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# Antimicrobial effect of pyocyanin produced by clinical isolate of *Pseudomonas aeruginosa*

**A thesis**

**Submitted to the Council of College of Medicine at University of Kerbala in Partial Fulfillment of the Requirements for the Master Degree in Medical Microbiology.**

**By**

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**((قَالُواْ سُبْحَانَكَ لاَ عِلْمَ لَنَا إِلاَّ مَا عَلَّمْتَنَا إِنَّكَ أَنتَ الْعَلِيمُ الْحَكِيمُ))**

صدق الله العلي العظيم

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We, the examiner committee certify that we´ve read the M.Sc. thesis entitled:

***Antimicrobial effect of pyocyanin produced by clinical isolate of Pseudomonas aeroginosa***

We have examined the student ( Jenan Kathem Mohammed Al-Asadi)

In its contents.In our opinion it meetsthe standards of thesis for the degree of Masters in Medical Microbiology and Immunology.

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# *Dedication*

**I dedicate my humble work to…..**

***Prophet of Mercy*** *and Liege of the Nation Prophet Muhammad (peace be upon him and his pure family) and to my Liege and my Lord, owner of era and time, Imam Al-Hujjah (May God hasten his relief) and to my Liege and My Lady, Mother of the Sons (peace be upon her).*

***To my pride*** *and honor of the present.. the absent, may God has mercy upon his pure soul.. my father.*

***To whom has*** *had sacrificed her health in my upbringing and education.. My dear mother (God save her).*

***To my dear*** *husband Anwar Al-Sa'adi.. My supporter and the source of my strength in this life.*

***To the twilight stars****.. My precious children (Zainab, Murtaja and Tuqa).*

***To my off*** *spring brothers and sisters.*

**Janan**
**2018**

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# Summary

Dermatophytose is caused by dermatophytes located in cutaneous layer of skin. It also infected nails and hair , it is consired a common fungal disease all of the world. This type of infection was determined among 18 patients (9 males and 9 females) involved in this study. Dermatophytes were isolated from 7(38.88%) of involved patients, while 11(61.11%) of them given a negative results. Distribution of fungal infection among genders showed the presence of two positive males and five positive females. Tinea capitis 2 (11.11%) was the only type of dermatophytoses that gave a positive culture for dermatophytes among males. Whereas, two of tinea capitis 2 (11.11%) and three of tinea corporis 3 (16.66%) were gave a positive culture of dermatophytes in females.

Pyocyanin (PCN), which is bule –green pigments mainly produced by *Pseudomonas aeruginosa* during the stationary phase of the growth curve bacteria and can easily solvent in water. PCN was successfully isolated from clinical isolate of *Pseudomonas aeruginosa* which is registered later in the data base of GenBank under the number MH382164. Chloroform method was used for purification of PCN from bacterial culture and its concentration was determined by spectrophotometer method.

Antimicrobial activity of pyocyanin (PCN) was determined against various species of bacteria and fungi. Antibacterial activity of PCN was tested against 14 strains of isolated bacteria by disk diffusion method. Diagnosis of isolates was performed depending on morphological characters, biochemical tests and by API-20 system. Isolated bacteria distributed between 8 strains of Gram negative and 6 strains of Gram positive. Antifungal action of PCN was

also tested against three strains of yeasts and seven isolates of dermatophytes which also registered in GenBank after molecular identification and sequences of Internal transcribed spacer (ITS) genes, including *Trichophyton interdigitale* (MH383047), *Epidermophyton floccosum* (MH383041), three strains of *Microsporum canis* (MH383044, MH383045, MH383046), and two strains of *Microsporum ferrugineum* (MH383042, MH383043). The MIC value of PCN was also determined against isolated organisms by CLSI methods.

The activity of PCN at 4 µg/ml on the isolated strains of bacteria was variable between Gram positive and negative. The most sensitive strains were *Staphylococcus epidermidis*, *Salmonella* spp., *Streptococcus pneumonia*, *Citrobacter freundii* and *Enterobacter cloacae*, which were showed a zone of inhibition 7, 5.6, 5.3, 5.1, and 5 mm, respectively. Other strains showed less sensitivity toward the 4 µg of PCN as with *Streptococcus agalactiae, Streptococcus pyogenes, Acinetobacter baumannii*, and *E. coli* with a zone of inhibition 2.2, 2.3, 2.3, and 2.4 mm, respectively. *Streptococcus agalactiae* needed less MIC value of PCN (3.5 µg/ml) to inhibit than other bacteria.

An antifungal effect of PCN at 4 µg/ml was tested against isolated fungi. *Microsporum canis* (MH383044), *Epidermophyton floccosum*(MH383041) and *Candida albicans* which revealed a zone of inhibition 6.9, 4.5, and 1.2 mm, respectively were the most sensitive fungi toward PCN with significant differences from most of fungi at $p < 0.05$. The isolates of *Microsporum ferrugineum*(MH383043), *Microsporum canis* (MH383046) and *Cryptococcus terreus* shown a variable sensitivity to PCN (2, 0.7, and 0.3 mm, respectively) with a significant differences from each other at $p < 0.05$. The MIC value of *M. canis* (MH383044) as the most sensitive fungal strain was determined at 3.8 µg/ml, while for *C.albicans* was 4 µg/ml.

Its effect on the antibacterial action of ampicillin and cefotaxime and on the action of griseofulvin as antifungal was also measured. Generally, the effect of PCN (4 µg/ml) on the antibacterial activity of ampicillin was variable on some isolated bacteria. It was decreased by the effects of PCN even at high concentration (8 µg/ml) as with *E. coli* and *E. cloacae* in comparison with ampicillin alone. Whereas, it enhanced ampicillin activity against *S. pyogenes* by decreasing MIC value from 6 to 4 µg/ml. The 2 µg/ml of PCN decreased the MIC action of ampicillin against all of bacterial strains to reach a concentration 2 µg/ml, except *Acinetobacter baumannii* which showed resistance to the largest MIC value (8 µg/ml) of ampicillin in compared with ampicillin alone.

At concentration 15 µg/ml of cefotaxime combined with PCN (4 µg/ml), all bacterial growth was also inhibited, except of *Streptococcus pyogenes, Acinetobacter baumannii* and *Shigella* spp. The lower concentration of cefotaxime (10 µg/ml) revealed no activity against all of bacteria strains, except of *Staphylococcus aureus*, *Citrobacter freundii*, *E. coli* and *Klebsiella pneumoniae*. The PCN didn't show any enhancement effects on the action of cefotaxime at high concentration (20 µg/ml) against all of isolated bacteria. However, the most activity of PCN at 2 µg/ml on the cefotaxime was indicated by elevating its MIC value from 10 to 15 µg/ml against *S. aureus, Citrobacter freundii,* and *E. coli* or from 20 to more than 25 µg/ml against *Acinetobacter baumannii*. The MIC of cefotaxime against other strains was not changed in the presence of PCN.

Antifungal activity of griseofulvin was decreased after mixing with 2 µg/ml of PCN against *M. canis* and *C. albicans* through elevation of MIC value to 100 µg/ml.

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# List of Abbreviations

| | |
|---|---|
| 1-OH-PHZ | 1-hydroxyphenazine |
| API ®20 E System | Analytical Profile Index for Enterobactaerace identification |
| API ® 20STAPH System | Analytical Profile Index for Staphylococci identification |
| API® 20 STRET System | Analytical Profile Index for Streptococci identification |
| API®20 C AUX System | Analytical Profile Index for Yeast identification |
| AHL | *N*-acyl homoserine lactones |
| AIDS | Acquired immunodeficiency disease syndrome |
| ANOVA | Analysis of variance |
| ATP | Adenosine triphosphate |
| BLAST | Basic Local alignment search tool |
| bp | Base paire |
| CF | Cystic fibrosis |
| CFU | Colony forming unit |
| CLSI | Clinical and Laboratory Standards Institute |
| DMSO | Dimethyle sulphoxid |

| | |
|---|---|
| DNA | Deoxyribonucleic acid |
| dntps | Deoxynucleotide triphosphates |
| eDNA | Extrcelluler deoxyribonucleic acid |
| EDTA | Ethylene Diamine Tetra Acetic Acid |
| Fe-SOD | encoding iron SOD |
| HAP | Hospital-acquired pneumonia |
| ITS1 | Internal transcribed spacer 1 |
| ITS2 | Internal transcribed spacer 2 |
| KOH | Potassium hydroxide |
| LB medium | Luria-Bertani medium |
| MDR | Multidrug resistant |
| µg/ml | Microgram per milliliter |
| MHA | Müller Hinton Agar |
| MHB | Müller Hinton Broth |
| MIC | Minimum inhibition concentration |
| µL | micro liter |
| mM | millimol |

| | |
|---|---|
| NADH | Nicotinamide adenine dinucleotide |
| NAD(P)H | Nicotinamide adenine dinucleotide phosphate |
| O.D. | Optical density |
| PCA | phenazine-1-carboxylic acid |
| PCN | Pyocyanin |
| PCR | Polymeras chain reaction |
| QS | Quorum sensing |
| RPM | Revolution per minute |
| SD | Standard deviation |
| SDA | Sabouraud's Glucose Agar |
| SDB | Sabouraud's Glucose Broth |
| SOD | Superoxide dismutases |
| TBE | Tris Borate – EDTA |
| U | Unit |
| XLD | Xylose Lysine Deoxycholate agar |

# *Chapter One*

# *Introduction*

# 1. Introduction:

*Pseudomonas aeruginosa* is a Gram negative bacilli, motile with an ability to grow at 42° C in addition to 37° C in aerobic conditions. It also give a positive result with oxidase and catalase testes (Gellatly and Hancock, 2013; Hameed *et al*., 2014;El-Fouly *et al*., 2015). The bacterium is widely distribution in various environments such as in water and soil ( Moore *et al*., 2002;Pitondo-Silva *et al.,* 2014 ). It also considers an opportunistic human pathogen which has the capacity to causes infection especially in individuals with immunocompromised system (Fujitani *et al*., 2011;Streeter and Katouli, 2016) and also can presence in hospital climate (Bavasheh and Karmostaji, 2017).

*P. aeruginosa* has the ability to secret several virulence factors that can use in their pathogenicity leading to invade and damage cells (Ciragil and Söyletir, 2004; Gellatly and Hancock, 2013). Pigments production could be considers one of these virulence factors (Ernst *et al.*, 2003 ; Lamont and Martin, 2003;Rodríguez-Rojas *et al*., 2009;Hameed *et al*., 2017). Pyocyanin (PCN) is one of the most important pigments produced by *P. aeruginosa* (Karpagam *et al.*, 2013; Jayaseelan *et al.*, 2014; Özyürek *et al.*, 2016). About 90% of *P. aeruginosa* isolated from patients found to produce PCN (Mohammed *et al.*, 2014), especially during stationary phase of the growth curve (Cabeen, 2014). PCN as one toxin is usually secreted from this organism to kill other competitor microorganisms and to help it to colonize tissues as found in immune deficiency individuals with cystic fibrosis or acquired immune deficiency syndrome (Ghoul *et al*., 2015; Bhagirath *et al*., 2016).

The PCN is defined as a blue–green soluble, phenize derivative (Norman *et al*.,2004; O'Malley *et al*., 2004a; O'Malley *et al*., 2004b; Liang *et al*., 2008;

Pierson III and Pierson, 2010 Karpagam *et al*., 2013; Jayaseelan *et al.*, 2014; Özyürek *et al*., 2016). It is one of the secondary metabolites that can act as antimicrobial agent to inhibit the growth of several microorganism such as bacteria and fungus (Reimer *et al*., 2000;El-Shouny *et al.*, 2011; Gharieb *et al.*, 2013 ). Gram positive bacteria were much more susceptible to PCN than Gram negative bacteria (El-Shouny *et al.*, 2011; Gharieb *et al.*, 2013). This effect is occurred by redox-active mechanism through changing the normal electron transport in respiratory chain and formation of the free oxygen radicals ( Ran *et al*., 2003; Lau *et al*., 2004; Wang and Newman, 2008; El-Fouly *et al*., 2015;). Another an important factor that assist PCN activity against other organism is its easily diffuse through the host cell membrane and undergo redox reactions with other molecules (Schwarzer *et al.*, 2008).

The synthesis of PCN is affected by several factors. Quorum sensing (QS) or also called auto inducers molecules is one of them (Lafayette, 2016). Another factors can also effect on enhancement of the production of PCN, including incubation period, pH, temperature, and carbon or nitrogen source (Onbasli and Aslim, 2008; Saha *et al.*, 2008; El-Shouny *et al*., 2011; Gharieb *et al*., 2013; Agrawal and Chauhan, 2016;Gahlout *et al.*, 2017).

Dermatophytoses or as it called tinea or ring worm are the most common disease with widely distributed all over the world (Dismukes *et al*., 2003; AL-Janabi, 2014). It caused by dermatophytes which included three genera; *Trichophyton* spp. , *Microsporum* spp. and *Epidermophyton* spp.(Weitzman and Summerbell, 1995;AL-Janabi, 2014 ). Infection can occurs in the skin of any part of the human body as indicated with name of the disease such as tinea capitis that occurs on head (Dismukes *et al.*, 2003; AL-Janabi, 2014). Dermatophytoses can develop after direct contact with infected animals or human or even from the contaminated soil (Mancianti *et al.*, 2002; AL-Janabi, 2014).

Prevalence of dermatophytoses types is different from one country to another (Ismael, 2011). It dependence on many factors which assess and increased the occurring and distributing of dermatophytosis such as the environmental condition, the healthy statue of exposure persons, unhygienic life style of the community, low socio-economic condition and overproduction (Al Sheikh, 2009; Gadadavar *et al*., 2018).

## Aims of the study:

1- Isolation of PCN from clinical isolated *Pseudomonas aeruginosa*.
2- Investigation for antimicrobial activity of PCN against different isolates of human pathogenic bacteria and fungi.
3- Study the effect of PCN on the activity of various antibacterial and antifungal agents.
4- Determination the common dermatophytoses occurring in Karbala city during the period of the study.

# *Chapter Two*

# *Review of literatures*

# 2. Review of literatures:

## 2.1. *Pseudomonas aeruginosa*

*P. aeruginosa* is a Gram-negative rod shape bacterium with oxidase positive (Hameed *et al.*, 2014). Its can produce Beta- hemolysis on blood agar, while it doesn't fermentation of lactose on MacConkey agar medium. Another characters, including production of a grape-like odor due to secretion of 2-aminoacetophenone and has the ability to grow at 42° C with production of blue–green phenazine pigment (El-Fouly *et al*., 2015). As other members of *Pseudomonas* genus, *P. aeruginosa* mostly considers free-living bacteria that widely distributed in soil (Pitondo-Silva *et al.,* 2014), water (Moore *et al.,* 2002), and hospital environments causing nosocomial infectious disease (Ibrahim *et al.,* 2000; Streeter and Katouli, 2016) and also found in intensive care unit (Bavasheh and Karmostaj, 2017).

*P. aeruginosa* is most frequently associated with various human infections ( Fujitani *et al*., 2011; Streeter and Katouli, 2016) such as urinary tract infection (Fluit *et al.*,2001; Mittal *et al.,* 2009; Bavasheh and Karmostaj, 2017) bacteraemia in hospitalized patients (Fluit *et al*., 2001; El-Solh *et al.,* 2012) and cystic fibrosis (CF) through its ability to permanent resident in the airways of the patients (Evans *et al.,* 1994; Nixon *et al.,* 2001; Ciragil and Söyletir, 2004;). It also considers a common agent of the hospital-acquired pneumonia (HAP), especially in immunocompromised individuals (Ibrahim *et al.,* 2000; Howell *et al.,* 2013;Rangel *et al*., 2015; Micek *et al.,* 2015) and mostly isolated from severe burns and wound infections (Estahbanati *et al*., 2002 ;Ressner *et al.,* 2008).Wound infections caused by multidrug resistant (MDR) *P. aeruginosa* have been associated with high morbidity and mortality rates worldwide (Estahbanati *et al*., 2002; Armour *et al.,* 2007; Ressner *et al.,*

2008). Moreover, infection with *P. aeruginosa* can produce a tissue necrosis through the rapidly progressive pseudomonal acute rhinosinusitis and lead to cause necrotizing sinonasal infections (Kuan *et al.*, 2017).

## 2.2. Virulence factors of *P. aeruginosa*:

*P. aeruginosa* have several virulence factors which play an important role in its pathogenesis in the host (Ciragil and Söyletir, 2004; Gellatly and Hancock, 2013) such as flagella (Veesenmeyer *et al.*, 2009; Byrd *et al.*, 2010; Mohammed, 2011) and type IV pili that located at a cell pole (Hahn, 1997; Hogardt and Heesemann, 2010; Persat *et al.*, 2015). It also secreted alginate (Wozniak *et al.*, 2003; Ciragil and Söyletir, 2004;Mittal *et al.*, 2006 Veesenmeyer *et al.*, 2009) which is an exopolysaccharide used to form biofilm (Déziel *et al.*, 2001; Wozniak *et al.*, 2003; Byrd *et al.*, 2010; Colvin *et al.*, 2012) and another four toxins include exoenzymes S, T, U, and Y (Goehring *et al.*, 1999;Galle *et al.*, 2012). Extracellular virulence factors is also produced by *P. aeruginosa* such as elastase, toxin A, rhamnolipids, lipopolysaccharide (LPS) and protease (Evans *et al.*,1994; Yates *et al.*, 2006; Zulianello *et al.*, 2006;Veesenmeyer *et al.*,2009;Andrejko *et al.*,2013). Lipases and phospholipase are other virulence factors of the bacterium with targeted lipids in the surfactant as well as host cell membranes (Stuer *et al.*,1986; Steinbrueckner *et al.*, 1995;König *et al.*, 1996; Veesenmeyer *et al.*, 2009).

