

Original Research Article

**Detection of Antimicrobial Activity of Pyocyanin Produced by
Pseudomonas aeruginosa Against Urinary Tract Pathogens**

Abstract:

This study was performed to determine the antimicrobial activity of pyocyanin produced from *Pseudomonas aeruginosa* against urinary tract infection pathogens. Five different isolates of *Pseudomonas aeruginosa* were isolated from wound infection and eye drops. The isolates were tested for the production of pyocyanin by growing in nutrient agar after incubation at 37°C for 18 hour. Considerable amount of blue pigment pyocyanin were produced by *Pseudomonas aeruginosa* after grown in lauryl broth medium and extracted by chloroform extraction method. Maximal pigment achieved after 72 hours and used in sensitivity test against isolated microorganisms " *S.aureus* , *S.epidermis*, *S.saprophyticus*, *E.coli*, *K.pneumoniae*, *P.aeruginosa*, *C.frundii* and *C.albicans* ". The antimicrobial activity of the pigment was evaluated using Mueller Hinton Agar medium. The isolated microbes from urinary tract infection have different percentages of microorganisms " *S.aureus*15%, *S.epidermis*5%, *S.saprophyticus*10%, *E.coli*40%, *K.pneumoniae*10%, *P.aeruginosa*5%, *C.frundii* 5% and *C.albicans*10% ". The pigment have properties that make it an important bioactive compound which has the ability to arrest the electron transport chain of bacteria and exhibit antibacterial activity towards gram positive and un capsulated gram negative by different zone of inhibition at different concentrations. The inhibition zone determined by using 25%,50%,75% and 100%. The *S.aureus* inhibition zone at 25% was 16mm,50% was 17.5mm,75% was 20mm and 21mm at 100% concentration , *S.epidermis* at 25% was 17mm,50% 20mm,75% was 21mm and 31mm at 100%, *S.saprophyticus* at 25% was 19mm, 50% was 23mm,75% was 25mm and 33mm at 100%. *E.coli* at 25% was 13mm,50% was 15mm,75% was 25mm and 22mm at 100%. *C.frundii* was resistant to 25% and 50% concentration and given inhibition zone 11,13mm at 75,100% concentration. Also *K.pneumoniae*, *P.aeruginosa*, and *C.albicans* were resistant to pyocyanin.

Key words: *Pseudomonas aeruginosa*, pyocyanin, Urinary tract pathogen.

Introduction

The Urinary System is the organs of the urinary system are the kidneys, ureters, urinary bladder and urethra. Kidneys are

one on each side of the abdomen , they function as filtration organ . They filters the blood from all metabolic waste products and water soluble wastes

specially the nitrogenous wastes forming the urine.(Ohno,*et al.*,1993 ; Eugene,*et al.*, 2009). Ureters function as transporting tubes; they drain the urine formed in the kidneys to the urinary bladder (Betty, *et al.*,2007; Christine ,*et al.*,1996) urinary bladder is an expandable sac, lies on the lower abdominal part. It functions as a storage sac (Betty, *et al.*,2007 ; Christine ,*et al.*,1996). The urethra is a tube that connects the urinary bladder to the outside of the body, where urine passes through to outside the body (Betty, *et al.*,2007 ; Christine ,*et al.*,1996).

Urinary Tract Infection (UTI) is describing the colonization of microbes in urine and the invasion in the tissue lining the urinary tract (Wyngaarden, *et al.*,1992) The infection can be caused by viruses and fungi but bacteria have always been responsible for this infection. Urinary tract infection can be uncomplicated when there is no structural, functional or underlying host illness , It can be complicated when underlying abnormalities occur (Gilbert, *et al.* ,2005;Connolly,*et al.* ,1999 and Nicolle,*L,E.*,1994) UTI caused by different bacteria gram positive and gram negative such as *Staphylococcus aureus*, *Staphylococcus saprophyticus*, *Staphylococcus epidermidis*, *Enterococci*, *Haemolytic streptococci*, *Escherichia coli*, *Proteus species*, *Pseudomonas aeruginosa*, *Klebsilla strain* (Cheesbrough,2000)

