , fungi were found to be insensitive.*
Keywords: *C- phycocyanin, Arthrospira platensis, antimicrobial activity, Extreme haloalkaline environment.*

I. Introduction

The broad spectrum antimicrobial potency of C-phycocyanin extract obtained from Spirulina spp isolate, exhibited uniqueness & novelty of the fluorescent phycobiliprotein molecule. Antimicrobial character of C- phycocyanin is rarely studied & reviewed earlier against the test potent pathogens examined. The Lonar crater (placed at 19°59′ N and 76°31′ E), in the Buldhana District, Maharashtra, India, is located in the Deccan Traps.[13] It is one of the few known terrestrial craters to be emplaced in basaltic target rock.[8] It has an average diameter of 1800 m and its depth is about 150 m, with a halo-alkaline lake occupying the floor of the crater.[12] Lonar Crater Lake is an unique alkaline extreme environment as its pH range varies from 10.5 to 12.5. The microbial diversity of the lake comprises bacteria, fungi and microalgae. The microbial biota of the lake shows variant characteristics such as salt tolerance, characteristic photosynthetic rate, pigmentation, etc. The microalgal flora of the crater lake exhibits Euglena, Eudorina, Pandorina, Chlorella, Oscillatoria, Anikistrodesmus, Spirulina, Pediastrum, etc.[5] The nutritional value of Spirulina is well known and is been used for human consumption since .Spirulina is one of the most concentrated natural sources of nutrition. Nowadays not only it's nutritional but also the vibrant biotechnological applications have been thoroughly investigated. Spirulina is rich in important metabolites like Poly β hydroxybutyrate, γ linolinate, C- phycocyanin, allophycocyanin, erythrocyanins, etc. C-Phycocyanin is a blue colored fluorescent water soluble phycobiliprotein, possess molecular weight up to 20000 D^0-23000 D^0. Conventionally C-phycocyanin has been used as natural food colourant in cosmetic industry & pharmaceutical industry. C-phycocyanin is known for its hepatoprotective and antioxidant properties.[1-14] The recent report reveals the C-phycocyanin from cyanobacterium Westiellopsis sps has exhibited the antibacterial activity against Pseudomonas sps, B.subtilis and Xanthomonas sps.[15] The anti-inflammatory and antihyperalgesic activity of C-phycocyanin have been reported.[6] Present study reveals the broad spectrum antimicrobial activity of C-phycocyanin against the potent pathogenic microorganisms.

II. Materials And Methods

Isolation & Identification: The samples of Lonar Crater Lake water were collected and used as inoculums for isolation purpose. The samples were inoculated in the Zarrouk's and CFTRI medium, with inoculum size of 20% for the enrichment purpose and kept under continuous illumination of 2500 flux at 27 ^{0} C for 12 days with manual agitation twice a day for 5 minutes for avoidance of the mat formation. The isolated culture was identified by microscopic examination of the morphology and 16S r-RNA cataloging. The sequence obtained from 16 S –r RNA cataloging revealed the 91 % resemblance with Arthrospira platensis Spp - 17 (Taxid No. 447706).

2.1Pathogenic Microorganisms: The pathogenic microorganisms both Bacterial as well as fungi were procured from National Chemical Laboratory, Pune, India. The details of the pathogenic microorganisms are shown in Table No.3. The obtained cultures were grown and maintained by regular sub culturing on Nutrient agar medium for every fortnight through out the investigation.

2.2 Determination of C-Phycocyanin: The content of C-Phycocyanin in the isolated Arthrospira platensis was determined by the method described by the Boussiba and Richmond.[4] The percent pure C-Phycocyanin is determined by equation given below:

% Pure C-PC= A_{620} x (10) x (100) / 7.3 X (mg. Sample) x % dry weight

Where –

7.3 is the extinction coefficient of C-PC at 620 nm

7.4 The purity of C-PC was monitored by A_{620}/A_{280} ratio.

2.3 Extraction of C-Phycocyanin: The C-Phycocyanin was extracted from cell mass of Arthrospira (Spirulina) platensis isolate by implementing the Freeze thaw method. The overnight freezing was performed at -4° C and then cell biomass was thawed at 37° C for 4 hours. The three repeated cycles of freeze thaw of 2-2 hours were given to the cell mass in Phosphate buffer of pH 7 to extract out the maximum C-PC from the cell mass. The extracted C-PC from 500 mg of the cell mass showed concentration up to 0.0348 mg/ml, it was then centrifuged and preserved at 4^0 C for further use.[7] The various concentration of C-PC used for the study are depicted in Table No.2.