Resistance to most antibacterial agents could be other virulence factors produced by *P. aeruginosa* (Lambert, 2002; Aloush *et al.*, 2006). There are three basic mechanisms make the bacteria resistance to the action of antimicrobial agents, including restricted uptake, efflux, drug inactivation and changes in targets (Lambert, 2002). The restricted uptake represented by the innate resistance of *P. aeruginosa* to all classes of antibiotics by lowering the permeability of such agents through bacterial cell wall (Lambert, 2002;

Breidenstein *et al.*, 2011). Efflux systems of bacteria are composed of three protein components working by eliminating the antibiotic molecules outside the bacterial cell (Lambert, 2002; Sadeghifard *et al.*, 2012). Third mechanism of drug inactivation and changes in targets is mainly resulted from mutational changes in target bacterial enzymes to become resistance to the action of selective inhibition of antibiotic (Lambert, 2002). Formation of biofilm by *P. aeruginosa* can also contribute to the protection of bacteria by the stimulation of the antibiotic resistance (Mulcahy *et al.*, 2008; Chiang *et al.*, 2013).

Pyocyanin (PCN), a blue water soluble pigment, is another virulence factor can produce by *P. aeruginosa* (Mathew *et al*., 2011; Hameed *et al.*, 2017). More pigments can also synthesis by *P. aeruginosa* such as yellow-green fluorescent pigments (pyoverdin) (Lamont and Martin , 2003; Hameed *et al.*,2017), reddish pigment (pyorubin) (Hameed *et al.*,2017) and pyomelanin which is appear as a dark brown pigment (Ernst *et al.*, 2003; Rodríguez-Rojas *et al.*, 2009).

## 2.3. Pyocyanin (PCN):

Pyocyanin (PCN) is a phenazine derivative compound, represents an extracellular blue-green pigment produced by active cultures of *P. aeruginosa* in large quantities and can easily emulsified in water (Norman *et al.*, 2004; O'Malley *et al.*, 2004a; O'Malley *et al.*, 2004b; Liang *et al.*, 2008; Pierson III and Pierson, 2010; Jayaseelan *et al.*, 2014; Karpagam *et al.*, 2013; Özyürek *et al.*, 2016). It recognized in 90% of isolated *P. aeruginosa* from patients with different disease in Baghdad (Mohammed *et al.*, 2014). Stationary phase is the main bacterial growth phase to synthesis PCN (Cabeen, 2014). The zwitter ion nature and the low molecular weight believed to give PCN an easily ability to permeate cell membranes (Reimer *et al.*, 2000). As phenazines compounds,

PCN considers the most synthesis one by *P. aeruginosa* (Saha *et al.*, 2008). Phenazine also biosynthesis by others different types of bacteria genera, including *Brevibacterium* spp., *Burkholderia* spp., and *Xanthomonas* spp., as well as the Gram-positive genus *Streptomyces* spp. and even the archaeal genus *Methanosarcina* spp. (Rao and Sureshkumar, 2000; Beifuss and Tietze, 2005). The main function of such compounds is used as a virulence factor to kill other competitor microorganisms and make producing bacteria colonization in tissue as seen in immunocompromised patients with cystic fibrosis (Ghoul *et al.*, 2015; Bhagirath *et al.*, 2016) and AIDS (Shepp *et al.*, 1994). The association of PCN with the pathogenesis of *P. aeruginosa* occurs via oxidative stress-dependent mechanism or by biofilm formation (Das *et al.*, 2015). The PCN promotes release extracellular DNA (eDNA) as a key player in biofilm formation by *P. aeruginosa* through inducing of cell lysis that mediated by hydrogen peroxide ($H_2O_2$) production (Das and Manefield, 2012). Also it intercalates with the nitrogenous bases of DNA and creates structural perturbation on the double-helix structure (Das and Manefield, 2012; Das *et al.*, 2015).

## 2.4. Synthesis of PCN:

The genetic studies have shown that genes containing in two identical *phzABCDEFG* operons is required for the synthesis of PCN (Mavrodi *et al.*, 2001; Gallagher *et al.*, 2002; Higgins, 2015). Each of this biosynthetic operon is sufficient for production of a single compound called phenazine-1-carboxylic acid (PCA) (Mavrodi *et al.*, 2001; Higgins, 2015). Subsequent conversion of PCA to PCN in *P. aeruginosa* is mediated by two novel phenazine-modifying genes, *phzM* and *phzS*, which encode putative phenazine-specific methyltransferase and flavin-containing monooxygenase, respectively (Fig. 1) (Mavrodi *et al.*, 2001).

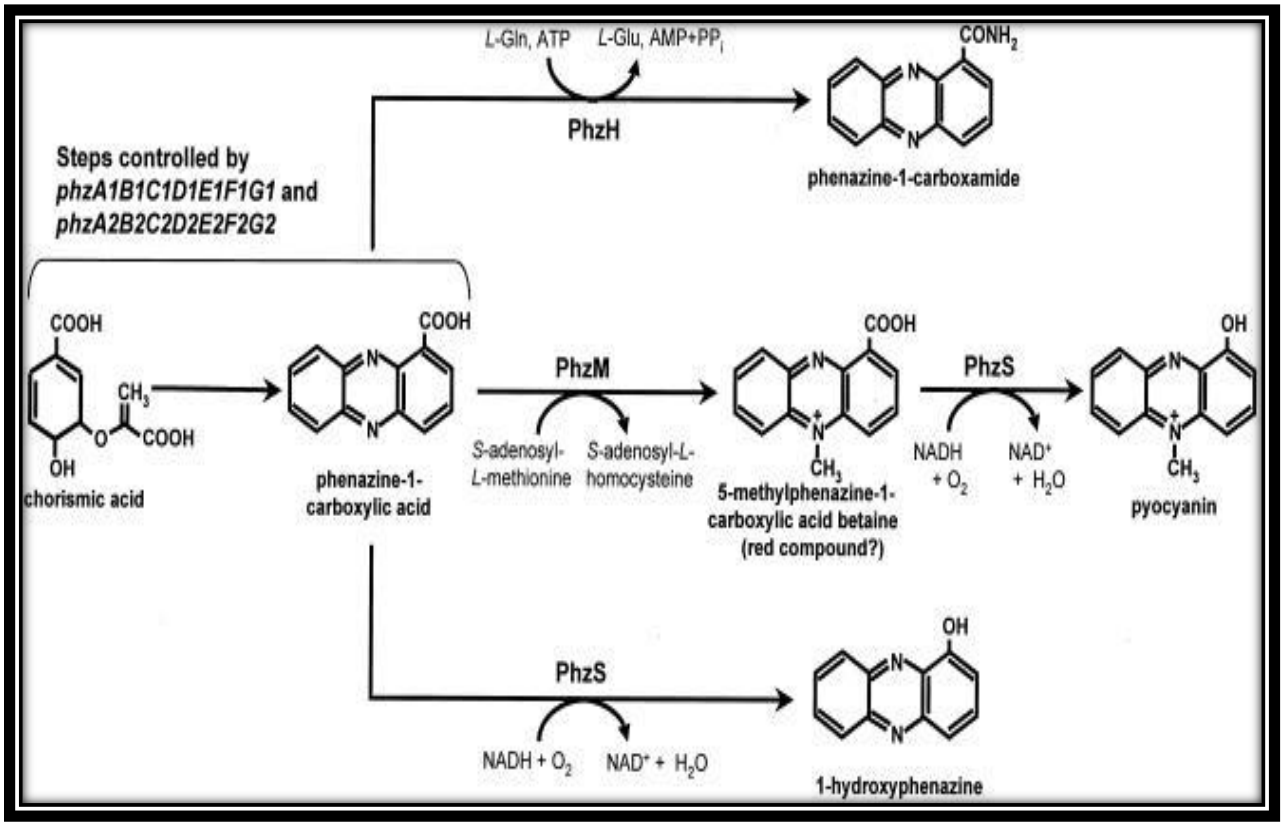



**Fig. (1): Proposed mechanism for the synthesis of PCN, 1-OH-PHZ, and phenazine-1-carboxamide in *P. aeruginosa* PAO1 (Mavrodi *et al.*,2001).**

PCN production is mostly regulated by quorum sensing (QS)(Lafayette, 2016) or cell-to-cell communication which is a small self-generated signal low weight molecules called autoinducers such as *N*-acyl homoserine lactones (AHL) in Gram negative bacteria (de Kievit and Iglewski, 2000; Fuqua *et al.*, 2001). The gene responsible for expression of such molecules is affected by various stimuli such as growth phase, culture density,oxygen and iron availability (Venkataraman *et al.*, 2010).

## 2.5. Conditions effect on PCN production:

There are many factors effected on the production of PCN pigment such as incubation period, pH, temperature, source of carbon or nitrogen during the growth of *P. aeruginosa* (Onbasli and Aslim, 2008; Saha *et al.*, 2008; El-Shouny *et al.*, 2011; Agrawal and Chauhan, 2016 ; Gahlout *et al.*, 2017). Production of PCN was usually began during the first 24 hour of bacterial growth until 72 hour at a temperature of 35$^{o}$ C (El-Shouny *et al.*, 2011; Agrawal and Chauhan, 2016; Gahlout *et al.*, 2017).

PCN was highly produced in media containing peptone as a nitrogen source (El-Shouny *et al.*, 2011). Das and Das (2015) suggested that peptone contains particular peptides or microelements or vitamins that could be essential for pigment synthesis. The maximum production rate of PCN was noted in media with mannitol as a carbon source, while its minimum rate was found in media with sucrose as carbon source (Agrawal and Chauhan, 2016). The pH factor is also contributed in synthesis of PCN when the maximum production was found at natural pH and could decrease at any further increasing of pH level (Agrawal and Chauhan, 2016).

## 2.6. Antimicrobial effect of PCN:

PCN is an effective antimicrobial agent against Gram negative and Gram positive bacteria. Gram positive bacteria are much more susceptible to PCN than are Gram negative bacteria (El-Shouny *et al.*, 2011; Gharieb *et al.*, 2013). In one study, Gram positive bacteria; such as *Staphylococcus aureus*, *Bacillus licheniformis, and Bacillus subtilis* showed more sensitive to PCN than Gram negative bacteria as with *E. coli*, while other species revealed either resistance as *Klebsiella pneumoniae* or intermediately affected as *Salmonella typhi*, and *Proteus mirabilis* (El-Shouny *et al.*, 2011). Gram positive bacteria such as

*Staphylococcus aureus*, *Streptococcus viridians*, and *Streptococcus pneumoniae* also showed more susceptible to the PCN of *P. aeruginosa* with MIC 0.06 mg/ml, than Gram negative bacteria (Gharieb *et al.*, 2013). While in another study, both of bacterial groups showed sensitivity against PCN as with *E. coli*, *Staphylococcus* spp. and *Bacillus* spp. (Abdul-Hussein and Atia, 2016). Also it found that *E. coli* affected by PCN more than *Klebsiella sp*, *S.aureus* and *Proteus* spp. (Sudhakar *et al.*, 2015). Different concentrations of PCN (5,10,15,20 and 25 mg/ml) exhibited a strong antimicrobial action on *Citrobacter* spp. (Saha *et al*., 2008), while the best inhibition of the growth of *S. aureus, S. saprophyticus, S. epidermidis, E. coli and C. freundii* is noted at concentrations (25, 50, 75, and 100%)(Mohammed and Almahde, 2017). *Streptococcus pneumoniae, S. aureus, Acinetobacter* spp., and *E. coli* in comparative study with standard antibiotic revealed sensitive to PCN compared with other bacteria such as *K. pneumoniae* and *Proteus vulgaris*, which did not affected by this pigment (Sweedan, 2010).However, *P.aeruginosa* can defense itself from the effect of the superoxide resulted from PCN activity by possesses two superoxide dismutases (SOD) (SodA for Mn-SOD and SodB for Fe-SOD), especially by Fe-SOD superoxide anion (Hassett *et al.*,1995).

In an animal model, Gharieb and his colleagues (2013) found that after applied of topical PCN to treat wounded skin of rabbits resulting from infection with *S. aureus, K. pneumoniae,* maximum healing and hair growing of the PCN treated area was observed after 14 -20 days in all cases compared to the untreated infected control. Also PCN have antimicrobial activity against several multidrug resistant pathogenic microbes.

In addition to antibacterial effects, PCN have antifungal activity through its ability to arrest the electron transport chain of fungi (Gharieb *et al.*, 2013;

Karpagam *et al.*,2013; El-Fouly *et al.*,2015; Abdul-Hussein and Atia, 2016; Özyürek *et al.*,2016). PCN found more effective against three species of *Candida* spp. and *Cryptococcus neoformans*, while its not effect on *Candida krusei* (Karpagam *et al*., 2013). Also it has antimicrobial activity against *Candida* spp., but not against *Aspergillus niger* (Özyürek *et al.*, 2016). About 35 µg/ml of PCN is needed to inhibit *Candida albicans* (El-Fouly *et al.*, 2015), while *Candida albicans* and other species such as *Candida tropicalis* and *Aspergillus niger* did not affected at higher concentration of PCN (Gharieb *et al.*, 2013; Mohammed and Almahde, 2017). Another study found that PCN have variable antifungal effects on different fungi ranging between effective against *Aspergillus niger*, *Aspergillus fumigatus* and *Cryptococcus neoformans*, moderate effects on *Candida tropicalis* and *C. albicans* and less effect on *C. krusi* (Abdul-Hussein and Atia , 2016). *In vivo* application of PCN is also studies. Topical treatments of PCN ointment on tinea corporis caused by *Trichophyton rubrum* revealed more efficiency to cure the disease with MIC 2000 µg/ml compared to fluconazole (El-Zawawy and Ali, 2016). The skin of rabbits infected with *Candida albicans* showed maximum healing and hair growing after treated with PCN ointment (5 mg) for 14 -20 days in all cases compared to the untreated infected control (Gharieb *et al.*, 2013).

Phenazine derivatives PCN not only kill bacteria and fungi but are also involved in killing of the nematode *Caenorhabditis elegans* through production of intermediates reactive oxygen (Mahajan-Mikios *et al.*, 1999).

## 2.7. Dermatophytes:

Dermatophytes contain three genera, including *Trichophyton* spp., *Microsporum* spp. and *Epidermophyton* spp. (Dismukes *et al.*, 2003; AL-Janabi, 2014). They also can divide into three groups based on the source of infection. First one is anthropophilic when infection transmitted from human

to another via direct contact (AL-Janabi, 2014) as with *Microsporum langeronii* which found it caused tinea corporis in most children of a public primary school of Antananarivo (Madagascar)(Carod *et al.*,2011) and *Trichophyton interdigitale* as a causative agent of tinea faciei (Pragya *et al.*, 2017).Zoophilic is another group of dermatophytes when the fungi transmitted from animals, domestic or wild, to human or other animals (AL-Janabi, 2014) as with *Microsporum canis* and *T. mentagrophytes* which mostly isolated from dogs and cats in Italy (Mancianti *et al.*, 2002). Third group of dermatophytes is geophilic which found in soil living on keratinous materials as saprophytes and can transmit to human after contact with contaminated soil as with *Microsporum gypseum* (AL-Janabi, 2014). The infection by anthropophilic type of dermatophytes usually recognized by the chronic nature with low inflammation, while an inflammation is high when disease caused by zoophilic or geophilic (Weitzman and Summerbell, 1995).

Dermatophytes have the ability to cause a cutaneous skin disease called dermatophytoses.This disease considers a common fungal infection in different parts of the human body which enrichment with keratin, especial hair, skin and nail (AL-Janabi,2014). It's also called ring-worm when the lesion appears as a ring shape with clear center and inflammatory edge (Dismukes *et al.*,2003;AL-Janabi,2014).Tinea is another term of dermatophytoses which could take a different name based on infected site of the human body such as tinea unguium (onychomycosis) in finger nails, tinea faciei on the face, tinea pedis (athlete's foot) on the feet, and tinea corporis on any glabrous skin (Weitzman and Summerbell, 1995; AL-Janabi, 2014). Infection usually occurs in both genders at different ages (Al Sheikh, 2009; Akcaglar *et al*., 2011; Ismael, 2011).

The ability of dermatophytes to use keratin protein, the main protein constituent of hair, nails and skin, is related to its production of a proteolytic

keratinase (Weitzman and Summerbell, 1995; AL-Janabi, 2014). However, more than twenty types of protease can produce by dermatophytes that play a role in invasion of keratinized structure and causes infection, but the role of them as a virulence factor is not specific (Achterman and White, 2012). Other types of enzyme can also produce by dermatophytes using for digestion Process such as alkaline phosphatase and N-acetyl-beta-glucosaminidase (Brasch *et al*.,1991).