Pseudomonas aeruginosa is an opportunistic pathogen of human (Nester, *et al.*,2001;Todar,*et al.*,2004) belonging to the family pseudomonadaceae. It is wide spread in the environment where in majority of which are responsible for nosocomial infections. In addition, they are also found associated with chronic infections in cystic fibrosis patients and wound infections especially of burns (Delden, *et al.*,1998). The biofilm formation on various antiseptics and disinfectants and the mechanism involving drug resistance toward various antibiotics has been one of the strategies in the emergence and the release of the resistant strain in the environment (Nikaido,*et al.*, 2009). However, Pseudomonads are known to produce pigments resolved to survive under oxidative stress imposed by environmental hazards and exhibits antagonism. This pigment is known Phenazines are heterocyclic compounds that are produced naturally and substituted at different points around their rings by different bacterial species (Price-Whelan, *et al.*, 2006). Pyocyanin is a water-soluble blue-green phenazine pigment produced in large quantities by active cultures of *Pseudomonas aeruginosa*. Pyocyanin (N-methyl-1-hydroxyphenazine) has antibiotic activity against a wide variety of microorganisms (Baron and Rowe,1981,;Flaishman,*et al.*,1990,

Anjaiah, *et al.*,1998, Norman, *et al.*,2004 , Liang, *et al.*,2008). The ability of opportunistic human pathogen; *P.aeruginosa* to acquire resistance to a broad range of antibiotics has made effective therapy more difficult. Several recent investigations have dealt with the problem of antibiotic resistance in *P.aeruginosa* (Diab, *et al.*,2004., El-Shouny,*et al.*,2006). A multidrug resistant *P. aeruginosa* strain has caused an outbreak in a neurosurgery ward (Sekigucki, *et al.*,2005).The increase in bacterial strains resistance, both to antibiotics and other disinfectants and germicides, led researchers to investigate other options, in treating both antibiotic-resistant and susceptible infections. In this concern, phenazines have been of great interest to pharmaceutical and clinical research groups for the last 50 years (Liang, *et al.*,2008, Laursen and Nielsen,2004, Dietrich, *et al.*,2006) .

Urinary tract infections include Urethritis, Cystitis, Ureteritis and Pyelonephritis, (Tad's,*et al.*,2007 ,Bale and Maston, 2007). Sign and Symptoms of UTIs is a frequency of urination, Burning pain or scalding sensation when urinating, feeling bladder is still full even after urination, pain above the pubic bone , blood in urine, discomfort or pressure in the lower abdomen, cramping in the pelvic area or back, strong- offensive odor, flank pain,

vomiting, nausea and fever and chills (Tad's,*et al.*,2007 ,Bale and Maston, 2007). Risk Factors for UTIs include Structure of female urethra, sexual intercourse, irregular urination, insufficient water intake, inadequate personal hygiene, catheter or tube placed in the bladder, menopause, age and medications(Tad's,*et al.*,2007 ,Bale and Maston, 2007). Complication of UTIs due to recurrent infections, permanent kidney damage, increased risk of women delivering low birth weight or premature infants and sepsis (Mackie and McCartney, 1996).

Urinary tract pathogens are *Staphylococcus aureus*, Is gram positive cocci, grape like cluster, facultative anaerobic and nonmotile. It grows well in a variety of commercial broth media, including trypticase soy broth, brain heart infusion broth and tryptose phosphate broth, with or without the addition of blood. Commonly used selective media include mannitol salt agar, lipase salt mannitol agar, Columbia colistin–nalidixic acid (CNA) agar and Baird–Parker agar base supplemented with egg yolk tellurite enrichment. These media inhibit the growth of gram-negative bacteria but allow the growth of Staphylococci and certain other Gram-positive bacteria (Stephen,*et al.*,2006), disease caused by *S.aureus* are skin infection, boils, impetigo, cellulites, systemic infection,