2.3.1 Disc diffusion Assay: This is the preliminary method implicated for analyzing the potency of the C-Phycocyanin against the selected pathogenic microorganisms. For this test Antibiotic Assay Medium (Himedia) was used to validate the antimicrobial activity of CPC. In this assay, routine standard spread plate technique was implemented to seed the 24 hrs old active culture of selected pathogens. The plates were incubated for 24 hrs at 37^0C. The antimicrobial activity in terms of inhibitory zone in mm against selected pathogens for respective concentration of C-PC extract was recorded to determine MIC. [3]

1) Preparation of the Disc: The discs of Whatman filter paper No.1 (5 mm) were taken and were sterilized in autoclave at 15 lbs for 20 minutes. On these sterile discs the C-PC extract aliquots ranging 10 µl, 20 µl, 30 µl, 40 µl, and 50 µl were applied with respective C-PC concentrations as depicted in Table No.2 with the help of micropipette and allowed to soak. The discs were further used for experimental purpose.
2) Agar Well Diffusion Assay: Agar well diffusion assay was employed to check the susceptibility of the test microorganisms against the C-Phycocyanin. Assay was conducted by using antibiotic sensitivity medium. The pH of the medium for bacterial cultures was maintained 7.1 and for fungi it was kept at 6.8. The selected pathogens were seeded and plates were prepared by using standard spread plate technique. Five mm well was bored on the agar base with the help of cork borer. For determination of MIC of C-PC, the C-PC extract was taken by using micropipettes in five different aliquots i.e. 10 µl, 20 µl, 30 µl, 40 µl, and 50 µl representing various concentration of C-PC as shown in Table No. 2 respectively. Then the plates were kept at 4^0 C for 5 minutes for proper diffusion. Afterwards the plates were incubated at 37^0 C for 24 hrs and the antimicrobial activity of C-PC was recorded as inhibitory zone.[16]
3) Turbidometric Assay: The turbidometric method is based on the inhibition of growth of a microbial culture in a fluid medium containing a uniform distribution of an antimicrobial compounds. The turbidometric assay was employed to evaluate the sensitivity of the test pathogen in liquid culture. In this assay of the 24 hrs old culture (1ml) was inoculated in sterile nutrient broth (10 ml) and to this the 10^{-1}, 20^{-2}, 30^{-3}, 40^{-4}, and 10^{-5} dilutions of C-PC extract (1ml) were added and allowed to incubate for 24 hrs at 37^0 C. After incubation the growth in terms of turbidity of the bacterial cultures was measured spectrophotometrically at 600 nm. [2] The readings were compared with that of the controls.
4) Comparative analysis of antimicrobial potential of C-PC with Standard Antibiotics:
The standard antibiotics used in the experimental design were procured from Lotus Pharmaceuticals, Ahmedabad. The standard antibiotics are pure drug used as active pharmaceutical ingredients. All the antibiotics were of ultrapure grade. The antimicrobial activity of the Standard antibiotics were against the test pathogen was assessed by multidisc diffusion assay. The multidisc of the differential Standard Antibiotics for Gram Positive and Gram Negative test pathogen were placed on the microbial lawn with help of the forceps. The discs were gently impregnated with forceps for proper diffusion. Plates were incubated at 37^0 C for 24 hrs. The results were recorded by measuring the inhibitory zone of respective standard antibiotics in mm. The data recorded was used for performing the comparative analysis with C-PC.[3]

TABLE 1 –Various standard antibiotics with their concentration used for comparative analysis.

Standard Antibiotics for Gm +ve	**Concentration** (micrograms)	**Standard Antibiotics for Gm -ve**	**Concentration** (micrograms)
C+S= Cefopera + Sulbactum	75/10	AMK = Amikacin	30
CFX= Ciprofloxacin	5	GNT= Gentamicin	10
LIV= Levofloxacin	5	PPR= Piperacillin	100
GNT = Gentamicin	10	CZI = Ceftazidime	30
CFR = Cefuroxime	30	CFP = Ciprofloxacin	75
GFX= Gatifloxacin	5	CFX= Ciprofloxacin	5
CFC= Cefaclor	30	OFX= Ofloxacin	5
CTR= Ceftriaxone	30	CFL= Cephalexine	30
CFD= Cefodroxyl	30	CTR = Ceftriaxone	30
A+S= Ampicillin + Sulbactum	10/10	CFI= Cefixime	5
OFX= Ofloxacin	5	CFM = Ceftamet	5
TTR= Tetracyclin	30	CFT= Cefotaxime	30
PPR= Piperacillin	100	--	--
CFT= Cefotaxime	30	--	--
LIN= Lincomycin	10	--	--
TOB= Tobramycin	10	--	--
LNZ= Linezolid	30	--	--

AMK= Amikacin	30	--	--
AZT= Azithromycin	15	--	--
SFX=Sparfloxacin	5	--	--

TABLE 2- Concentration of C-PC in the respective amount of extract used in experimental design.