In addition to enzymes, several factors can associate with high prevalence of dermatophytoses such as high temperature and humidity in tropical and sub-tropical regions (Gadadavar *et al.*,2018) and type of geographical region when the infection is most common in rural areas than in urban regions (Al Sheikh, 2009). The chronic diseases or disorder in the human body is also play a role in distribution of infection as found among patients with diabetes (AL-Janabi, 2014). Other factors such as overcrowding and unhygienic life style of the community with low socio-economic background are contributed for developing of dermatophytoses (Gadadavar *et al.*, 2018). Patients living in low socio-economic conditions consider highly infected with this organisms than who living in middle and high socio-economic statues and tinea infections are significantly occurred in the developed countries due to receive immunosuppressive drugs and diseases like AIDS (Negi *et al.*, 2017). Moreover, close contact with animals, using of antibiotics and steroid drugs and also living in community are also increased infection (Weitzman and Summerbell ,1995).

The prevalence of dermatophytoses is differed from country to another or even in the same country. Among 67 Iraqi patients with various dermatophytoses infections, tinea pedis (45.1%) was the most common infection, followed by tinea manuum (22.2%), tinea capitis (11.8%), tinea corporis (7.8%), tinea unguium (5.9%), and both of tinea faciei and tinea

cruris (3.57%) (Ismael, 2011). Tinea capitis showed the most clinical type of dermatophytes (1.01%) among school children in Menoufia governorate of Egypt ( Farag *et al.*, 2018). In Saudi Arabia, tinea corporis and tinea cruris were the most common infections in the Eastern province due to stretched of this region along with the Arab Gulf region (Al Sheikh, 2009). About 76% of 52 children of Madagascar suffering from tinea corporis and 64% from tinea capitis (Carod *et al.*, 2011).

Distribution the species of dermatophytes is also differed from one country to another. A study of five years in Kuwait showed that *Trichophyton mentagrophytes* is the most prevalence (39%) species among 2730 patients, followed by *Microsporum canis* (16%) and *Epidermophyton floccosum* (6.2%) (Yehia *et al.*, 2009). *Trichophyton mentagrophytes* is also revealed highest percentage of infection (57.14%) than other species, including *Trichophyton rubrum, T. verrucosum, T. schoenleinii, T. violaceum, Microsporum canis* and *M. audouinii* (Ismael, 2011).

# *Chapter Three*

# *Materials and Methods*

# 3. Materials and Methods

## 3.1. Materials:

### 3.1.1. Apparatuses and Equipment

All of apparatus and instruments used in the experiments of the current study were illustrated in tables 1 and 2.

**Table (1): Apparatuses used in the study**

| No. | Apparatuses | Company | country of origin |
|---|---|---|---|
| 1 | Autoclave | Hirayama | Japan |
| 8 | Biological safety cabinet | Lab Tech | Korea |
| 2 | Bunsen Burner | Jenway | Germany |
| 3 | Centrifuge | Hettich | |
| 4 | Cooling centrifuge | Hitachi | |
| 7 | Compound microscope | Leica | |
| 5 | Different sizes of Micropipettes | Slamed | |
| 6 | Digital balance | Kern | |
| 10 | Eppendorf tubes | Merck | |
| 9 | Gel documented | Cleaver Scientific Ltd | U.K. |

| | | | |
|---|---|---|---|
| 14 | Haemocytometer | Marienfeld | Germany |
| 15 | Horizontal Electrophoresis system | Cleaver Scientific Ltd | UK |
| 17 | Incubator | Fisher scientific | Germany |
| 16 | Oven | Fisher scientific | |
| 18 | pH-meter | WTW inolab | |
| 11 | Refrigerator | Vestel | Turkey |
| 20 | Spectrophotometer | APEL | Japan |
| 12 | Thermal PCR | Cleaver Scientific Ltd | U.K. |
| 19 | Vortex | Gemmy | U.S.A. |
| 13 | Water bath | GFL | Germany |

**Table (2): Equipment used in the study**

| No. | Equipment | Company | country of origin |
|---|---|---|---|
| 1 | Eppendorf tubes | Merck | Germany |
| 2 | Funnel 250 ml | Marienfeld | Germany |
| 3 | Glass slides | Supertek | India |
| 4 | Gloves | Salalah Medical Supplies | Oman |
| 5 | Inoculating loop | Loop Shandon | England |
| 6 | L- shape spreader glass loop | RunLab | China |
| 7 | Microscope cover glass | Supertek | India |
| 8 | Micro-filter paper | Jiao Jie | China |
| 9 | Tissues | Kardelen | Turkey |
| 10 | Pyrex Beakers | Marienfeld | Germany |
| 11 | Pyrex Cylinders | | |

| | | | |
|---|---|---|---|
| 12 | Pyrex conical flask | | |
| 13 | Petri dish | PlastLab | Lebanon |
| 14 | Plastic microdilution plate (96 wells) | CITOTEST Labware Co., Ltd | China |
| 15 | Syringe 5 ml | MEDI | China |
| 16 | Syringe Filters 0.22 µm | Bio Basic Inc | Canada |

### 3.1.2. Chemical and biological materials

Different types of chemical and biological materials were used in current study (tables 3 and 4).

**Table (3): Chemical and biological materials used in the study**

| No | Chemicals and biological materials | Company | country of origin |
|---|---|---|---|
| 1 | AccuPower® PCR Premix | Bioneer | Korea |
| 2 | Agarose | Reagent World | USA |
| 3 | Ampicillin sodium | Strides | India |

| | | | |
|---|---|---|---|
| 4 | Bacitracin disc | Bioanalyse | Turkey |
| 5 | Boric acid | Fisher scientific international | U.K |
| 6 | Cefotaxime | LDP | Spain |
| 7 | Chloramphenicol | Kontam | China |
| 8 | Chloroform | Himedia | India |
| 9 | Dettol | Al samma Al mushmesa | Jordan |
| 10 | Dimethyl sulfoxide | Qualikems | India |
| 11 | DNA Ladder (100 -3000) bp | Geneaid | Tawian |
| 12 | Ethidium Bromide | BioTech | U.S.A. |
| 13 | Ethanol (99.9%) | Scharlau | Spain |
| 14 | Ethylene diaminetetra Acetic acid | BDH | England |
| 15 | Glucose | Samarra | Iraq |
| 16 | Gram Stain | Jourilabs | Sorachim Switzerland |

| | | | |
|---|---|---|---|
| 17 | Griseofulvin | Sigma-Eldritch | Germany |
| 18 | Hydrogen peroxide (3%) | Solvochem | U.K. |
| 19 | Lactophenol cotton blue stain | Himedia | India |
| 20 | MacFarland 0.5 | BioMerieux | France |
| 21 | MacFarland 2 | BioMerieux | France |
| 22 | Optochin disc | Bioanalyse | Turkey |
| 23 | Potassium Hydroxide | Himedia | India |
| 24 | Sodium chloride solution (0.85%) | BioMerieux | France |

| | | | |
|---|---|---|---|
| 25 | Tetramethyl-p-phenylenediaminedihydrochloride | Scharlau | Spain |
| 26 | Tris base | Himedia | India |

**Table (4): Biological kits**

| No. | Kit name | Company | country of origin |
|---|---|---|---|
| 1 | Presto™ Mini gDNA Bacteria | Geneaid Biotech | Taiwan |
| 2 | FavorPrep™ Fungi/Yeast Genomic DNA Extraction Mini Kit | FavorgenBiotchCorp. | |

## 3.1.3. Culture Media

All of cultured media that already prepared were purchased as mention in table (5), except Sabouraud's glucose agar (SGA), Sabouraud's glucose broth and Luria-Bertani medium.

**Table (5): Culture media**

| No. | Media | Manufacturing Company | country of origin |
|---|---|---|---|
| 1 | Agar agar | HIMIDIA | India |
| 2 | Brain Heart Infusion Broth | | |
| 3 | Blood Base Agar | | |
| 4 | Luria-Bertani medium | Prepare in laboratory | |
| 5 | MacConkey Agar | HIMIDIA | India |
| 6 | Mannitol Salt Agar | | |
| 7 | Müller-Hinton Agar | | |
| 8 | Müller-Hinton Broth | | |
| 9 | Nutrient Agar | | |
| 10 | Peptone water | | |
| 11 | Sabouraud's Glucose Agar | | |

| | | | |
|---|---|---|---|
| 12 | Sabouraud's Glucose Broth | Prepare in laboratory | |
| 13 | Xylose Lysine Deoxycholate agar | HIMIDIA | India |
| 14 | Yeast Extract Powder | | |

### 3.1.4. Rapid multi-system tests

Different types of Api 20 system were used for diagnosis of isolated bacteria and fungi (table 6).

**Table (6): Rapid multi-system tests**

| Kit name | Manufacture company | Country |
|---|---|---|
| API ®20 E System | BioMérieux | France |
| API ® 20 STAPH System | | |
| API® 20 STRET System | | |
| API®20 C AUX System | | |

### 3.1.5. Molecular requirements

The main components of PCR reaction were purchased from Bioneer Corp. company (Daejeon, Korea) as a PCR-PreMix kit. The components of kit was illustrated in table (7). The sequence of primers that used in PCR was illustrated in table (8).

**Table (7): PCR premix components**

| Component | Concentration |
|---|---|
| Taq DNA polymerase | 1U |
| dNtps (dATP, dCTP, dGTP, dTTP) | 250 µM for each one |
| Reaction Buffer | 1X |
| $MgCl_2$ | 1.5 mM |
| Stabilizer and tracking dye | - |

**Table (8): Primers sequences used for PCR.**

| Organism name | Gene name | Primer | Reference of Primer sequences |
|---|---|---|---|
| ***Pseudomonas aeruginosa*** | **16S rRNA** | **F-5´-AGAGTTTGATCCTGGCTCAG-3´**<br>**R-5´GGTTACCTTGTTACGACTT-3´** | El-Fouly *et al.*, 2015 |
| **Dermatophytes** | **ITS 1** | **5´-TCCGTAGGTGAACCTGCGG-3** | Hsiao et *al.*, 2005 |
| | **ITS 2** | **5´-GCTGCGTTCTTCATCGATGC-3** | |

F: Forward primer; R: Reverse primer

### 3.1.5.1. Extraction of bacterial DNA

DNA extraction of bacteria was performed by using Presto™ Mini gDNA Bacteria Kit (table 9).

**Table (9): Components of DNA extraction kit of bacteria**

| Component | GBB100 |
|---|---|
| GT Buffer | 30 ml |
| GB Buffer | 40 m |
| W1 Buffer | 45 ml |
| Wash Buffer | 25 ml |
| Lysozyme* | 110 mg |
| Proteinase K | 11 mg X 2 |
| Elution Buffer | 30 ml |
| GD Columns | 100 |
| 2 ml Collection Tubes 8 200 | 200 |

* Lysozyme should be stored at -20ºC for extended periods.

### 3.1.5.2. Extraction of dermatophytic DNA

DNA of dermatophytes was extracted by using FavorPrep™ Fungi/Yeast Genomic DNA Extraction Mini Kit (table 10).

**Table (10): Components of DNA extraction kit of dermatophytes**

| Component | Cat.No:FAFYG 001 |
|---|---|
| Bead Tube | 50 pcs |
| FA Buffer | 60ml |
| FB Buffer | 32 ml |
| TG1 Buffer | 22 ml |
| TG2 Buffer | 15 ml |
| W1 Buffer [a](concentrate) | 22 ml |
| Wash Buffer [b](concentrate) | 10 ml |
| Elution Buffer | 7 ml |
| Lyticase solution | 550µ×5 |
| Proteinas K[C] | 11mg |
| TG Mini Column | 10 pcs ×5 |
| Collection Tube | 100 pcs |
| Elution tube | 50 pcs |

a: Ethanol volume for W1 Buffer.
b: Ethanol volume for Wash Buffer.
c: dd$H_2O$ volume for Proteinase K.

# 3.2. Methods

## 3.2.1. Preparation of Culture Media:

### 3.2.1.1. Preparation of ready-made media

Blood Base Agar, Nutrient Agar, MacConkey Agar, Xylose Lysine Deoxycholate agar (XLD), Mannitol Salt Agar, Müller-Hinton Agar and Müller-Hinton Broth media are prepared according to the instructions of the manufacturing company. Sterilization of culture media and solutions are achieved by autoclaving at 121˚C/1 pound for 15 minutes.

### 3.2.1.2. Media prepared in the laboratory:

#### 3.2.1.2.1. Luria-Bertani (LB) broth

LB broth was prepared by dissolving 10 g peptone, 10 g NaCl, and 5 g yeast extract in a conical flask (1 liter) containing 950 ml of distill water. The components were mixed by shaking until they dissolved. pH was adjusted to 7.0 with 5N NaOH (~ 0.2ml) and also adjusted the final volume of the solution to one liter of $H_2O$. A flask was sterilized by autoclave for twenty minutes at 121˚ C/1 pound. Media was left to cool at 45˚ C and kept in refrigerator until used.

#### 3.2.1.2.2. Sabouraud's Glucose Agar (SGA)

SGA was prepared by dissolving 10 g peptone, 20 g glucose, and 15 g agar in a conical flask (1 liter) containing 950 ml of distill water. The components were mixed by shaking until they dissolved. Final volume of the solution was completed to one liter of $H_2O$. A flask was sterilized by

autoclave for twenty minutes at 121° C/1 pound. Chloramphenicol (0.05 g/l) was added into media after cooling to 45° C to prevent the growth of contaminated bacteria. Media was poured in sterilized Petri dishes or as a slant in sterilized test tube and kept in refrigerator until used.

### 3.2.1.2.3. Sabouraud's Glucose Broth (SGB)

As with SGA, SGB media was prepared by dissolving 10 g peptone, and 20 g glucose in a conical flask (1 liter) containing 950 ml of distill water. The components were mixed by shaking until they dissolved. Final volume of the solution was completed to one liter of $H_2O$. A flask was sterilized by autoclave for twenty minutes at 121° C/1 pound. Chloramphenicol (0.05 g/l) was added into media after cooling to 45°C to prevent the growth of contaminated bacteria. Media was kept in refrigerator until used.

## 3.2.2. Preparation of solution and reagents:

### 3.2.2.1. Preparation of 1X TBE buffer

1X TBE ((Tris-BoricAcid-EDTA) was prepared by dissolving 89 mM tris-base (pH 7.6), 89 mM boric acid, and 2 mM EDTA in a conical flask (1 liter) containing 950 ml of distill water. The components were mixed by shaking until they dissolved. Final volume of the solution was completed to one liter of water (Green and Sambrook, 2001).

### 3.2.2.2. Rehydration of primers

The primers listed in table (8) were used in present study. These primers were provided in a lyophilized form. Rehydration of them was performed by dissolving in sterile deionized water to obtain a stock concentration of 100

pmol, and then stored in the deep freezer until used in PCR amplification. The suitable concentrations from stock primer were prepared as needed.

### 3.2.2.3. Preparation of ampicillin concentrations

For preparation a stock solution of ampicillin (10 µg/ml), 10 µg of ampicillin pure powder was dissolved in 1ml of sterile distill water to obtain 10 µg/ml. Serial dilutions (0.125, 2, 4, 6, and 8 µg/ml) were prepared from this stock (CLSI-M07-A10, 2015).

### 3.2.2.4. Preparation of cefotaxime concentrations

Stock solution of cefotaxime (30 µg/ml) was prepared by dissolving 30 µg of cefotaxime pure powder in 1 ml of sterile distills water. A serial dilution (0.625, 10, 15, 20, and 25 µg/ml) were prepared from this stock (CLSI-M07-A10, 2015).

### 3.2.2.5. Preparation of griseofulvin concentrations

Stock solution of griseofulvin (1600 µg/ml) was prepared by dissolving 1600 µg of griseofulvin pure powder in 1 ml of DMSO. Serial dilutions (25, 50, and 100 µg/ml) were prepared from this stock (CLSI-M38-A2, 2008).

### 3.2.2.6. Preparation of oxidase test:

The oxidase test is prepared by dissolving 1 g of substrate tetramethyl-p-phenylenediamine dihydrochloride into 80 ml of distill water, followed by completed the volume into 100 ml. Then, stored in a dark container in the refrigerator at 8° C until usage.

### 3.2.2.7. Preparation of KOH (20%):

For preparation a solution of 20% KOH, 20 g of potassium hydroxide was dissolved in 80 ml of sterile distilled water and then completed the volume to 100 ml. The solution was mixed well and finally store in clean container at room temperature.

## 3.2.3. Isolation of microorganisms:

### 3.2.3.1. Isolation of *Pseudomonas aeruginosa*

*P. aeruginosa* was isolated from urine of female with UTI (7 years) admitted to pediatric teaching hospital, Karbala-Iraq in June 2017. Specimen was cultured on blood base agar, MacConkey agar and nutrient agar and incubation at 37° C for 18-24 hours. The bacteria were Gram negative rod shape when stained with Gram stain, beta hemolysis on blood agar, pale colonies or non-lactose ferment on MacConkey agar with fruit odor, and producing a greenish-blue color on nutrient agar. Biochemical tests such as oxidase test was also performed as followed:

### 3.2.3.2. Oxidase test:

The oxidase test was used for identification of *Pseudomonas* spp. and other Gram negative bacteria with the production of the cytochrome oxidase enzyme. A piece of filtered paper was saturated with a few drops of oxidase reagent and then a colony of the test organism was taken from nutrient agar and smeared on this piece of filtered paper. A result was read within 10 seconds by the appearance of a dark purple color, meaning that the organism was oxidase producing and leading to convert the substrate tetramethyl-p-phenylenediamine dihydrochloride into indophenol with a dark purple color as a final product (Tille, 2014).