wound infection, bacteremia, (Kenneth,2009), Meningitis, endocarditis, septic arthritis, food poisoning, staphylococcal scalded skin syndrome and toxic shock syndrome (Saez-Liorens and McCracken, 2003). *Staphylococcus epidermidis* is a gram-positive cocci, facultative anaerobe and one of over 40 species belonging to the genus *Staphylococcus* (Schleifer and Kloos, 1975), it is part of the normal human flora, typically the skin flora, and less commonly the mucosal flora. (Fey and Olson, 2010), although *S.epidermidis* is not usually pathogenic, patients with compromised immune systems are at risk of developing infection, these infections are generally hospital-acquired (Levinson, 2010), *S.epidermidis* is a particular concern for people with catheters or other surgical implants because it is known to form biofilms that grow on these devices (Salyers,*et al.*, 2002) being part of the normal skin flora, *S.epidermidis* is a frequent contaminant of specimens sent to the diagnostic laboratory (Queck and Otto,2008). *Staphylococcus saprophyticus* is gram positive, is globular shaped, a facultative anaerobe, largely commensals it is second pathogen associated with UTIs (Pead,*et al.*, 1985) causing 10-20% of all UTIs in sexually active young women .like other Staphylococci, *S.saprophyticus* colonizes the urinary tract of humans and

is isolated from urine samples, young women are more susceptible to colonization in the urinary tracts and sexual intercourse promotes its spread (Raz,*et al.*,2005), *S.saprophyticus* is not normally present in the body (Pead,*et.a.*,1985) It is also isolated from the carcasses of dead animal (Raz,*et al.*,2005), first isolated coagulase-negative Staphylococci with antigen 51 from women with acute UTI by Torres Pereira A. (Torres Pereira,1962) later this antigen was classified as *S.saprophyticus*, it was identified by absence of coagulase and resistance to antibiotic Novabiocine (Raz,*et al.*,2005), disease caused by *S. saprophyticus*; Candidal infection and stones in the kidney and ureter are associated with *S.saprophyticus* infection, other more severe diseases pyelonephritis, endocarditis septicemia, and nephrolithiasis .(Raz, *etal.*,2005). *Escherichia coli* is gram negative bacteria, non spore- forming, short rod belong to family enterobacteriaceae aerobic, facultative anaerobic bacteria, Found as normal flora in gastrointestinal tract of human and animal, Disease caused by *E.coli* are Urinary tract infection, Meningitis, Sepsis and Pneumonia(Cheesbrough,2000). *Pseudomonas aeruginosa* is gram-negative rod, motile, an obligate aerobic, the bacterium is ubiquitous in soil and water,

and on surfaces in contact with soil or water, its metabolism is respiratory and never fermentative, but it will grow in the absence of O_2 if NO_3 as terminal electron acceptor is available as a respiratory electron acceptor, *Pseudomonas aeruginosa* is oxidase positive (Collee,*et al.*,1996), it produces a green fluorescent pigment (fluorescein) and a blue pigment (pyocyanin), which gives colonies and infected wound dressings a greenish-blue coloration, *Pseudomonas aeruginosa* grows well on simple bacteriological media, and most strains elaborate the blue phenazine pigment pyocyanin which together impart the characteristic blue-green coloration to agar cultures (Mark Gladwin,*et al.*,2014), *Pseudomonas aeruginosa* is an opportunistic pathogen, meaning that it exploits some break in the host defenses to initiate an infection, it cause urinary tract infection, skin infection, respiratory infection, external ear infection, eye infection, and septicaemia (Cheesbrough,M.,2000), it also produces a sweet grape-like scent, so wound dressings and agar plates are often sniffed for organism identification, *Pseudomonas aeruginosa* has weak invasive ability, healthy people just don't get infections with this guy, However, once inside a weakened patient, the story changes. It elaborates numerous exotoxins including exotoxin A, which has the same