Amount of Extract	Concentration of C-PC
10 µl	0.348 µg
20 µl	0.696 µg
30 µl	1.044 µg
40 µl	1.392 µg
50 µl	1.74 µg

TABLE 3- Pathogenic Microorganisms utilized in the experimental design.

Sr .No.	Name of Microorganism	ATCC No.
1.	Bacillus cerus	13601
2.	Bacillus subtilis	6633
3.	Escherichia coli	25922
4.	Pseudomonas aeuroginosa	9027
5.	Salmonella typhi	23564
6.	Shigella spp.	*
7.	Staphylococcus aureus	9144
8.	Aspergillus flavus	1003
9.	Aspergillus niger	10594
10.	Fusarium spp	*
11.	Mucor spp	*
12.	Rhizopus oryzae	62073
* = Laboratory isolates		

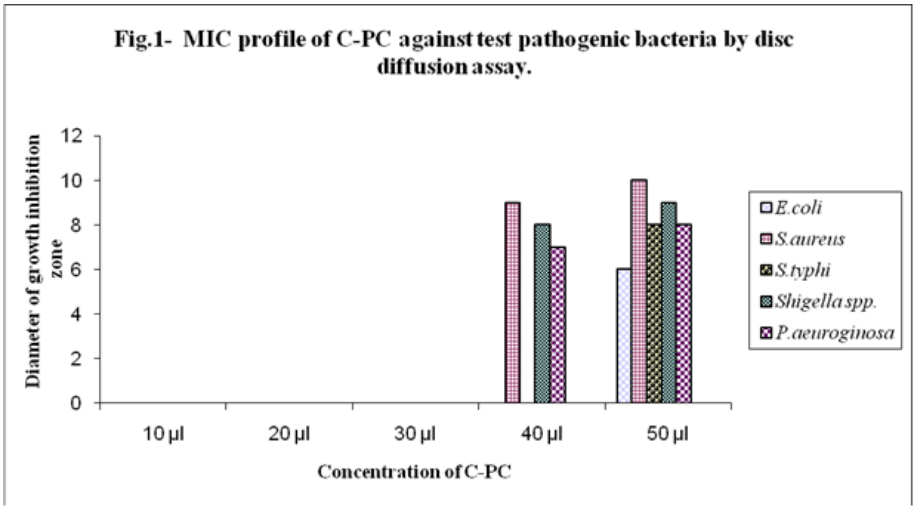
III. Result And Discussion

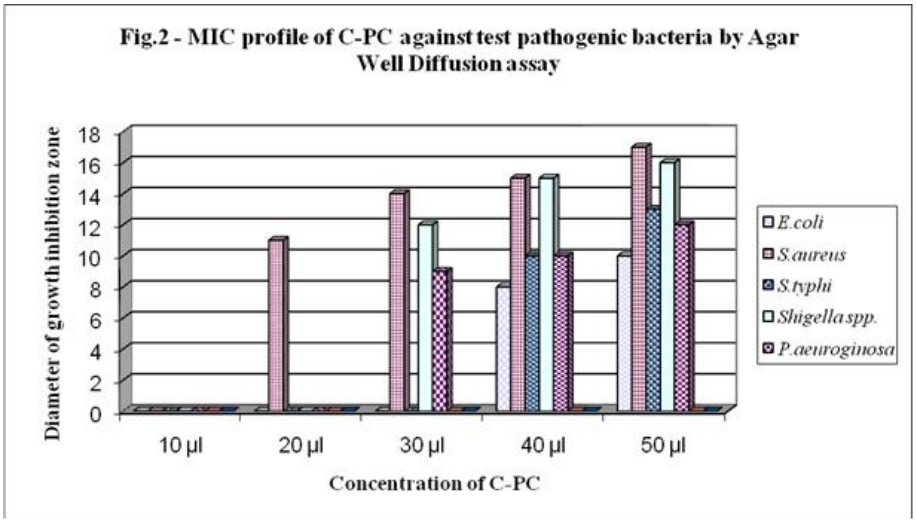
The potential of C-PC as antimicrobial agent is being analyzed by various assays. Fig. 1 depicts the inhibitory activity of C-PC against the pathogenic bacteria by implementing disc diffusion assay. When the results were analyzed in term of inhibitory zone obtained using given concentration of C-PC it was observed that MIC for S. aureus, Shigella spp and Pseudomonas aeuroginosa was found to be 40 µl. The recorded diameters of zone of inhibition were 9mm, 8mm and 7 mm respectively. The MIC for E. coli and S. typhi was 50 µl exhibiting 6mm and 8mm diameters of zone of inhibition. The Minimum inhibitory concentrations for the test pathogens using Agar well diffusion assay. The most sensitive bacterium was found to be S. aureus , as MIC for S. aureus was noted 20 µl. The MIC of C-PC for Shigella spp. and P. aeuroginosa was recorded 30 µl. For E. coli and Shigella spp. the MIC was found to be 40 µl canvassing 8mm and 10 mm diameter of zone of inhibition as recorded in fig 2.