### 3.2.3.3. Confirmatory identification of *P. aeruginosa*

#### 3.2.3.3.1. Diagnosis by API 20 Systems:

API 20 system consists of strip with dehydrated substrates in separated twenty micro tubes. These tests are reconstituted by adding an aliquot of API system to each micro tube and been inoculated with bacterial suspension which was adjusted with MacFarland 0.5. Strip was incubated for 18-24 h at 35-37˚ C. After incubation, metabolism produces color change that is either spontaneous or revealed by the reagents addition. Identification of specific species is made by using indicators in differential charts supplied by the manufacturer. The reactions were read according to the reading table and the identification was obtained by referring to the analytical profile, which is read as NO.2206046.

#### 3.2.3.3.2. Molecular diagnosis:

#### A- DNA extraction:

The DNA of *P. aeruginosa* was extracted according to the manufacturer's instructions supplied with the extraction kit. The first step before extraction method, reagents of the kit were prepared as follow:

1- **Wash Buffer:** Absolute ethanol (100 ml) was added to the bottle of wash buffer, then mixed by shaking for a few seconds. The bottle must be closed tightly after each usage to avoid ethanol evaporation.
2- **Proteinase K:** Deionized water ($ddH_2O$) (1.1 ml) was added into the vial of proteinase K, and then vortex to ensure that proteinase K is completely dissolved. Once it was completely dissolved, it centrifuged for a few seconds to spin it down. For extended periods, the $ddH_2O$ and

proteinase K mixture should be stored at 4º C. Fresh dd$H_2O$ was used only as ambient $CO_2$ can quickly cause acidification.

**The DNA extraction was performed as in the following protocol:**

**1. Sample Preparation**

Bacterial cells (up to 1 x $10^9$) were transferred to 1.5 ml micro- centrifuge tube. It was centrifuged for one minute at 14,000-16,000 RPM and then discards the supernatant. The 180 µl of GT buffer was added and re-suspend the cell pellet by vortex or pipette. Then, 20 µl of proteinase K was added. Finally, the tube was incubated at 60º C for at least 10 minutes. During incubation, the tube was inverted every three minutes.

**2. Lysis**

A 200 µl of GB buffer was added to the sample and mixed by vortex for 10 seconds. Tube was incubated at 70º C for at least 10 minutes to ensure the sample lysate is clear. During incubation, tube was inverted every three minutes. At this time, pre-heat the required elution buffer (200 µl per sample) to 70º C (prepare for step 5 DNA elution).

**3. DNA Binding**

To the last prepared tube, about 200 µl of absolute ethanol was added to the sample lysate and mixed immediately by vigorously shaking. If precipitate appears, suspension should be break up as much as possible with a pipette. A GD column must prepare by placed it in a 2 ml collection tube. Then, the mixture (including any insoluble precipitate) was transferred into the GD column and centrifuged at 14,000-16,000 RPM for two minutes. The 2 ml collection tube containing the flow was discarded and placed the remaining GD column in a new 2 ml collection tube.

**4. Wash**

About 400 µl of W1 buffer was added to the GD column, followed by centrifuged at 14,000-16,000 RPM for 30 seconds and then the flow- through was discarded. The GD column was placed back into the 2 ml collection tube. 600 µl of wash buffer was added to the GD column after making sure ethanol was added. GD column was centrifuged at 14,000-16,000 RPM for 30 seconds and then discard the flow-through. GD column was later put back in the 2 ml collection tube and centrifuged again for 3 minutes at 14,000 -16,000 RPM to dry the column matrix.

**5. Elution**

A volume of 100 µl of elution solution was finally added to the GD column. If fewer samples used, elution volume (30-50 µl) can reduced to increase the DNA concentration. If higher DNA yield is required, DNA elution step can repeat to increase DNA recovery and the total elution volume to approximately 200 µl.

## B-Application of polymerase chain reaction (PCR) for diagnosis of *P. aeruginosa*:

Diagnosis of *P. aeruginosa* was completed by using polymerase chain reaction (PCR) through amplification of 16S rRNA gene. PCR mixture sets up in a total volume of 20 µl, including 5 µl of PCR master mix, 1 µl of each primer while 1µl of template DNA is being used. The rest of the volume is completed with sterile deionized distilled water. At the beginning, the extracted DNA, primers and PCR master premix were thawed at room temperature, vortexed and centrifuged briefly for bring the contents to the bottom of the tubes. Negative control contains all material, except template

DNA was also used. PCR reaction tubes were placed into thermocycler PCR instrument which programmed according to the PCR conditions as mentioned by EI-Fouly *et al*. (2015) (table 11).

**Table (11): The PCR condition for amplification of 16S rRNA gene of bacteria**

| Gene | PCR step | Temp. | Time | Repeat |
|---|---|---|---|---|
| **16S rRNA** | Initiation | 95 °C | 1 min | 1 |
| | Denaturation | 95 °C | 30 sec. | 35 cycles |
| | Annealing | 53.6 °C | 1 min | 1 |
| | Extension | 72 °C | 1 min | 1 |
| | Final extension | 72 °C | 90 sec | 1 |

### 3.2.3.3.3. Preparation of Agarose gel

Agarose gel is used for separation and visualization DNA of various sizes. Agarose gel was prepared in 1% concentration for quality of the extracted DNA, by dissolving 1g of agarose powder in 100 ml of 1X TBE buffer (electrophoresis buffer) in conical flask (150 ml). It mixed perfectly to dissolve the agarose powder in the buffer to create a suspension. Agarose suspension was melted until homogenized by using microwave for one minute until all of the small translucent agarose particles are dissolved. A molten gel was left to cool until 60° C, followed by adding 0.5 µg/ml Ethidium bromide which is used for visualization of the DNA before pouring it into the gel tank. Cooled agarose was poured on electrophoresis tray which contains a comb. After the final solidification, the comb was carefully removed to obtain wells.

### 3.2.3.3.4. Electrophoresis method:

A ready prepared tray with agarose was immersed in the electrophoresis tank which was full with 1 X TBE buffer. Each well within agarose was loaded with 10 µl of each DNA sample. A10 µl of 100 bp DNA ladder was also loaded in the first left well of the agarose electrophoresis gel. The electrophoresis tank closed with its special lid and powered on with a 70 V and 60 A of electric current for 30 min. The final product was examined to show the gene band under short wavelength of UV by gel documentation instrument (Green and Sambrook, 2001).

### 3.2.3.3.5. Sequences of PCR product:

All of PCR final products were sent to the Bioneer company (Korea) for sequencing based on the same primers that used in PCR amplification. The obtaining sequences were uploaded to the BLAST web site of GenBank (https://www.ncbi.nlm.nih.gov/GenBank/) for comparative with other universal sequence for determining the species of our bacterial isolate. After diagnosis of bacterial species, our bacterial sequence was submitted again to the GenBank for registering the isolated bacteria within its data.

## 3.2.4. Production of pyocyanin (PCN):

*P. aeruginosa* was cultured in 150 ml of LB medium in 250 ml conical flask and incubated at 37 ° C for 1-5 days. The conversion of media into green color is a good indicator for production of PCN (Fig. 2 and 3).

**Fig. (2): Culture of *P. aeruginosa* with PCN production in nutrient agar**

### 3.2.4.1. Extraction of PCN:

PCN was isolated from grown bacteria in broth culture (Fig. 3) by filtration through filtered paper as first step, then centrifuged at 6000 RPM for 10 min. The superannuated was filtered again with 0.2 µm microfilter to get PCN without bacterial cells. To extract PCN from filtered liquid media, equal amount of chloroform was added to media with PCN (1:1) to obtain two layers. The upper brown layer was discarded, while second lower layer with blue color saved to get PCN. After two to three time of isolation step repeating, extracted PCN was dried by oven at 50° C to evaporate the chloroform and concentrate PCN which appears as a dark green color (El-Fouly *et al*., 2015).

**Fig. (3): PCN with deep green color production by *P.aeruginosa* in LB medium**

### 3.2.4.2. Determination of PCN concentration:

The concentration of PCN was determined by measuring the absorption of PCN solution by spectrophotometer at wavelength 520 nm according to the following formula: (El-Fouly *et al*., 2015)

**Concentration of PCN (µg/ml) =$O.D_{520}$ × 17.072.**

Where:

$O.D_{520}$: optical density at 520 nm

## 3.2.5. Isolation of other bacteria:

Different strains of bacteria were isolated from patients with various diseases during admitted to the Al- Kafeel hospital, and Al-Imam Al-Hussein Medical city, Karbala-Iraq in December 2017. The isolated strains were consisted of 14 strains distributed between 8 strains of Gram negative, including *Acinetobacter baumannii, Citrobacter freundii, E. coli, Enterobacter cloacae, Klebsiella pneumoniae, Proteus mirabilis, Salmonell*a species, *Shigella* species and 6 strains of Gram positive, including *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus agalactiae* and *Enterococcus faecalis*. Gram negative bacteria were successful isolated by using blood agar and MacConkey agar, while Gram positive bacteria were isolated by culturing on blood agar and mannitol salt agar. All isolated bacteria were incubated at 37° C for 18-24 hours at aerobic condition, except *Streptococcus pneumonia* which was incubated at 5% $CO_2$.

### 3.2.5.1. Diagnosis of isolated bacteria:

#### 3.2.5.1. 1. Presumptive diagnosis

The morphological characters are the first step for diagnosis of isolated bacteria. These characters are clearly identified by using a Gram stain. The main purpose from Gram stain is to differentiation between bacteria which take a blue color (crystal violet stain) as Gram positive or those take a pink color (Safranin stain) as Gram negative. The usage of this stain is also useful to determine the shape, size, and arrangment of bacterial cells (Tille, 2014). Biochemical tests were also used for diagnosis of isolated bacteria.

### 3.2.5.1.2. Biochemical tests

**1- Bacitracin susceptibility test**

This test is used for diagnosis and differentiation between *Streptococcus pyogenes* which is sensitive to the bacitracin (positive result) and *Streptococcus agalactiae* which is resistance to this antibiotic (negative results). The protocol of bacitracin susceptibility test was performed as mention by Tille (2014). About 2-3 colonies were taken from a culture of isolated bacteria that incubated at 37° C for 24 hours. These colonies were cultured on blood agar plates by streaking. A disk of bacitracin (0.04 U) was inoculated on the cultured plate by sterilized forceps. The cultured plates were incubated at 37° C for 24 hours with 5% to 10% $CO_2$. Zone of inhibition was determined. Any inhibition zone around the disk is considered positive result, whereas the absence of inhibition is considered resistance or negative result.

**2- Optochin disc test:**

Optochin (ethyl hydrocupreine hydrochloride) is an antibiotic have the ability to react with the ATPase enzyme which effect on production of adenosine triphosphate (ATP) in microorganisms. The sensitivity of microorganism to optochin in disk form has a useful role in the differentiation of alpha-hemolysis *Streptococcus* species such as *S. pneumoniae* (positive test) from other *Streptococcus* species which are resistant to the optochin. The protocol was performed as mention by Tille (2014) which includes preparation of culture of selective bacteria by streaking 2-3 colonies of it on blood agar. Optochin disk was gently added on the inoculated media by sterilized forceps, then incubated at 35° C for 18-24 hours in 5% to10 % of $CO_2$. Any inhibition zone around the disk with the diameter of ≥ 14 mm is considered positive result.

**3- Catalase Test:**

The catalase test depends on the ability of catalase enzyme produce by microorganism to breakdown the hydrogen peroxide into water and oxygen which can be indicated by the production of the bubbles in the air. This test mainly used to differentiate *Staphylococcu*s spp. (positive test) from other Gram-positive cocci such as *Streptococcus* spp. (negative test). The protocol of test was performed as mentioned by Cheesbrough (2005). A few colonies of selected bacteria was transferred from grown culture by wooden stick rod to the clean glass slid, then a drop of 3% $H_2O_2$ was added on the colonies and mixed with each other. The air bubbles were noted to record the result.

**4- Coagulase test:**

Coagulase is an enzyme causing plasma to clot by converting fibrinogen to fibrin clot. This test is used to differentiate *Staphylococcu*s *aureus* which produces coagulase enzyme from other *Staphylococcu*s species that are non-coagulase production. Tube test was performed in this study as mentioned by Tille (2014). Several colonies from culture of *Staphylococcus* spp. was mixed with 0.5 ml of human plasma into a sterile tube. Tube was incubated at 35-37° C in ambient air for 4 hours. The visible clot will indicate the positive result, while negative result indicated by non clotting appearance. If the result is negative after 4 hours, the tube must be incubated again at room temperature overnight and check it for clot formation.

## 3.2.5.1.3. Confirmatory identification:

Complete diagnosis of isolated bacteria was performed by using different types of API-20 system, including API-20E medium for diagnosing of Enterobacteriaceae, API-20 Staph medium for *Staphylococcus* species, and API-20 Strep medium for *Streptococcus* species (section 3.2.3.3.1).

### 3.2.6. Isolation of Dermatophytes:

A total of 18 patients with different types of dermatophytoses, including 9 males (4.5-40 years) and 9 females (12-45 years) were involved for isolation of dermatophytes during admitted to Al-Imam Al-Hussein Medical city, Karbala-Iraq from June to December 2017. Dermatophytes were isolated by collecting skin specimens from involved patients. The infected lesions were clinically diagnosed by specialized dermatologists of the hospital consultation department. Skin samples were collected by scraping the edge of lesion after cleaned with 70% ethyl alcohol. Each skin specimen was divided into two parts. First part was microscopically examined with 20% KOH for detection of fungal conidia and/or hypha within the skin scales. Another specimen part was cultured on Sabouraud's glucose agar (SGA) to incubate at 28°C for 1-2 weeks. Grown fungi were microscopically examined with lactophenol cotton blue for determine the morphological characters of macroconidia, microcondia, and hypha arrangement. Colonies texture, color of front and reverse side of colonies, and growth rate were also determined.

#### 3.2.6.1. Confirmatory diagnosis of fungi:

##### 3.2.6.1.1. Diagnosis of yeasts

In addition to morphological features, diagnosis of isolated yeasts was confirmed by using API® 20 C AUX system. This system consists of strips containing dehydrated substrates in individual twenty micro tubes. These tests are reconstituted by adding an aliquot of Api®20 C AUX medium to each micro tube that has been inoculated with the fungal suspension measured by MacFarland 2.The strip was read after 48 -72 hours (± 6 hours) of incubation. The reactions were read according to comparison with supplementary reading

table and the identification was obtained by checking with reference of analytical profile.

### 3.2.6.1.2. Diagnosis of dermatophytes

Complete diagnosis of dermatophytes was performed using of molecular approach, including DNA extraction, PCR reaction and gene sequences.

### A- DNA extraction:

Extraction of dermatophytic DNA was performed according to the manufacturer's instructions of FavorPrep$^{TM}$ Fungi/Yeast Genomic DNA Extraction Mini Kit. The extraction protocol as follow:

1- About 1~ $5\times10^6$ of cultures fungal cells was transferred to 1.5 ml micro-centrifuge tube.
2- 1ml of FA buffer was added to the cells with resuspended by pipetting.
3- The cells were descended by centrifuging at 5,000 RPM for 2 minutes, then supernatant was completely discarded.
4- The cells were resuspended in 550 µl of FB buffer and added 50 µl of lyticase solution, then it well mixed by vortex and incubated at 37° C for 30 minutes.
5- The cells were descended by centrifuging at 5,000 RPM for 10 minutes, then supernatant was completely removed.
6- The 350 µl TG1 buffer was added and mixed well by pipetting, followed by transfer the sample mixture to a bead tube and mixed well by vortex for 5 minutes.
7- About 20 µl of proteinase K (10 mg/ml) was added and mixed well by vortex, then incubated at 55° C for 15 minutes with vortex for 30 seconds every 5 minutes of incubation.

8- The cells were descended by centrifuging at 5,000 RPM for one minute, followed by transferring 200 µl of supernatant to a new 1.5 ml microcentrifuge tube.

9- A 200 µl of TG2 buffer was added and mixed well by pipetting, followed by added a 200 µl of ethanol (96-100%) and also mixed well by pipetting.

10- The TG mini column was placed in collection tube. Then, the sample mixture included any precipitate was transferred carefully to the TG mini column.

11- The tubes were centrifuged at 11,000 RPM for 30 seconds and placed the TG Mini Column to a new collection tube.

12- A 400 µl of W1 buffer was added to the TG mini column and centrifuged at 11,000 RPM for 30 seconds followed by discarding the flow-through and placed the TG mini column back to the collection tube.

13- A 750 µl of wash buffer was added to the TG mini column, then it centrifuged at 11,000 RPM for 30 seconds and discarded the flow-through. The TG mini column was placed back to the collection tube.

14- Tubes were centrifuged at full speed (15,000 RPM) for 3 more minutes to dry the column.

15- The TG mini column was placed into elution tube, then added 50 ~ 100 µl of elution buffer to the membrane center of the TG mini column and left it stand for 3 minutes.