mechanism of action as diphtheria toxin (stops protein synthesis) but is not antigenically identical. Some strains also possess a capsule that is antiphagocytic and aids in adhesion to target cells for example in the lungs. (Mark Gladwin,*et al.*,2014). Pyocyanin is phenazine antibiotic pigment produced by *P.aeruginosa*, the antibiotic action of pyocyanin pigment against different bacteria and yeast was investigated , *P.aeruginosa* is the only known organism of the pseudomonads and other glucose–nonfermenting gram– negative bacilli capable of producing the very distinctive water-soluble pigment pyocyanin (Daly,*et al.*,1984), it is considered as a product of secondary metabolites, pyocyanin has phenazine nucleus that means it belongs to the phenazine family (electron acceptor), which stimulates redox cycling in bacteria, and human epithelial and liver cells, Pyocyanin has a variety of pharmacological effects on eukaryotic and prokaryotic cells and has bactericidal activity against many bacteria such as, *Staphylococcus aureus*, *Staphylococcus saprophyticus*, Enterococci, *Haemolytic streptococci*, *Escherichia coli*, *Proteus species*, *Klebsilla strain* which may allow *P.aeruginosa* an advantage over competing bacteria occupying the same niche (Da Silva and Almeida, 2006), the inhibitory action of pyocyanin is the result

of its unique redox potential, it was also proposed that, during respiration, pyocyanin becomes reduced and univalently reduces oxygen to the toxic superoxide radical, the resistance of various bacteria to pyocyanin would therefore be dependent upon the levels of superoxide dismutase and catalase possessed by the organism and on the presence of oxygen (Hassan and Fridovich,1980), pyocyanin is a redox-phenazine compound that kills mammalian and bacterial cells through the generation of reactive oxygen intermediates, *P. aeruginosa* resists pyocyanin because of the limited redox cycling of this compound and that under conditions favoring pyocyanin production; catalase and superoxide dismutase activities are increased (Hassett,*et al.*,1992). *Klebsiella pneumonia* is gram-negative, non motile, encapsulated, facultative anaerobic lactose-fermenting, rod-shape bacterium, although found in the normal flora of the mouth, skin and intestines (Ryan and Ray,2004), it naturally occurs in soil, and about 30% of strains can fix nitrogen in anaerobic conditions (Postgate,1998), members of the *klebsiella* genus typically express two types of antigens on their cell surfaces, the first is O antigen, is a component of the lipopolysaccharide (LPS), of which nine varieties exist, the second is K antigen, a capsular

polysaccharide with more than 80 varieties (Podschum and Ullmann,1998), both contribute to pathogenicity and form the basis for serogrouping. *Klebsilla pneumonia* is distinguished by being indole negative and by its ability to grow on both melezitose and 3-hydroxybutyrate. *Citrobacter freundii* is a species of facultative, aerobic, gram-negative bacilli of the Enterobacteriaceae family; the bacteria are long rod-shaped with a typical length of 1–5 µm, most *C.frendii* cells are surrounded by several flagella used for locomotion, but a few are not mobile, it can be found in soil, water, sewage, food, and the intestinal tracts of animals and humans, the genus *Citrobacter* was discovered in 1932 by Werkman and Gillen. Cultures of *C.frendii* were isolated and identified in the same year from soil extracts (Wang, *et al.*,2000), as an opportunistic pathogen *C.frendii* is responsible for a number of significant infections, it is known to be the cause of a number of nosocomial infections of the respiratory tract, urinary tract, blood, and many other normally sterile sites in patients (Whalen,*et al.*,2007). *Candida albicans* is a diploid fungus that grows both as yeast and filamentous cells and a causal agent of opportunistic oral and genital infections in humans, (Ryan and Ray 2004; Denfer and Hube,2007), *Candida albicans* is commensal and a

constituent of the normal gut flora comprising microorganism that live in the human mouth and gastrointestinal tract, the potential of *C.albicans* as a urinary tract pathogen is dependent in part on successful colonization of body sites near to or with access to the urinary tract. Lower UTI with *C.albicans* usually occurs in patients with urinary catheters, typically after antibiotic therapy, although candidal and bacterial infections frequently occur simultaneously, *C.albicans* prostatitis occurs in frequently in patients with diabetes, usually after instrumentation, Renal candidiasis is usually spread hematogenously and commonly originates from the GIT, at high risk are patients with