The percent decrease in the growth of the test pathogens due to C-PC was determined by turbidometric method. Turbidometric assay as shown in fig.3 revealed the sensitivity B. subtilis and B. cereus towards the C-PC, the sensitivity of these bacteria was not observable in disc and agar well diffusion method. The findings of this assay definitely confirm the broad spectrum of C-PC as an antimicrobial agent. The percent decrease in the growth of E.coli was maximum 66.34 % against the upmost dilution of C-PC. The growth of B. subtilis was retarded by 60% in 10^{-4} dilution and it was recorded 58.5 % for the B. cereus by 10^{-5} dilution of C-PC. The growth of S. typhi was least affected only up to 20% decrease was observed.

The activity of C-PC was compared with the standard antibiotics. In the present investigation the standard antibiotics used against the Gram positive and Gram negative bacteria are specifically different as these microbes differ in all perspectives such as pathogenesis, distribution and genetic characteristics.[10] Fig-4 depicts that among the Gram positive, S. aureus was most sensitive towards the Piperacillin (PPR) showing 18mm diameter of zone of inhibition. C-PC also showed nearly the same effectiveness showing 17mm diameter of inhibitory zone. In fact C-PC proved superior to all rest of the standard antibiotics against the S. aureus. The B. subtilis and B. cereus showed the most sensitiveness towards Piperacillin(22 mm) and Tobramycin (19 mm) respectively. It should be noted that both of these organisms didn't exhibited sensitivity towards C-PC in disc diffusion and agar well diffusion assay. S. aureus showed resistance towards Cefaclor , Lincomycin and Linezolid and B. cereus was resistant to Cefaclor. In case of Gram negative bacteria chosen for the study, all of them showed sensitivity for C-PC as illustrated in fig-5. The previous studies have reported the capability of Gram negative bacteria to develop antibiotic resistance and are more pathogenic in nature. Amikacin, Gentamicin, Piperacillin and Ceftriaxone showed the effectiveness for E.coli, the most prominent was found to be Amikacin. E.coli showed sensitivity to C-PC developing 10 mm diameter of inhibitory zone. C-PC was more effective than PPR and CFT in case of E.coli. Ciprofloxacin among the effective antibiotics against Pseudomonas aeuroginosa gave maximum activity 14 mm zone of inhibition whereas for C-PC it was found to be 12mm. S. typhi was the most resistant against all the standard antibiotics used for comparative analysis in the present study; except the Gentamicin creating 14 mm of inhibitory zone, in case of C-PC it was 12 mm. The optimum activity of C-PC for Gram negative bacteria was recorded against Shigella spp. generating 16 mm inhibitory zone. The relative percent inhibition of C-PC compared to standard antibiotics with optimum lethalness for respective test pathogen is illustrated in fig.6. In case of E. coli the relative percent inhibition of C-PC with respect to