16- The TG mini column was centrifuged at full speed (15,000 RPM) for one minute to elute total DNA.

17- Total DNA was stored at 4°C or -20 °C.

## B- PCR amplification:

Molecular diagnosis of dermatophytes was performed using polymerase chain reaction (PCR) through amplification of ITS 1and ITS 2 genes. PCR mixture sets up in a total volume of 20 µl, including 5 µl of PCR master mix, 1µL of each primer while 1 µl of template DNA is being used. The rest of the volume is completed with sterile deionized distilled water. At the beginning, the extracted DNA, primers and PCR master mix were thawed at room temperature, vortex and centrifuged briefly for bring the contents to the bottom of the tubes. Negative control contains all material except template DNA was also used. PCR reaction tubes were placed into thermocycler PCR instrument which programmed according to the PCR conditions mentioned by Hsiao *et al*. (2005) with some modification (table 12).

**Table (12): The PCR condition for amplification of dermatophytic genes**

| Genes | PCR step | Temp. | Time | cycles |
|---|---|---|---|---|
| **ITS1 and ITS 2** | Initiation | 95 °C | 1 min | 1 |
| | Denaturation | 95 °C | 30 sec | 35 cycles |
| | Annealing | 60 °C | 1 min | 1 |
| | Extension | 72 °C | 90 sec | 1 |
| | Final extension | 72 °C | 10 min | 1 |

The agarose preparation, electrophoresis process, and sequences are similar with what mentioned in sections (**3.2.3.3.3, 3.2.3.3.4**, and **3.2.3.3.5**)

## 3.2.7. Antimicrobial effect of PCN:

### 3.2.7.1. Antimicrobial effect of PCN on bacteria:

Disc diffusion method was used to determine the antimicrobial effect of PCN extracted from *P. aeruginosa* on the isolated bacteria according to protocol mentioned by CLSI-M02-A12 (2015). A number of discs with 6 mm in diameter were prepared from filtered paper and sterilized. They impregnated in a chloroform solution containing 4 µg/ml of PCN. Inoculums of isolated bacteria were prepared in two different methods. First one is specific for *Streptococcus* spp. by inoculated a few colonies of bacteria that grown for 18-24 hours into 2 ml of normal saline. The turbidity of the suspension was adjustment with 0.5 McFarland standard to get approximately $1\text{-}2\times10^8$ cfu/ml. The second method is used to inoculate other types of bacteria. At least 3-5 pure colonies were selected from overnight grown culture to inoculate in 4-5 ml of brain heart broth and incubated at 35±2° C for 2-6 hours until it achieves the turbidity of the 0.5 McFarland standard. Finally adjust the turbidity actively growth with sterilized normal saline and the suspension turbidity was adjusted to reach $1\text{-}2\times10^8$ cfu/ml. From each of previous inoculation methods, 100 µl of standardized bacteria was spread on Müller-Hinton agar by L-shape glass spreader. The inoculated media was left for about 3-5 min to lead a perfectly well observation into medium. A disk with PCN was put on the inoculated plate by sterilized forceps. Plates were incubated for 24 hours at 37° C ± 2° C. A dicks with chloroform were used as a controls. The zone of inhibition was measured in mm around effective disk.

### 3.2.7.2. Antimicrobial effect of PCN on fungi:

The disc diffusion method for testing antimicrobial effects of PCN against yeast was also performed according to CLSI-M44-A2 (2009), while for dermatophytes by Nweze *et al*. (2010). Standardization of yeast cell count was performed as with bacteria to obtain 1-2×$10^8$ cfu/ml (section **3.2.7.1**). The isolated dermatophytes were sub-cultured first on SGA and incubated at 30° C for 1-2 weeks. Standard cell number of dermatophytes was performed using hemocytometer technique. A suspension of dermatophyte cells was prepared through taking a few amount of fungal colony by loop and suspended in sterilized normal saline or distill water, followed by shaking by vortex for fragmentation of fungal cells. A drop from prepared suspension was loaded into the hemocytometer after fixed a cover slip on it to check the number of fungal conidia and/or hyphal fragment. The fungal suspension was adjusted to 1x$10^6$ cell/ml which considered a standard concentration for all isolated dermatophytes. A 100 µl of standard fungal count was inoculated on SGA by spreading with L-shape glass loop and left to fix on media for 3 min. A number of discs with 6 mm in diameter were prepared from filtered paper. They impregnated in a chloroform solution containing different concentrations of PCN (4 µg/ml). These disks were put on the inoculated plate by sterilized forceps. Plates were incubated for one week at 30 °C. A dicks with sterilized distill water and chloroform were used as a controls. The zone of inhibition was measured in mm around effective disk.

### 3.2.7.3. Minimum inhibitory concentration (MIC) of PCN on isolated bacteria:

The MIC of PCN in bacteria was determined based on the dilution method for bacteria mentioned by CLSI-M07-A10 (2015). Isolated bacteria were sub-cultured in Müller-Hinton broth (MHB) for 24 hours at 37° C. A standard count of bacterial cells was prepared by inoculating a few colonies of grown bacteria in 4-5 ml of MHB and incubated at 37° C for 2-6 hours. The turbidity of grown bacteria was adjusting to become equal to 0.5 McFarland standard which containing approximately $1\times10^{8}$ cfu/ml. This process was used with all of isolated bacterial, except *Streptococcus* spp. which adjustment its count with 0.5 McFarland standard by direct inoculation with MHB from 24 hours grown culture. A series concentrations of PCN (3.0, 3.5 and 3.8 µg/ml) were prepared from a stock one (4 µg/ml). A plastic microdilution plate (96 well) was used to determine the MIC value of PCN. A 100 µl from each standard count of bacterial suspension was added into a well, followed by adding 100 µl of specific concentrations of PCN. Several controls were used within a microdilution plate, including MHB with only bacteria, MHB without bacteria, and distilled water. An aluminum foil was used to cover the inoculated plate to prevent contents dryness and contaminated during incubation periods. The plate was incubated at 35° C for 24 hours. Results were read as the presence or absence of bacterial growth that can be seen visually.

### 3.2.7.4. Minimum inhibitory concentration (MIC) of PCN on Fungi:

The MIC of PCN on fungi was determined based on the broth dilution method mentioned by CLSI-M27-A3 (2008) for yeasts and CLSI-M38-A2 (2008) for dermatophytes. All of isolated fungi were sub-cultured in SGA for activation. Yeasts were incubated at 37° C for 24 hours, while dermatophytes were incubated at 28° C for 1-3 weeks. Standard cell count was prepared by taking a few amounts of fungal cells from grown colony by loop to suspend in 2 ml of SGB. The turbidity of yeast cells was adjusted with 0.5 McFarland standard to be contained approximately $1\times10^8$ cfu/ml, while dermatophytic cells was adjusted to $1\times10^6$ cfu/ml by using hemocytometer technique. A serial concentrations of PCN (3.0, 3.5 and 3.8 µg/ml) were prepared from a stock one (4 µg/ml). Two of plastic microdilution plates (96 well) were used to determine the MIC value of PCN; one for yeast and another for dermatophytes. Each well of the plates received100 µl from standard count of each fungal suspension, followed by adding 100 µl of specific concentration of PCN. Several controls were used within a single microdilution plate, including SGB with only fungi, SGB without fungi, and distilled water. An aluminum foil was used to cover the inoculated plate to prevent contents dryness and contaminated during incubation periods. The plate containing yeast was incubated at 35° C for 24 hours, while plate of dermatophytes was incubated at 30° C for 72 hours. Results were read as the presence or absence of fungal growth that can visually determine.

### 3.2.8. PCN with antibacterial agents:

The effect of PCN on the antibacterial activity of two antibiotic agents, ampicillin and cefotaxime, were determined. The PCN was used in two concentrations (2 and 4 µg/ml) to mix with various concentrations of each of antibacterial agents. The concentration 2 µg/ml of PCN was mixed with 5 concentrations of ampicillin (0.125, 2, 4, 6, and 8 µg/ml) and with 5 concentrations of cefotaxime (0.625, 10, 15, 20, and 25 µg/ml). While, PCN at 4 µg/ml was mixed with 4 concentrations of ampicillin (2, 4, 6, and 8 µg/ml) and with 4 concentrations of cefotaxime (10, 15, 20, and 25 µg/ml). The activity of PCN mixed with antibacterial agents on isolated bacteria was measured as MIC level as mentioned by dilution method of CLSI-M07-A10 (2015). A stock solution of antibacterial agent was prepared by the following formula:

$$\boldsymbol{Weight(mg)} = (volume(ml)Xconcentration(\mu g/ml))/Potency\ (\mu g/mg)$$

Where the potency of ampicillin is 10 µg/ml and of cefotaxime is 30 µg/ml.

Two plastic microdilution plates (96 well) were used to determine the MIC value; one for mixed PCN with ampicillin and another for mixed PCN with cefotaxime. From standard bacterial count prepared in section **(3.2.7. 3)**, 100 µl from each standard count of bacterial suspension was added into a well, followed by adding 100 µl of specific concentrations of mixture. Several controls were used within each of microdilution plate including, MHB with only bacteria, MHB without bacteria, only PCN (4 or 2 µg/ml), antibacterial agent only, a mixture of PCN with antibacterial agent without bacteria, and distilled water. An aluminum foil was used to cover the inoculated plates to prevent contents dryness and contaminated during incubation periods. The

plates were incubated at 37° C for 24 hours. Results were read as the presence or absence of bacterial growth that can visually determine.

### 3.2.9. PCN with antifungal agent:

The activity of griseofulvin as antifungal agent on isolated fungi after mixed with PCN (2 µg/ml) was measured according to the broth dilution method mentioned by CLSI-M38-A2 (2008) and CLSI-M27-A3 (2008). Various concentrations of griseofulvin (25, 50, and 100 µg/ml) were prepared as mentioned in section **(3.2.2.5)**. From standard fungal count prepared in section **(3.2.7.4)**, two of plastic microdilution plates (96 well) were used to determine the MIC value of mixture (PCN with griseofulvin); one for yeasts and another for dermatophytes. Each well of the plates received 100 µl from standard count of each fungal suspension, followed by adding 100 µl of specific concentrations of mixture. Several controls were used within a single microdilution plate, including SGB with only fungi, SGB without fungi, only PCN, griseofulvin only, DMSO, mixture of PCN and griseofulvin without fungi and distilled water. An aluminum foil was used to cover the inoculated plate to prevent contents dryness and contaminated during incubation periods. The plate containing yeast was incubated at 37° C for 24 hours, while plate of dermatophytes was incubated at 30° C for 72 hours. Results were read as the presence or absence of fungal growth that can visually determine.

### Statistical analysis:

Data of all tests were expressed as mean ± SD. The values were analyzed statistically with one way ANOVA by using Excel application of Window 10. The minimum level of ($p$) value was < 0.05 concerts as significant level.

# *Chapter Four*

# *Results*

# 4. Results:

## 4.1. Isolation of bacteria

Fifteen bacterial strains were isolated from different parts of the human body (table 1). They were distributed between eight different strains from urine samples (two Gram positive and six Gram negative), two different strains from stool samples (Gram negative), one strain from each of cerebrospinal fluid (*Streptococcus pneumonia*e), nasal swab (*Staphylococcus aureus)*, throat swab (*Streptococcus pyogenes*), vaginal swab (*Streptococcus agalactiae*), and wound swab (*Acinetobacter baumannii*) (table 13). *Pseudomonas aeruginosa* which was isolated from urine sample was registered in GenBank under code number MH382164 after made sequences of 16S rRNA gene (Fig.4).

**Table (13): Isolated bacteria from the human specimens**

| No. | Strain | Specimen |
|---|---|---|
| 1 | ***Staphylococcus epidermidis*** | **Urine** |
| **2** | ***Enterococcus faecalis*** | |
| **3** | ***Citrobacter freundii*** | |
| **4** | ***E. coli*** | |
| **5** | ***Enterobacter cloacae*** | |
| **6** | ***Klebsiella pneumoniae*** | |
| **7** | ***Proteus mirabilis*** | |
| **8** | ***Pseudomonas aeruginosa*** | |
| **9** | ***Salmonella* spp.** | **Stool** |
| **10** | ***Shigella* spp.** | |
| **11** | ***Streptococcus pneumoniae*** | **Cerebrospinal fluid (CSF)** |
| **12** | ***Staphylococcus aureus*** | **Nasal swab** |
| **13** | ***Streptococcus pyogenes*** | **Throat swab** |
| **14** | ***Streptococcus agalactiae*** | **Vaginal swab** |
| **15** | ***Acinetobacter baumannii*** | **Wound swab** |

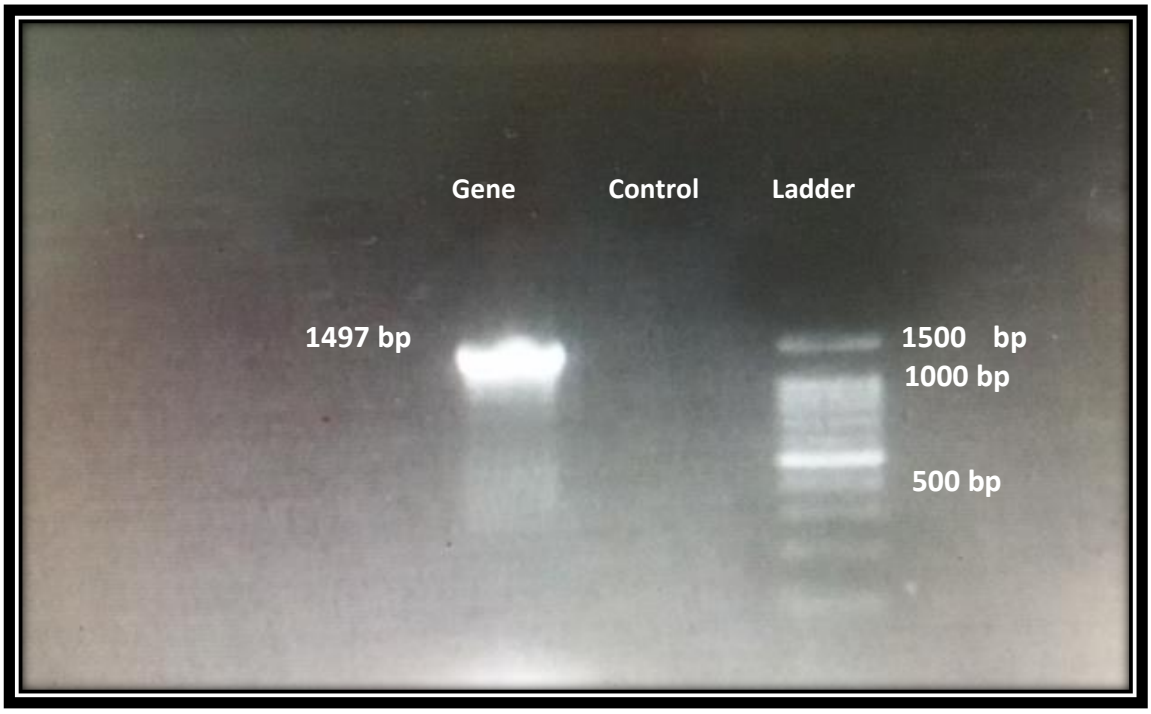



**Fig. (4): PCR product of 16S rRNA gene of *P. aeruginosa* with ladder DNA at the right side.**

## 4.2. Isolation of fungi

Ten fungal strains were isolated from patients with different skin infection. Dermatophytes were singly isolated from 7 patients with dermatophytoses, while 3 yeasts were isolated from 3 patients with other fungal skin infections. Dermatophytic strains were diagnosed after detection of ITS 1& ITS 2 genes by PCR (Fig. 5). Sequencing of these purified genes was performed which used to complete diagnosis process via using BLAST aligning section of GenBank after entering of gene sequences. All of isolated strains were gotten a code No. in GenBank after successful registering in this universal web site. *Trichophyton interdigitale* which was isolated from patient with tinea corporis was registered in GenBank under code No. MH383047 (Fig. 6, 7 and 10). Two strains of *M. ferrugineum* which were isolated from patients with tinea capitis were registered in GenBank under code No.

MH383042 and MH383043 (Fig. 6,7,8, 9 and 11). Three strains of *M. canis* which were isolated from patients with tinea corporis and tinea capitis were registered in GenBank under code No. MH383044, MH383045, and MH383046 (Fig. 8, 9, and 12). *Epidermophyton floccosum* which was isolated from patient with tinea corporis was registered in GenBank under code No. MH383041 (table 14) (Fig. 6, 7 and 13).