Matrials and Methods:

This study was conducted in Khartoum state, forty samples of urine were collected from Khartoum Educational Hospital. Collection and transport of sample According to cheesbrough, 2000 aseptically patients were given a urine container to collect midstream of urine .Instruction was given to the patient to get midstream urine, the sample was labeled very well . After the collection , 1% boric acid was added to the sample as the preservative until transported to laboratory .

Solid media “Nutriant, Blood, CLED, Sabouraud Dextrose, Mannitol Salt, Eiosin

diabetes and those who are immunocompromised because of tumor, AIDS, chemotherapy, or immunosuppressants. Renal transplantation increases the risk because of the combination of indwelling catheters, stents, antibiotics, anastomotic leaks, obstruction, and immunosuppressive therapy (Ryan and Ray 2004). Most are asymptomatic, where *Candida* can cause urethral symptoms ,mild urethral itching, dysuria, watery discharge in men is uncertain. Among lower UTIs, cystitis due to *Candida* may result in frequency, urgency, dysuria, and suprapubic pain and hematuria (Ryan and Ray 2004).

Methyline Blue, Mueller Hinton and DNase Agar” was cultured by streaking tested organism with sterile loop in the surface of the media, semi solid media “Kligler Iron Agar” was cultured by inserted sterile straight wire containing tested organism in tube and stabbed in media, Broth media “Peptone water, MR – VP and Citrate medium” was cultured by inserted sterile loop containing tested organism in tube. Results of cultural characteristics, primary and secondary tests for bacterial identification were recorded, also *C.albicans* was isolated and conferred by Germ Tube Test.

Pyocaynin was prepared by growing *P.aeruginosa* in LB media, and then sub cultured on tryptic soy broth, and incubated at 37°C for 18 hrs , centrifuged 3000 rpm for 30min , and supernant was taken. An aliquot of 5ml from culture then extracted into 1ml of chloroform, and then reextracted into 1ml of 0.2 N HCl. The absorbance of this solution was measured

at 520 nm (Essar, *et al.*; 1990).The different pathogenic bacteria were cultured on brain-heart broth 24 hr , and the bacteria was cultured on a nutrient agar by spreader and left to dry for 15min after that wells made by cork borer, and filled with 0.1µl of pyocyanin extraction and incubated for 24 hrs ,and the results were recorded.

Result

Staphylococcus aureus , *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Escherichia coli* ,*Citrobacter freundii* , *Klebsilla pneumoniae* ,*Pseudomonas. aeruginosa* ,*Candida albicans* were isolated from urine, Incedance of UTI by *E.coli* is more high than incedance by othermicroorganisms .*S.epidermidis* and *P.aeruginosa* have low incedance and

associated with catheter as shown in table (1).

Effect of pyocyanin against isolated pathogens

Pyocyanin inhibited the growth of *S.aureus*, *S.saprophyticus*, *S.epidermidis*, *E.coli* and *C.frendii* indicated by zone of inhibition, and did not affect the growth of *K.pneumoniae* *P.aeruginosa* and *C.albicans* as shown in table (2), Figure (1 – 4).

Table (1): Percentage of microorganism.

Isolated Microorganism	Percentage %
<i>S.aureus</i>	15%
<i>S.saprophyticus</i>	10%
<i>S.epidermidis</i>	5%
<i>E.coli</i>	40%
<i>P.aeruginosa</i>	5%

<i>K.pneumoniae</i>	10%
<i>C.frendii</i>	5%
<i>C.albicans</i>	10%
Isolated Microorganism	Percentage %
<i>S.aureus</i>	15%
<i>S.saprophyticus</i>	10%

Table (2): Effect of pyocyanin against isolated pathogens.