Amikacin was found to be 62.5%.The maximum relative percent inhibition of C-PC was recorded 94.44% with Piperacillin against S. aureus. Among the test pathogens the most resistant towards the standard antibiotics was found to be S. typhi, its sensitivity is only towards Amikacin among the standard antibitics and amazingly the relative percent inhibition of C-PC as compared to Amikacin against S. typhi was 92.85 %. Antifungal activity of C-PC was studied against Fusarium spp, Aspergillus niger, Apergillus flavous, Mucor spp. And Rizopus spp, by all three assays mentioned earlier, neither of them shown the sensitivity towards C-PC, possibly larger concentration of C-PC could inhibit the fungal growth which needs to be further investigated to understand fungicidal capability of this novel C-PC molecule. The side effect of synthetic antibiotics and decrease in the immunity & hyper allergic responses in some cases against chemical antibiotics are well known. The common incidences of gaining primary resistance in pathogens against higher levels of broad spectrum of synthetic drugs which showed lethal effect on normal beneficial human microbiota is area of concern. Therefore current investigation on medicine and pharmaceutical research emphasizes on antibiotic efficacy of natural nutraceuticals[9]. We suggest C-Phycocyanin of Arthrospira platensis strain from Lonar reflects antibiotic potentials which can be a candidate molecule to be exploited as an alternative to known antibiotics.





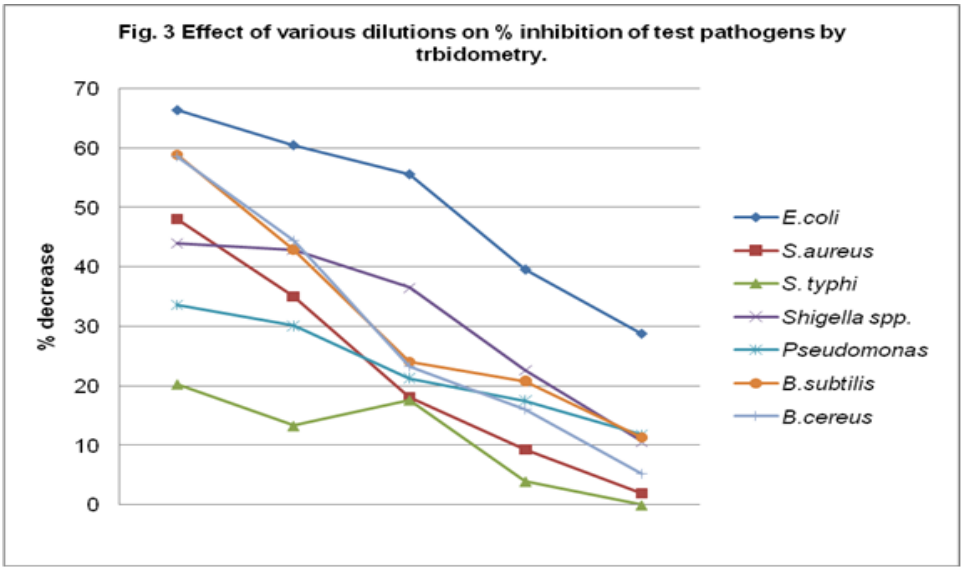


Fig. 3 Effect of various dilutions on % inhibition of test pathogens by trbidometry.
% decrease
70
60
50
40
30
20
10
0
E.coli
S.aureus
S. typhi
Shigella spp.
Pseudomonas
B.subtilis
B.cereus

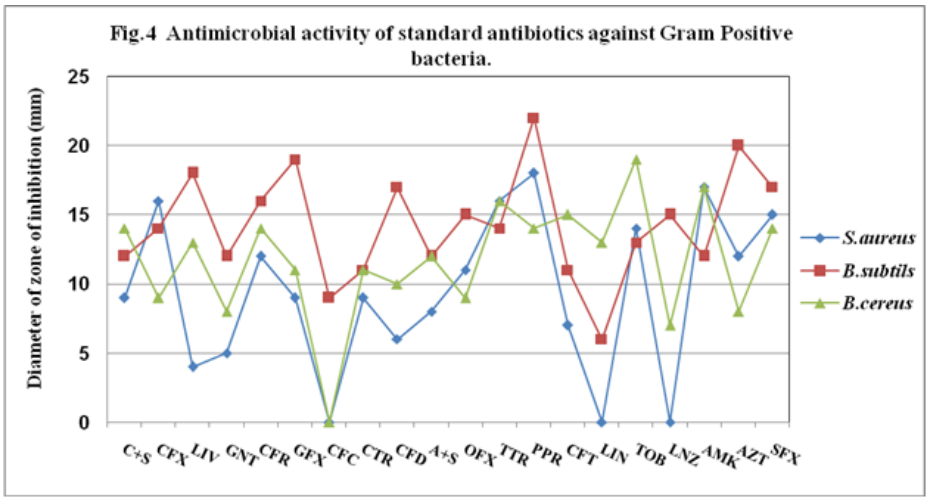


Fig.4 Antimicrobial activity of standard antibiotics against Gram Positive bacteria.
Diameter of zone of inhibition (mm)
25
20
15
10
5
0
S.aureus
B.subtils
B.cereus
C+S CFX LIV GNT CFR GFX CFC CTR CFD A+S OFX TTR PPR CFT LIN TOB LNZ AMK AZT SFX