**Table (14): Isolated fungi from the human specimens**

| No. | Strain | Specimen | GenBank cod No. |
|---|---|---|---|
| 1 | ***Trichophyton interdigitale*** | **Tinea corporis** | MH383047 |
| 2 | ***Microsporum ferrugineum*** | **Tinea capitis** | MH383042 |
| 3 | ***Microsporum ferrugineum*** | **Tinea capitis** | MH383043 |
| 4 | ***Microsporum canis*** | **Tinea capitis** | MH383044 |
| 5 | ***Microsporum canis*** | **Tinea corporis** | MH383045 |
| 6 | ***Microsporum canis*** | **Tinea corporis** | MH383046 |
| 7 | ***Epidermophyton floccosum*** | **Tinea corporis** | MH383041 |
| 8 | ***Cryptococcus albidus*** | **Skin scrap** | - |
| 9 | ***Cryptococcus terreus*** | **Skin scrap** | - |
| 10 | ***Candida albicans*** | **Scalp scrap** | - |


**Fig. (5) : Product gene of PCR for 7 strains of dermatophytes with ladder DNA at the end of right side.**

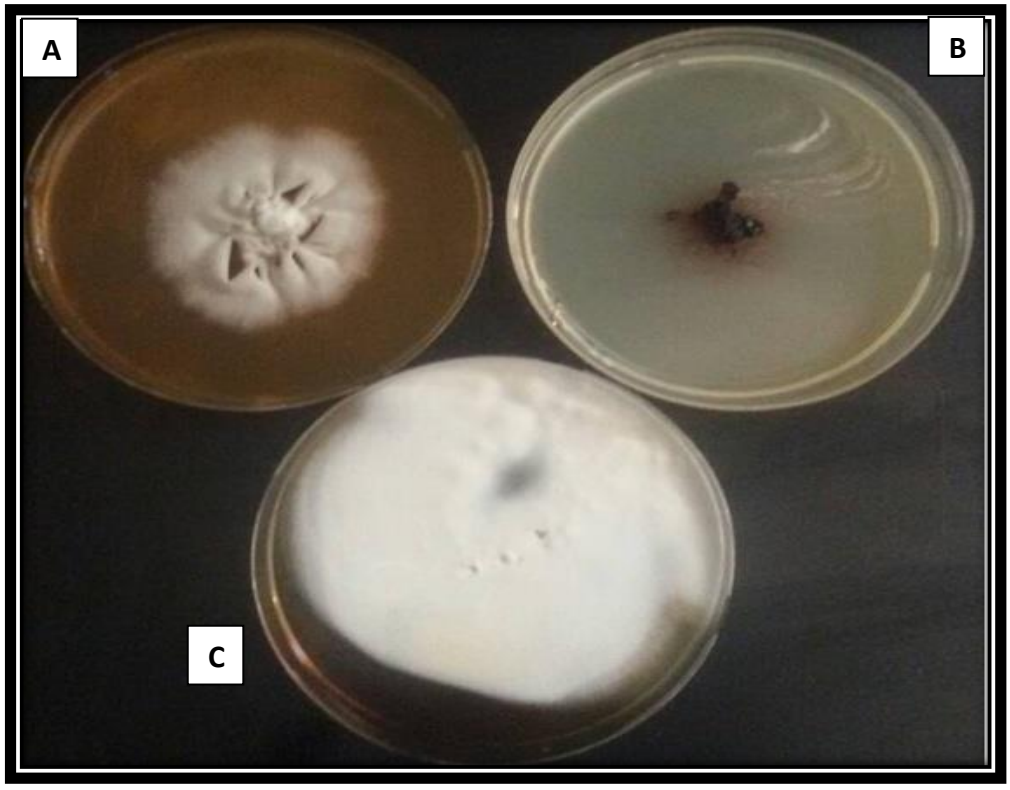
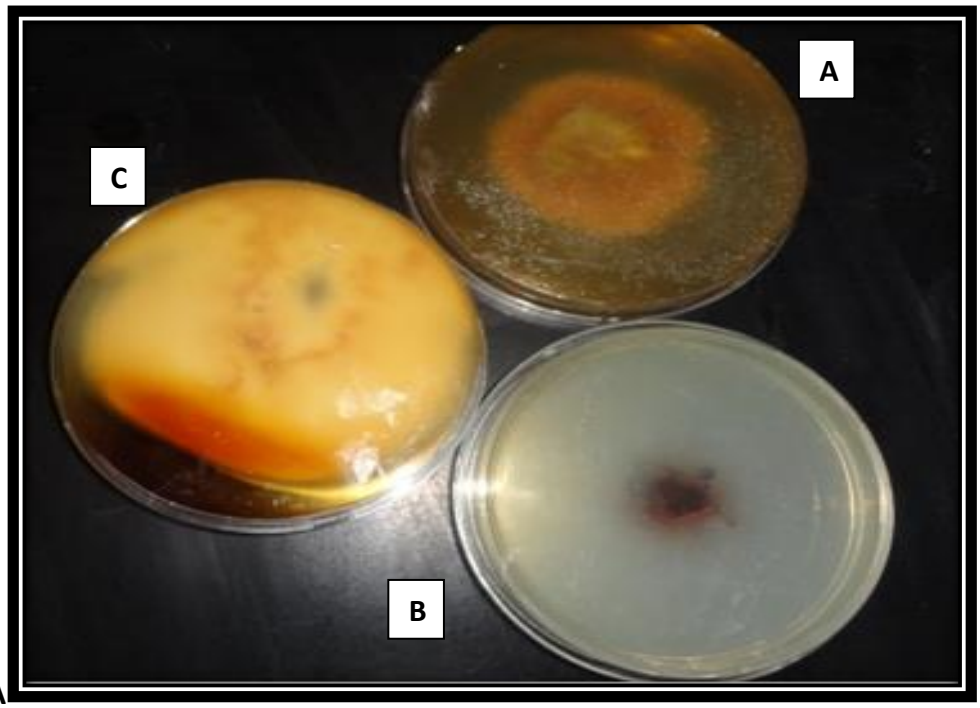



**Fig (6): Colony of isolated dermatophytes**

**a: *E. floccosum*, *b: M. ferrugineum* (MH383042), c: *T. interdigitale***




A

**Fig (7): Revise side of the colony of isolated dermatophytes**

***a:* *E. floccosum*, b: *M. ferrugineum* (MH383042), c: *T. interdigitale***


**Fig (8): Colony of isolated dermatophytes**

**a: *M. canis* (MH383045), *b: M. canis* (MH383044), c: *M. canis* (MH383046), d: *M. ferrugineum* (MH383043)**

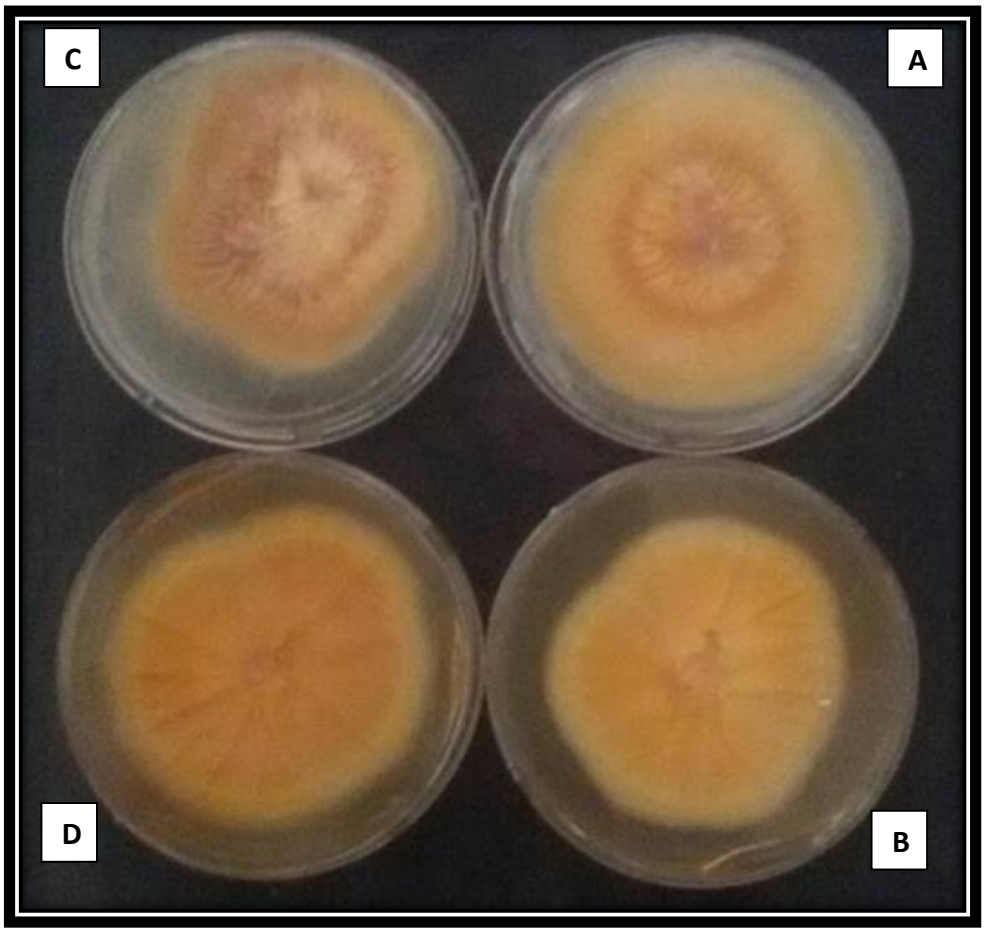



**Fig. (9): Revise side of the colony of isolated dermatophytes**

***a: M. canis* (MH383045), b: *M. canis* (MH383044)**

**c: *M. canis* (MH383046), d: *M. ferrugineum* (MH383043)**

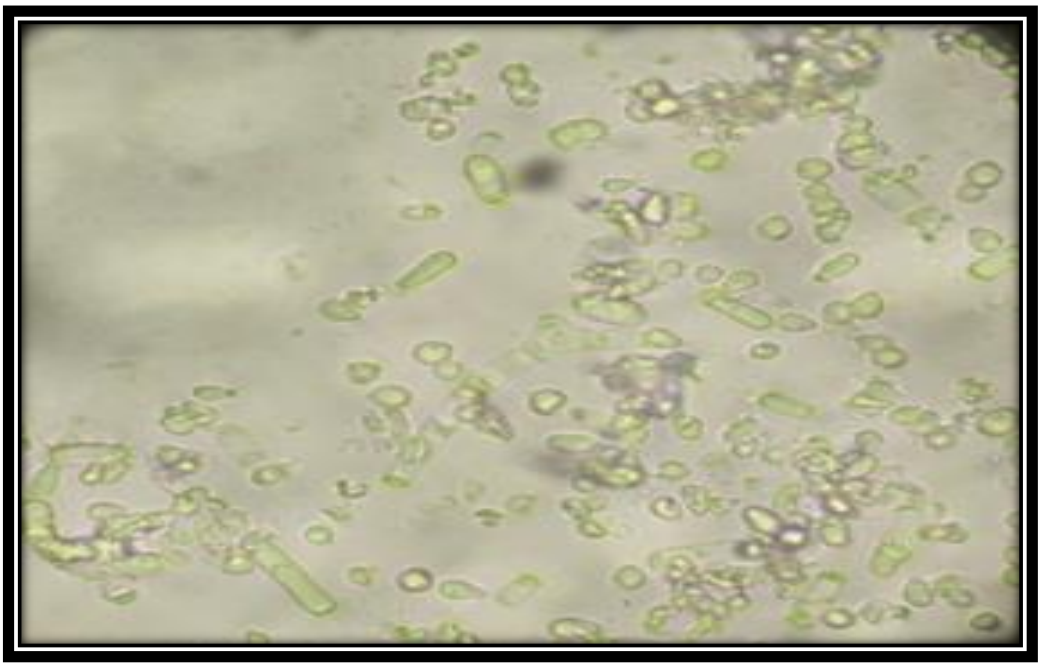


**Fig. (10): Microconidia and macroconidia of *T. interdigitale* (MH383047) (X100)**

**Fig. (11): Macroconidia of *M. ferrugineum* (MH383043) (X100)**

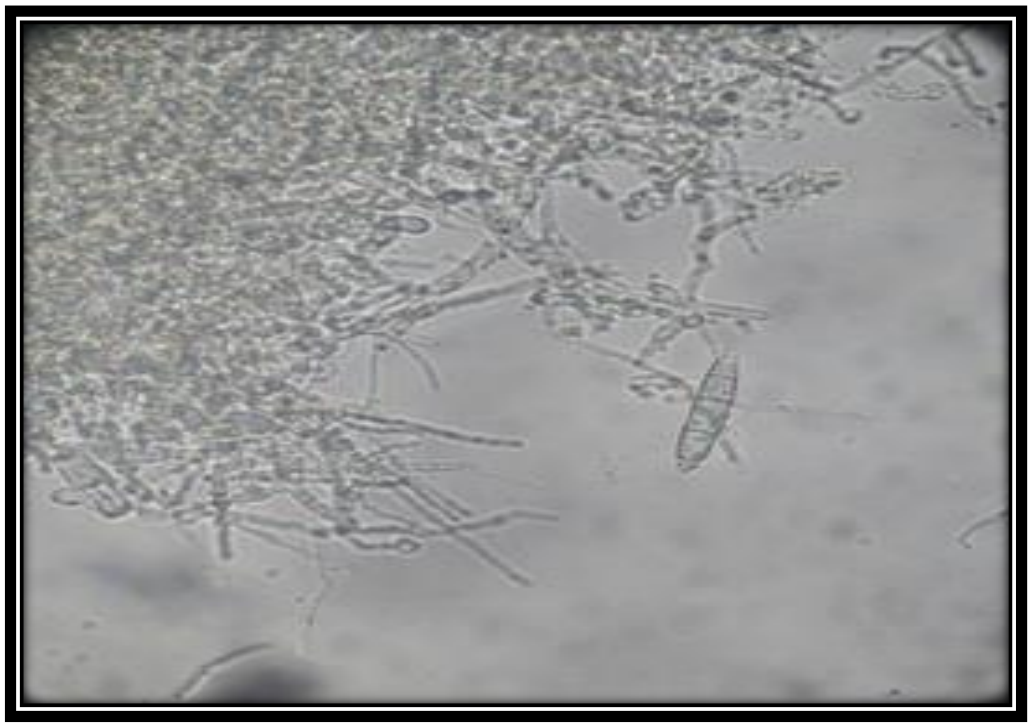


**Fig. (12): Macroconidia of *M. canis* (MH383046) (X100)**

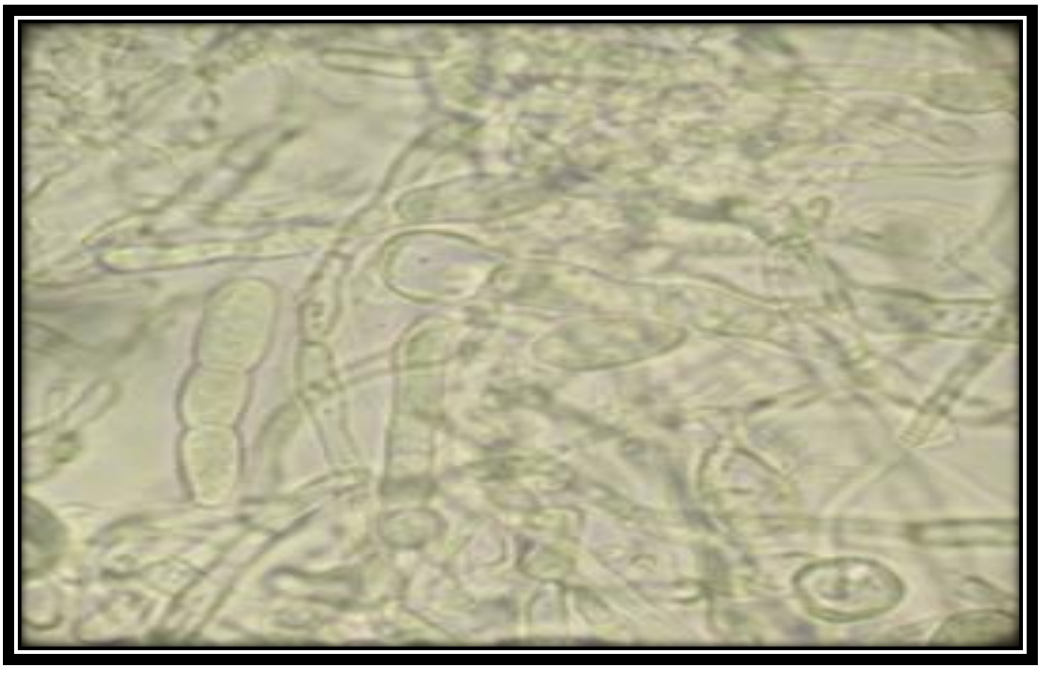


**Fig. (13): Macroconidia and hypha of *E. floccosum* (MH383041)(X100)**

## 4.3. Determination of dermatophytoses:

The age of patients with dermatophytoses was ranged between 4.5 and 45 years. Distribution of fungal infection among genders revealed that there were two positive males and five positive females; while other eleven patients (61.11%) showed a negative results. The different tinea types among both genders showed no significant difference ($P < 0.05$) among females, while no such difference was found among males (table 15).

Tinea capitis (11.11%) was the only type of dermatophytoses that gave a positive culture of dermatophytes among males. While two of tinea capitis (11.11%) and three of tinea corporis (16.66%) were gave a positive culture of dermatophytes in females (table 15).

The statistical comparison between the positive cases of dermatophytoses revealed that females with tinea corporis have significant differences at $P < 0.05$ from all of other male and female cases. Meanwhile, cases of tinea pedis in both of male and female were showed no significant differences at $p < 0.05$ from each other, while it was significant between positive and negative cases of male and female. On the other hand, there were no significant differences between negative cases with each other at $p < 0.05$ (table 15).