Microorganism	Diameter of zone by mm			
	Concentration of pyocyanin			
	25 %	50 %	75 %	100 %
<i>S.aureus</i>	16	17.5	20	21
<i>S.epidermis</i>	17	2	21	31
<i>S.saprophyticu</i>	19	23	15	33

<i>S</i>				
<i>E.coli</i>	13	15	21	22
<i>K.pneumoniae</i>	0	0	0	0
<i>C.freundii</i>	0	0	11	13
<i>P.aeruginosa</i>	0	0	0	0
<i>C.albicans</i>	0	0	0	0

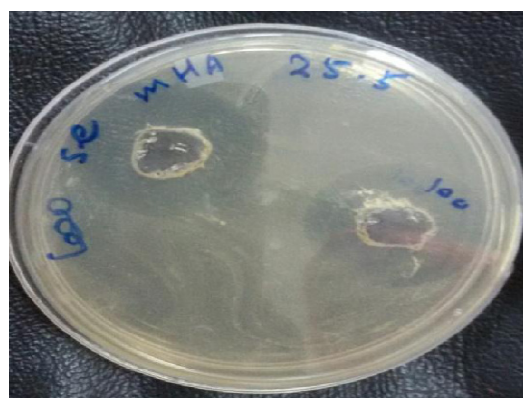
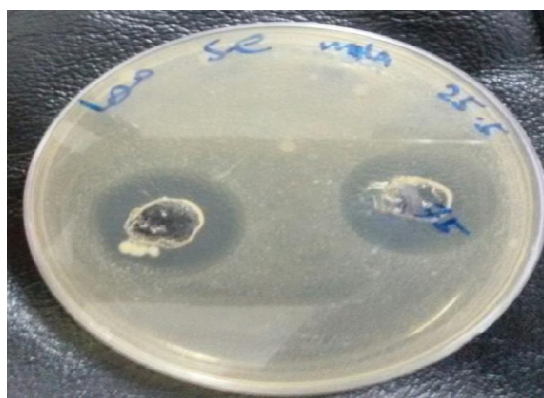


Figure (1): Left and right show inhibition zone of pyocyanin (75 and 100) % concentration against *S.epidermidis* .