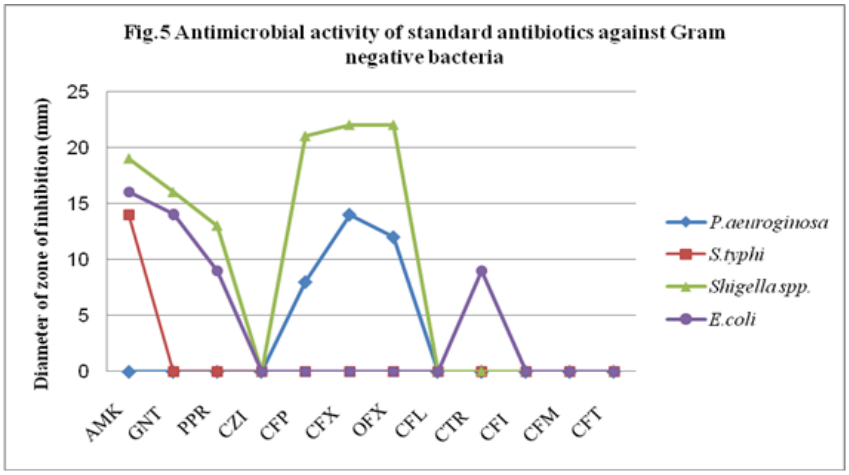
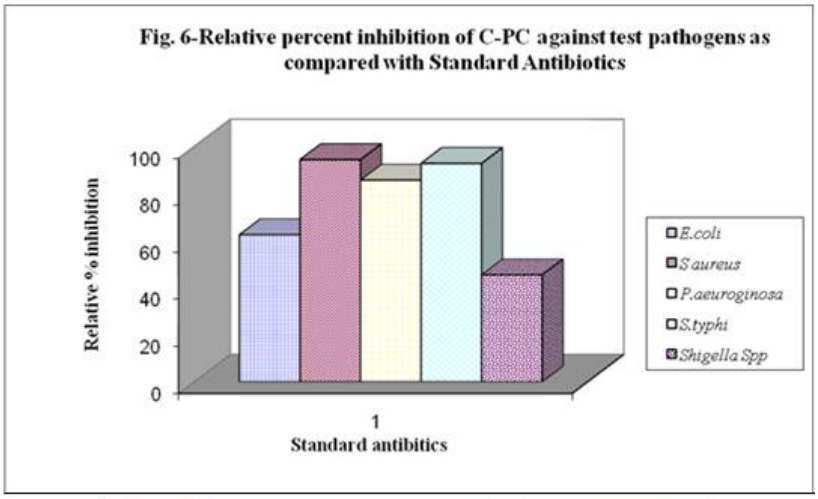
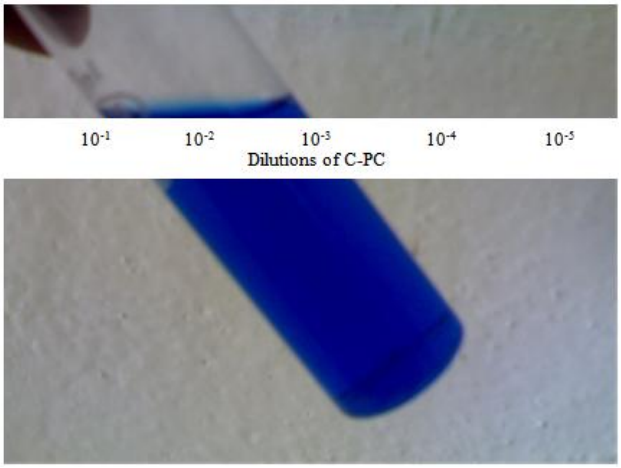


Fig.5 Antimicrobial activity of standard antibiotics against Gram negative bacteria
Diameter of zone of inhibition (mm)
25
20
15
10
5
0
P.aeuroginosa
S.typhi
Shigella spp.
E.coli
AMK GNT PPR CZI CFP CFX OFX CFL CTR CFI CFM CFT





Extracted C-PC from the isolate

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