**Table (15): No. of patients with Dermatophytoses**

| Gender | Dermatophytoses type | Age (years) | Fungal isolation | | Total No. |
|---|---|---|---|---|---|
| | | | Positive | Negative | |
| Male | Tinea capitis | 4.5-8 | 2 (11.11%) | 1 (5.55%) | 3 (16.66%) |
| | Tinea corporis | 35 | $0^{(a)}$ | 1 (5.55%) | 1 (5.55%) |
| | Tinea cruris | 20 | $0^{(b)}$ | 2 (11.11%) | 2 (11.11%) |
| | Tinea pedis | 15-40 | $0^{(c,e)}$ | $3^{(e)}$ (16.66%) | 3 (16.66%) |
| Female | Tinea capitis | 12-24 | 2 (11.11%) | 1 (5.55%) | 3 (16.66%) |
| | Tinea corporis | 16-45 | $3^{(a,b,c,d,g)}$ (16.66%) | $0^{(g)}$ | 3 (16.66%) |
| | Tinea pedis | 15-40 | $0^{(d,f)}$ | $3^{(f)}$ (16.66%) | 3 (16.66%) |
| Total No. | | | 7 (38.88%) | 11 (61.11%) | 18 |

**Similar letter means significant differences at $p < 0.05$**

## 4.4. Antibacterial effects of PCN:

The PCN had been shown antimicrobial activity against different strains of bacteria at concentration 4 µg/ml. After completion of bacterial strain diagnosis, the activity of PCN on the isolated strains of bacteria was variable between Gram positive and negative. The zone of inhibition size ranged between 2.2 and 7 mm. The most sensitive strains for 4 µg of PCN were *S. epidermidis*, *Salmonella* spp., *S. pneumonia*, *Citrobacter freundii* and *E. cloacae*, which were showed a zone of inhibition 7, 5.6, 5.3, 5.1, and 5 mm, respectively. Other strains showed less sensitivity toward the 4 µg of PCN as with *S. agalactiae*, *Streptococcus pyogenes*, *Acinetobacter baumannii*, and *E. coli* with a zone of inhibition 2.2, 2.3, 2.3, and 2.4 mm, respectively (table 16)(Fig.14).

The sensitivity of *S. epidermidis* which showed a large diameter of inhibition zone was revealed significant differences at $p < 0.05$ from other nine bacterial isolates. Meanwhile, *Salmonella* spp. as the second largest sensitive bacteria toward PCN was showed significant differences at $p < 0.05$ from four bacterial isolates (table 16).

**Table (16): Effect of PCN (4 µg/ml) on isolated bacteria determined by disc diffusion method**

| No. | Strain | Zone of inhibition (mm) |
|---|---|---|
| 1 | ***Staphylococcus aureus*** | **$2.9^{(a)}$ ± 2.9** |
| 2 | ***Staphylococcus epidermidis*** | **$7^{(b,a)}$ ± 5.0** |
| 3 | ***Streptococcus pneumoniae*** | **$5.3^{(a,c)}$ ± 4.6** |
| 4 | ***Streptococcus pyogenes*** | **$2.3^{(d,b,c)}$ ± 2.5** |
| 5 | ***Streptococcus agalactiae*** | **$2.2^{(e,b,c,e)}$ ± 4.0** |
| 6 | ***Enterococcus faecalis*** | **$2.9^{(b,c)}$ ± 2.1** |
| 7 | ***Acinetobacter baumannii*** | **$2.3^{(b,c,g)}$± 2.9** |
| 8 | ***Citrobacter freundii*** | **$5.1^{(b,d,e,g,h)}$± 4.7** |
| 9 | ***E. coli*** | **$2.4^{(b,c,h,i)}$ ± 1.8** |
| 10 | ***Enterobacter cloacae*** | **$5^{(d,e,g,i)}$ ± 5.3** |
| 11 | ***Klebsiella pneumoniae*** | **$2.5^{(b)}$ ± 4.6** |
| 12 | ***Proteus mirabilis*** | **$2.8^{(b,c)}$ ± 4.3** |
| 13 | ***Salmonella* spp.** | **$5.6^{(d,e,g,i)}$ ± 5.3** |
| 14 | ***Shigella* spp.** | **$2.7^{(b,c)}$ ± 3.8** |

**Mean ± SD**

**Similar letter means significant differences at $p < 0.05$**

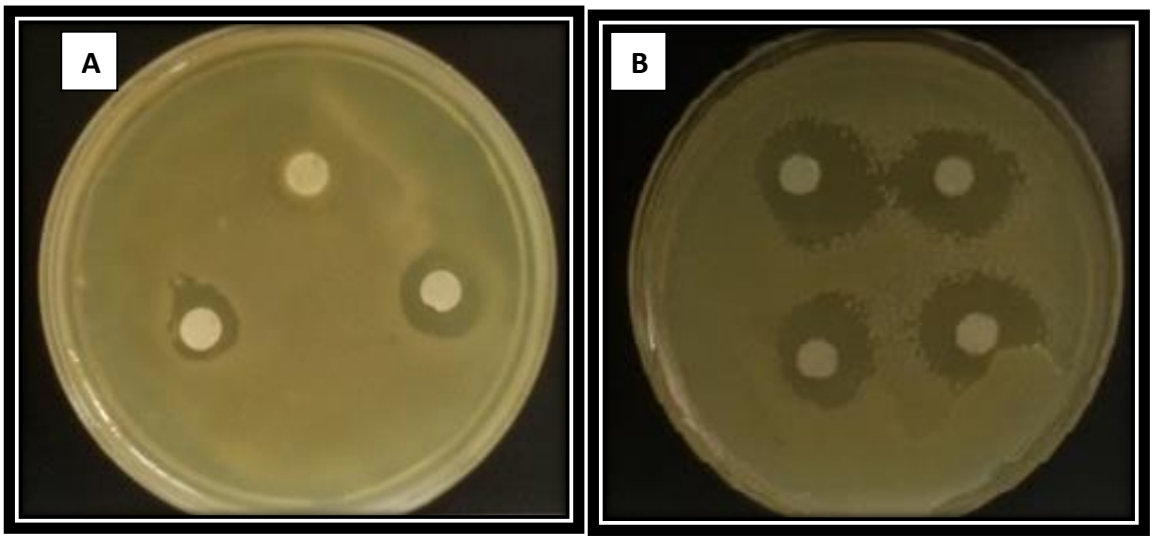



**Fig. (14): Zone of inhibition around a disk of PCN (4 µg/ml) for:**

**a: *S. pneumoniae*; b: *S. epidermidis***

## 4.5. Antifungal effects of PCN:

Test of PCN at concentration 4 µg/ml against isolated fungi revealed the ability of such compound to inhibit the growth of 6 fungi, including 4 dermatophytes and 2 yeasts. *M. canis* (MH383044), *E. floccosum* and *C. albicans* which revealed a zone of inhibition 6.9, 4.5, and 1.2 mm, respectively were the most sensitive fungi toward PCN with significant differences from most of fungi at $p < 0.05$. *M. ferrugineum* (MH383043), *M. canis* (MH383046) and *Cryptococcus terreus* were given a variable sensitivity to PCN (2, 0.7, and 0.3 mm, respectively) with a significant differences from each other at $P < 0.05$ (table 17) (Fig.15).

**Table (17): Effect of PCN (4 µg/ml) on isolated fungi determined by disc diffusion method**

| Isolate NO. | Strain | Zone of inhibition (mm) |
|---|---|---|
| 1 | *E. floccosum* (MH383041) | $4.5^{(a,c)}$ ±9.0 |
| 2 | *M. ferrugineum* (MH383042) | $0^{(a,b)}$ ± 0 |
| 3 | *M. ferrugineum* (MH383043) | $2^{(d)}$ ± 3.1 |
| 4 | *M. canis* (MH383044) | $6.9^{(b,c,d)}$ ± 4.5 |
| 5 | *M. canis* (MH383045) | $0^{(a,d,e)}$ ± 0 |
| 6 | *M. canis* (MH383046) | $0.7^{(d)}$ ± 2.6 |
| 7 | *T. interdigitale* (MH383047) | $0^{(a,c)}$ ± 0 |
| 8 | *Cryptococcus albidus* | $0^{(a,d,f)}$ ± 0 |
| 9 | *Cryptococcus terreus* | $0.3^{(d)}$ ±2.3 |
| 10 | *Candida albicans* | $1.2^{(b,c,d,e,f)}$ ± 1.1 |

**Mean ± SD**
**Similar letter means significant differences at $p < 0.05$**

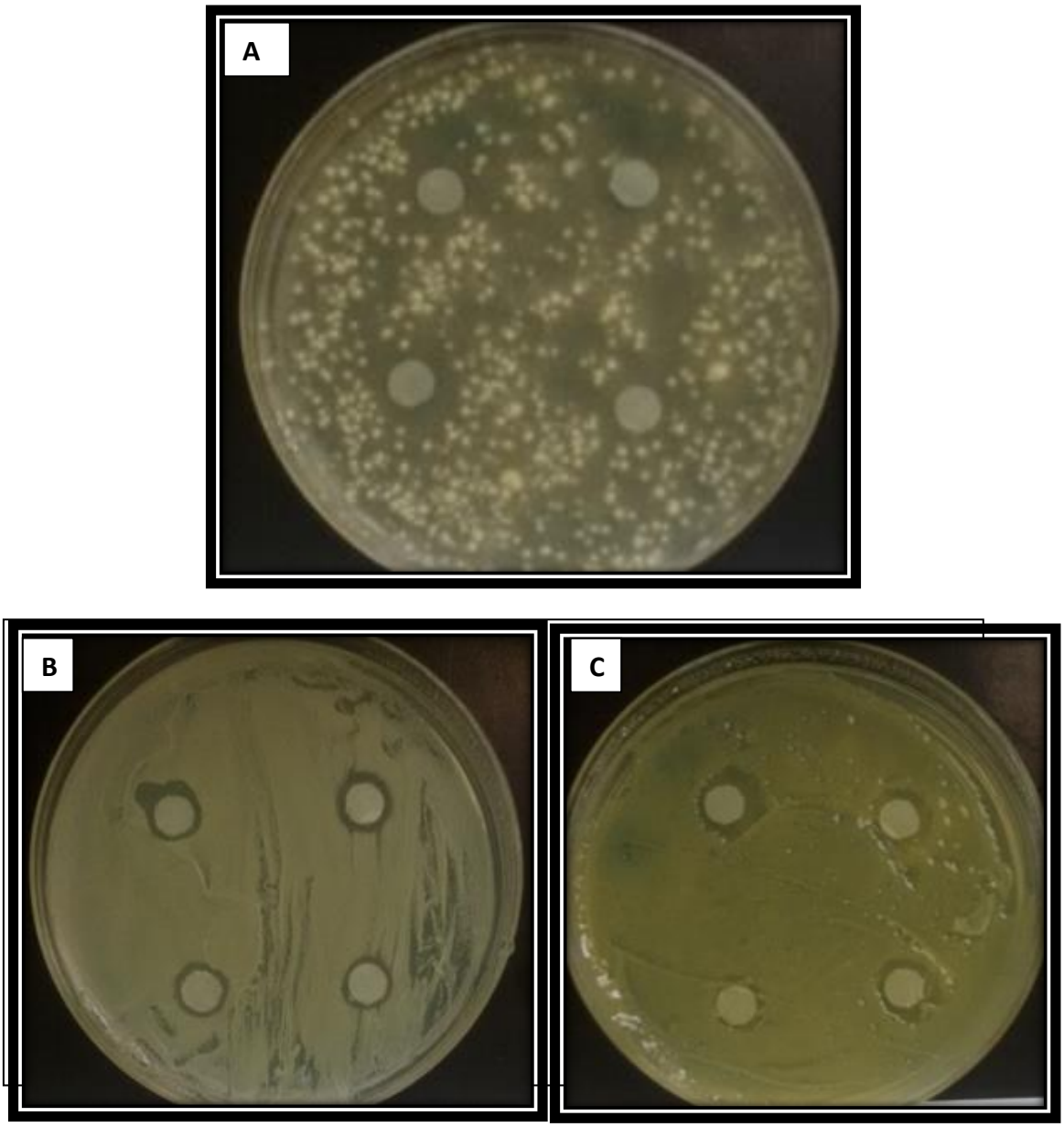



**Fig. (15): Zone of inhibition around a disk of PCN (4 µg/ml) for:**

**a:** ***M. canis*** **(MH383044), b:*C. albicans*; c:** ***C. terreus***

## 4.6. MIC value of PCN on isolated microorganism:

The MIC value of PCN on isolated bacteria was measured at various concentrations. From 14 isolates, *Streptococcus agalactiae* was the most sensitive bacteria to PCN which needed 3.5 µg/ml of it to inhibit, while the MIC of each of *Enterococcus faecalis* and *E. coli* was 3.8 µg/ml. Meanwhile, all of other 11 strains were sensitive to 4 µg/ml of PCN (table 18).

The MIC value of *Microsporum canis* (MH383044) as the most sensitive fungal strain was determined to be at 3.8 µg/ml, while for *C. albicans* was 4 µg/ml (table 19).

**Table (18): MIC value of PCN on isolated bacteria**

| No. | Strain | Antibacterial concentration (µg/ml) | | | |
|---|---|---|---|---|---|
| | | 4 | 3.8 | 3.5 | 3.0 |
| 1 | *Staphylococcus aureus* | _ | + | + | + |
| 2 | *Staphylococcus epidermidis* | _ | + | + | + |
| 3 | *Streptococcus pneumoniae* | _ | + | + | + |
| 4 | *Streptococcus pyogenes* | _ | + | + | + |
| 5 | *Streptococcus agalactiae* | _ | _ | _ | + |
| 6 | *Enterococcus faecalis* | _ | _ | + | + |
| 7 | *Acinetobacter baumannii* | _ | + | + | + |
| 8 | *Citrobacter freundii* | _ | + | + | + |
| 9 | *E. coli* | _ | _ | + | + |
| 10 | *Enterobacter cloacae* | _ | + | + | + |
| 11 | *Klebsiella pneumoniae* | _ | + | + | + |
| 12 | *Proteus mirabilis* | _ | + | + | + |
| 13 | *Salmonella* spp. | _ | + | + | + |
| 14 | *Shigella* spp. | _ | + | + | + |

+: growth; -: no growth

**Table (19): MIC value of PCN on isolated fungi**

| Strain | Antifungal concentration (µg/ml) | | | |
|---|---|---|---|---|
| | **4** | **3.8** | **3.5** | **3.0** |
| ***Microsporum canis* (MH383044)** | – | – | + | + |
| ***Candida albicans*** | – | + | + | + |

+: growth; -: no growth

## 4.7. Determination the effect of PCN on ampicillin activity:

The role of PCN (4 µg/ml) on the ampicillin represented by enhanced the activity of this agent against *S. pyogenes* by decreasing MIC value from 6 to 4 µg/ml. Otherwise, PCN elevated the MIC value of ampicillin from 4 to 6 µg/ml against another strain of bacteria as with *Citrobacter freundii* or from 4 to 8 µg/ml for *Proteus mirabilis*, *Salmonell*a spp., and *Shigella* spp. The elevation of MIC of ampicillin from 6 to 8 µg/ml by PCN was also showed against *S. aureus*, *S. epidermidis*, *S. pneumoniae*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Salmonell*a spp. and *Shigella* spp. The MIC value of ampicillin elevated to more than 8 µg/ml against *E. coli* and *Enterobacter cloacae* in the presence of PCN. Meanwhile, there was no effect of PCN on the MIC value of ampicillin against *S. agalactiae*, *Enterococcus faecalis*, and *Acinetobacter baumannii* (table 20).

Generally, the effect of PCN on the antibacterial activity of ampicillin was variable. After mixed with 4 µg/ml of PCN, ampicillin action against bacteria was decreased even at high concentration (8 µg/ml) as with *E. coli* and *E. cloacae* in comparison with ampicillin alone. This was also showed against four other bacterial strains (*S. aureus*, *S. epidermidis*, *S. pneumoniae*,

and *Klebsiella pneumoniae*) when they less affected by ampicillin at 6 µg/ml compared with ampicillin alone (table 20). Moreover, the MIC value of ampicillin was increased from 4 to 6 µg/ml after mixed with PCN (4 µg/ml) against four isolated bacteria, including *Citrobacter freundii*, *Proteus mirabilis*, *Salmonell*a spp. and *Shigella* spp. (table 20).

**Table (20): MIC of ampicillin with pyocyanin (4 µg/ml)**

| Strain | Antibacterial concentration (µg/ml) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | PCN (4 µg/ml) and Ampicillin | | | | Ampicillin alone | | | | PCN alone |
| | 8 | 6 | 4 | 2 | 8 | 6 | 4 | 2 | 4 |
| ***Staphylococcus aureus*** | - | + | + | + | - | - | + | + | - |
| ***Staphylococcus epidermidis*** | - | + | + | + | - | - | + | + | - |
| ***Streptococcus pneumoniae*** | - | + | + | + | - | - | + | + | - |
| ***Streptococcus pyogenes*** | - | - | - | + | - | + | + | + | - |
| ***Streptococcus agalactiae*** | - | + | + | + | - | + | + | + | - |
| ***Enterococcus faecalis*** | - | - | + | + | - | - | + | + | - |
| ***Acinetobacter baumannii*** | - | + | + | + | - | + | + | + | - |
| ***Citrobacter freundii*** | - | - | + | + | - | - | - | + | - |
| ***E. coli*** | + | + | + | + | - | - | - | + | - |
| ***Enterobacter cloacae*** | + | + | + | + | - | + | + | + | - |
| ***Klebsiella pneumoniae*** | - | + | + | + | - | - | + | + | - |
| ***Proteus mirabilis*** | - | + | + | + | - | - | - | + | - |
| ***Salmonell*a spp.** | - | + | + | + | - | - | - | + | - |
| ***Shigella* spp.** | - | + | + | + | - | - | - | + | - |

**+:** growth; **-:** no growth

The 2 µg/ml of PCN decreased the MIC action of ampicillin against all of bacterial strains to reach a concentration 2 µg/ml, except *Acinetobacter baumannii* which showed resistance to the largest MIC value (8 µg/ml) of ampicillin in compared with ampicillin alone (table 21).