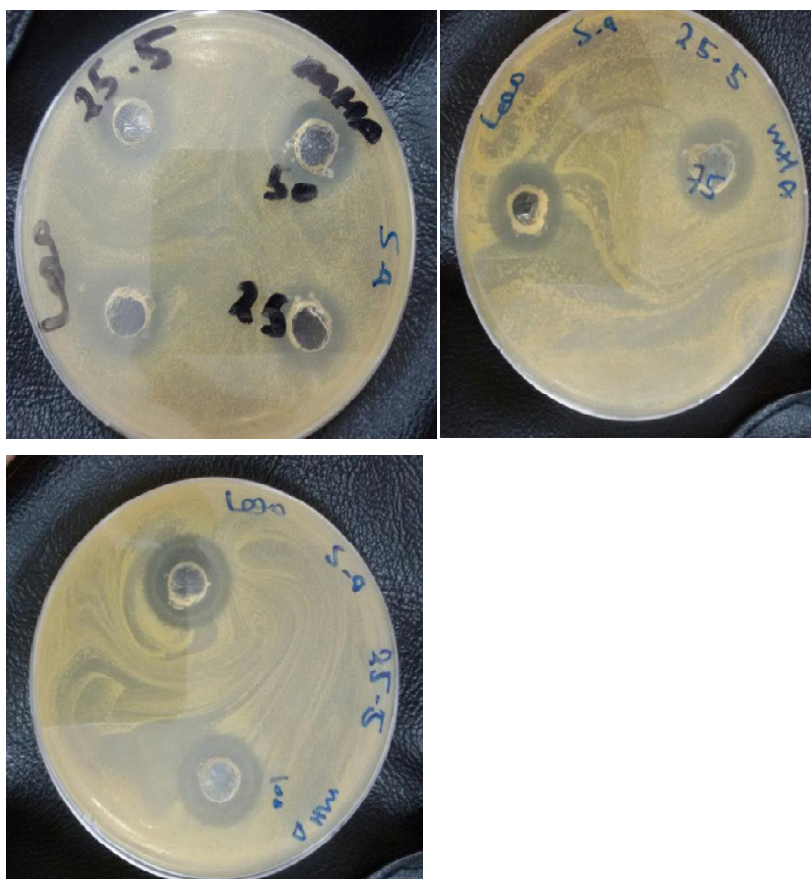


Figure (2): Left, right and middle show inhibition zone of pyocyanin (25, 50, 75 and 100)% concentration against *S. aureus*.

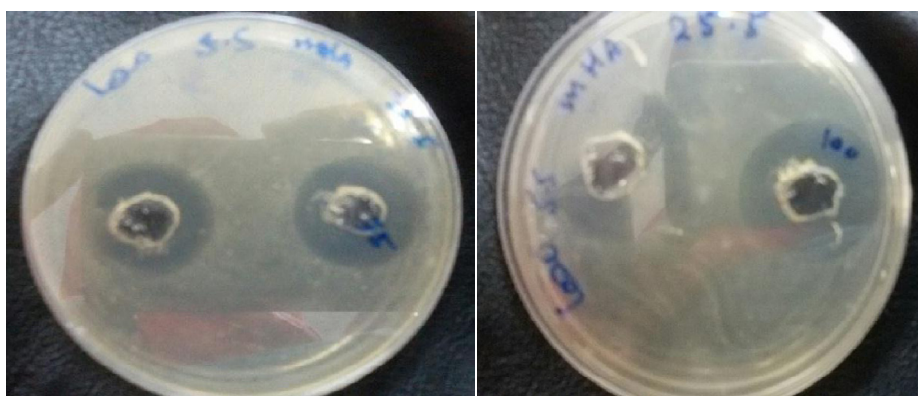


Figure (3): Left and right show inhibition zone of pyocyanin (75 and 100) % concentration against *S. saprophyticus*.



Figure (4): show inhibition zone of pyocyanin 100% concentration against *E.coli*.



Figure (5): Left and right show inhibition zone of pyocyanin (75 and 100) % concentration against *C.freundii*.

Discussion

According to the result the pyocyanin added at different concentrations (25,50,75, and 100) %, best results were showed by using 75%,100% concentration of pyocyanin that inhibited the growth of gram positive and uncapsulated gram negative bacteria by appearance of large zones around wells ,This result confirm the

finding of (El-shouny ,*et al.*,2011) but difference is due to difference in concentration of pyocyanin and technique used also disagreed with (Sundaramurthy Karpagam,*et al.*,2013) reported inhibition of *candida albicans* because they used high concentration and different technique. The results are in agreement with (Enass ,G. and Sweedan,2010) pyocyanin has

antibacterial activity, and can use as antibiotic against pathogenic bacteria.

This study concluded that pyocyanin possess antibiotic activity against gram positive and uncapsulated gram negative isolated pathogens of UTIs and more efficiency with high concentration than at low concentration. pyocyanin has antibacterial activity, and can be used as antibiotic against pathogenic bacteria. as shown by (J. of university of anbar for pure science 2010) .

Recommendations

1. Emphasis with the commitment by good personal hygiene to reduce urinary tract infection.
2. More research is needed for antimicrobial activity of pyocyanin against other microorganisms.
3. Further investigation under controlled circumstances will be needed to discover other procedures for pyocyanin production as an antibiotic.
4. Experimental research finding in vivo to study physiological changes of pyocyanin as antibiotic.

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