Generally, The concentration of 2 µg/ml of either of PCN or ampicillin was revealed no activity against all bacterial strains when they used alone. Otherwise, the PCN elevated the antibacterial action of ampicillin against three bacterial strains (*S. pyogenes, S. agalactiae*, and *Enterobacter cloacae*) by decreased MIC value from 6 to 2 µg/ml. Whereas, the same results was showed against eight tested bacteria (*S. aureus, S. epidermidis, S. pneumoniae, Enterococcus faecalis, Klebsiella pneumoniae, Proteus mirabilis, Salmonell*a spp., *Shigella* spp.) by decreased the ampicillin concentration from 4 to 2 µg/ml. *Acinetobacter baumannii* showed more resistance to ampicillin at high concentration (8 µg/ml) after mixed with PCN. Meanwhile, there were no differences between the effect of ampicillin alone at concentration 0.125 µg/ml or with PCN on all of bacteria (table 21).

**Table (21): MIC of ampicillin with PCN (2 µg/ml)**

| Strain | Concentration (µg/ml) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | PCN (2 µg/ml) and Ampicillin | | | | | Ampicillin alone | | | | | PCN alone |
| | 8 | 6 | 4 | 2 | 0.125 | 8 | 6 | 4 | 2 | 0.125 | 2 |
| ***Staphylococcus aureus*** | - | - | - | - | + | - | - | + | + | + | + |
| ***Staphylococcus epidermidis*** | - | - | - | - | + | - | - | + | + | + | + |
| ***Streptococcus pneumoniae*** | - | - | - | - | + | - | - | + | + | + | + |
| ***Streptococcus pyogenes*** | - | - | - | - | + | - | + | + | + | + | + |
| ***Streptococcus agalactiae*** | - | - | - | - | + | - | + | + | + | + | + |
| ***Enterococcus faecalis*** | - | - | - | - | + | - | - | + | + | + | + |
| ***Acinetobacter baumannii*** | + | + | + | + | + | - | + | + | + | + | + |
| ***Citrobacter freundii*** | - | - | - | - | + | - | - | - | + | + | + |
| ***E. coli*** | - | - | - | - | + | - | - | - | + | + | + |
| ***Enterobacter cloacae*** | - | - | - | - | + | - | + | + | + | + | + |
| ***Klebsiella pneumoniae*** | - | - | - | - | + | - | - | + | + | + | + |
| ***Proteus mirabilis*** | - | - | - | - | + | - | - | + | + | + | + |
| ***Salmonella* spp.** | - | - | - | - | + | - | - | + | + | + | + |
| ***Shigella* spp.** | - | - | - | - | + | - | - | + | + | + | + |

**+:** growth; **-:** no growth

## 4.8. Determination the effect of PCN on cefotaxime activity:

The PCN at 4 µg/ml showed an ability to elevate the MIC value of cefotaxime against four strains of bacteria (*S. aureus*, *Citrobacter freundii*, *E. coli*, and *Klebsiella pneumoniae*) from 10 to 25 µg/ml. Also this elevation was showed against other four bacterial strains (*S. epidermidis*, *S. pneumoniae*, *S. agalactiae*, and *Proteus mirabilis*) from 15 to 25 µg/ml and against *Enterococcus faecalis*, *Enterobacter cloacae*, and *Salmonell*a spp. from 15 to 20 µg/ml. The MIC value of cefotaxime also increased by the effect of PCN from 20 to 25 µg/ml against *S. pyogenes*. Meanwhile, there were no effects of 4 µg/ml of PCN on the MIC value of cefotaxime against *Acinetobacter baumannii*, and *Shigella* spp. at MIC 20 µg/ml (table 22).

Generally, PCN alone exhibited the ability to inhibit the growth of all bacteria strain at 4 µg/ml, whereas the cefotaxime alone at concentrations 25 and 20 µg/ml showed inhibitory action on the growth of all of isolated bacteria. At concentration 15 µg/ml of cefotaxime alone, all bacterial growth was also inhibited, except of *Streptococcus pyogenes*, *Acinetobacter baumannii* and *Shigella* spp. The lower concentration of cefotaxime (10 µg/ml) revealed no activity against all of bacteria strains, except of *S. aureus*, *Citrobacter freundii*, *E. coli* and *Klebsiella pneumoniae* (table 22).

Mixing of 4 µg/ml of PCN with high concentration of cefotaxime (25 µg/ml) didn't effect on the antibacterial activity of cefotaxime against all isolated bacteria in comparison with it alone. This result was changed at less concentrations of cefotaxime when PCN inhibited the activity of cefotaxime against nine strains at concentration 20 µg/ml, including *S. aureus*, *S. epidermidis*, *S. pneumoniae*, *S. pyogenes*, *S. agalactiae*, *Citrobacter freundii*, *E. coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis*. The same effected can

also be seen at concentration 15 µg/ml of cefotaxime against all isolated strains, except of *S. pyogenes*, *Acinetobacter baumannii*, and *Shigella* spp. Also the inhibition activity of PCN was clear on the concentration 10 µg/ml of cefotaxime against four strains of bacteria (*S. aureus*, *Citrobacter freundii*, *E. coli*, and *Klebsiella pneumoniae* )(table 22).

The PCN didn't show any enhancement effects on the action of cefotaxime at high concentration (20 µg/ml) against all of isolated bacteria. Meanwhile, there was no effect of PCN on the cefotaxime activity at concentration 20 µg/ml against *Acinetobacter baumannii* and *Shigella* spp. The PCN was also showed no activity against the antibacterial effect of cefotaxime at concentration 10 µg/ml on all of isolated bacteria, except *S. aureus*, *Citrobacter freundii*, *E. coli*, and *Klebsiella pneumoniae* (table 22).

**Table (22): MIC of cefotaxime with PCN (4 µg/ml)**

| Strain | Concentration (µg/ml) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | PCN (4 µg/ml) and cefotaxime | | | | Cefotaxime alone | | | | PCN alone |
| | 25 | 20 | 15 | 10 | 25 | 20 | 15 | 10 | 4 |
| ***Staphylococcus aureus*** | - | + | + | + | - | - | - | - | - |
| ***Staphylococcus epidermidis*** | - | + | + | + | - | - | - | + | - |
| ***Streptococcus pneumoniae*** | - | + | + | + | - | - | - | + | - |
| ***Streptococcus pyogenes*** | - | + | + | + | - | - | + | + | - |
| ***Streptococcus agalactiae*** | - | + | + | + | - | - | - | + | - |
| ***Enterococcus faecalis*** | - | - | + | + | - | - | - | + | - |
| ***Acinetobacter baumannii*** | - | - | + | + | - | - | + | + | - |
| ***Citrobacter freundii*** | - | + | + | + | - | - | - | - | - |
| ***E. coli*** | - | + | + | + | - | - | - | - | - |
| ***Enterobacter cloacae*** | - | - | + | + | - | - | - | + | - |
| ***Klebsiella pneumoniae*** | - | + | + | + | - | - | - | - | - |
| ***Proteus mirabilis*** | - | + | + | + | - | - | - | + | - |
| ***Salmonella* spp.** | - | - | + | + | - | - | - | + | - |
| ***Shigella* spp.** | - | - | + | + | - | - | + | + | - |

**+:** growth; **-:** no growth

The most activity of PCN at 2 µg/ml on the cefotaxime was elevated its MIC value from 10 to 15 µg/ml against *S. aureus*, *Citrobacter freundii*, and *E. coli* or from 20 to more than 25 µg/ml against *Acinetobacter baumannii*. The MIC of cefotaxime against other strains was not changed in the presence of PCN (table 23)

Generally, the cefotaxime alone showed inhibition activity against all of isolated bacteria at high concentrations (20, and 25 µg/ml), while it inhibited 7 strains (*S. epidermidis*, *Enterococcus faecalis*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Salmonell*a spp. and *Shigella* spp.) at 15 µg/ml. Other five strains of bacteria (*S. aureus, S. pneumoniae*, *S. agalactiae, Citrobacter freundii,* and *E. coli*) were inhibited at 10 µg/ml of cefotaxime (table 23).

The MIC value of high concentrations of cefotaxime (15, 20, and 25 µg/ml) that was mixed with 2 µg/ml of PCN showed no effect on all isolated bacteria, except with *Acinetobacter baumannii* in comparison with cefotaxime alone. At 10 µg/ml of cefotaxime, antibacterial action of this antibiotic decrease by elevating MIC value after mixing with PCN on some isolates as with *S. aureus, Citrobacter freundii,* and *E. coli*, while other not affected by PCN such as *S. epidermidis*, *S. pneumoniae*, *S. pyogenes*, *S. agalactiae*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Salmonell*a spp. and *Shigella* spp. Meanwhile, antibacterial activity of cefotaxime increased in the presence of PCN at concentration 10 µg/ml against *Enterococcus faecalis*. However, the lower concentration of cefotaxime (0.625 µg/ml) showed no change after mixing with PCN (table 23).

**Table (23): MIC of cefotaxime with pyocyanin (2 µg/ml).**

| Strain | Concentration (µg/ml) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | PCN (2 µg/ml) and cefotaxime | | | | | Cefotaxime alone | | | | | PCN alone |
| | 25 | 20 | 15 | 10 | 0.625 | 25 | 20 | 15 | 10 | 0.625 | 2 |
| ***Staphylococcus aureus*** | - | - | - | + | + | - | - | - | - | + | + |
| ***Staphylococcus epidermidis*** | - | - | - | + | + | - | - | - | + | + | + |
| ***Streptococcus pneumoniae*** | - | - | - | - | + | - | - | - | - | + | + |
| ***Streptococcus pyogenes*** | - | - | + | + | + | - | - | + | + | + | + |
| ***Streptococcus agalactiae*** | - | - | - | - | + | - | - | - | - | + | + |
| ***Enterococcus faecalis*** | - | - | - | - | + | - | - | - | + | + | + |
| ***Acinetobacter baumannii*** | + | + | + | + | + | - | - | + | + | + | + |
| ***Citrobacter freundii*** | - | - | - | + | + | - | - | - | - | + | + |
| ***E. coli*** | - | - | - | + | + | - | - | - | - | + | + |
| ***Enterobacter cloacae*** | - | - | - | + | + | - | - | - | + | + | + |
| ***Klebsiella pneumoniae*** | - | - | - | + | + | - | - | - | + | + | + |
| ***Proteus mirabilis*** | - | - | - | + | + | - | - | - | + | + | + |
| ***Salmonella* spp.** | - | - | - | + | + | - | - | - | + | + | + |
| ***Shigella* spp.** | - | - | - | + | + | - | - | - | + | + | + |

**+:** growth; **-:** no growth

## 4.9. Determination the effect of PCN on griseofulvin activity:

The MIC of PCN at 2 µg/ml and griseofulvin at 100, 50 and 25 µg/ml was tested alone or as a mixture against more sensitive species of fungi. The PCN played an effective role in the enhancement of the MIC value of griseofulvin against *M. canis* and *C. albicans* from 25 to 100 µg/ml.

In general, the action of PCN alone at 2 µg/ml showed no activity against tested fungi, while griseofulvin alone as a standard antifungal agent had the ability to prevent the growth of both fungi at all tested concentrations (100, 50 and 25 µg/ml). This result was changed after mixing of griseofulvin with PCN. The griseofulvin inhibited the growth of *M. canis* and *C. albicans* at higher concentration (100 µg/ml) only (table 24).

**Table (24): MIC of griseofulvin with pyocyanin (2 µg/ml)**

| Fungal strain | Concentration (µg/ml) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Griseofulvin and PCN (2 µg/ml) | | | Griseofulvin alone | | | PCN alone |
| | 100 | 50 | 25 | 100 | 50 | 25 | 2 |
| ***M. canis*** | - | + | + | - | - | - | + |
| ***C. albicans*** | - | + | + | - | - | - | + |

**+:** growth; **-:** no growth

# *Chpter Five*

# *Discussion*

# 5. Discussion:

## 5.1. Dermatophytoses:

Dermatophytoses or ringworm disease is one of the most common infectious skin diseases worldwide. Tinea is another name for such infection which mostly used for this disease (AL-Janabi, 2014). Determination the anatomically location of the disease on the human body can be known by appending the Latin term designating the body site after the word tinea, e.g. tinea pedis for ringworm of the foot (Weitzman and Summerbell, 1995). Keratinous part of the skin tissues is more infected area as in superficial skin layer, nail and hair (Weitzman and Summerbell ,1995; Dismukes *et al.*, 2003; AL-Janabi,2014). Dermatophytes are the main causative agents of dermatophytoses which include three genera *Trichophyton* spp., *Microsporum* spp., and *Epidermophyton* spp. (Weitzman and Summerbell ,1995; Dismukes *et al.*, 2003; AL-Janabi, 2014). These fungi form restricted infection within superficial layer of the skin and could not deeply penetrate due to inability to tolerance the body temperature (37º C) and other inhibitory compounds of the blood (AL-Janabi, 2014). However, skin infection could be caused by a single species of dermatophyte or by different species producing clinically identical lesions (Weitzman and Summerbell, 1995).

This study included, seven patients from 18 suspected cases were diagnosed as a positive for dermatophytoses in which the infection distributed between tinea capitis and tinea corporis. Tinea capitis was the most infection type among involved patients. Mohammed *et al.* (2015) also mentioned that tinea capitis in Baghdad city was the common dermatophytoses diseases among patients, while tinea corporis was the less one. Whereas, tinea capitis recorded to be the less type of dermatophytoses among patients in Samarraa city of Iraq and in Bursa city of Turkey with 8.3% and 0.18%, respectively,

while tinea corporis showed highest ratio (45% and 4.50%, respectively) (Akcaglar *et al.*, 2011; Bander *et al.*, 2012). However, the order of tinea capitis was variable among patients in other Iraqi cities. In Al-Najaf city, it represented 18.47% of cases after tinea corporis (47.77%), while tinea cruris was the less type (4.46%) (Al-Hmadani *et al.*, 2014). This was also found in Thi-Qar city when tinea capitis (17.27%) noted to be less than tinea corporis (42.73 %) and the lower percentage was tinea faciei (3.63 %)(Najem *et al.*, 2016). The same results was obtained in Diala city when tinea corporis was the most common type (44.6%), followed by tinea capitis (39.2%), tinea faciei (10.8%), tinea pedis (2.3%), and tinea cruris (1.5%) (Abdul Hussan *et al.*, 2014). In Saudi Arabia, tinea capitis and tinea corporis were noted at high percentage in age group 0-15 years (Al Sheikh, 2009). Moreover, tinea capitis found to be at third level after other types of dermatophytoses in various Iraqi cities such as in Erbil city which came after tinea pedis and tinea manuum (Ismael, 2011) and at fifth level in Hilla city after tinea unguium, tinea cruris, tinea corporis and tinea pedis (Abed Ali *et al*, 2017) and at fourth level in Al-Hindya (Karbala) after tinea pedis, tinea unguium and tinea manuum (Mohammed and Al-Daamy, 2012).

Among our involved patients, females showed high percentage of dermatophytoses than males. This was also noted in various Iraqi cities as in Al-Najaf, Al- Hindya (Karbala), Samaara, and Hilla (Bander, 2012; Mohammed and Al-Daamy, 2012;; Al-Hmadani *et al.*, 2014; Abed Ali *et al.*, 2017) or in other Arab countries such as in Saudi Arabia (Al Sheikh, 2009). Meanwhile, males in Baghdad were high infected with dermatophytoses than females (Mohammed *et al.*, 2015).

Seven strains of dermatophytes were successfully diagnosed by using the sequences of ITS 1 and ITS 2 genes. They have now a registration code in GenBank as isolates of dermatophytes in Karbala-Iraq (as mentioned in result

section). The sequences are deposited in the GenBank database. *Microsporum canis* was the most common isolates of dermatophytes, followed by *Microsporum ferrugineum*, and one isolate of each of *Trichophyton interdigitale*, and *Epidermophyton floccosum*. Two strains of *Cryptococcus* spp. and one of *Candida albicans* were also isolated.

*M. canis* had also registered to be the most common isolates in two of Iraqi cities, Al-Najaf and Thi-Qar cities. In Al-Najaf, it showed highest frequency (31.8 %), while *M. audouinii* and *Trichophyton schoenleinii* was the lowest frequency (4.71%) (Al-Hmadani *et al.,* 2014). The percentage of *M.canis* (40.91 %) distribution in Thi-Qar was higher than other dermatophytes, followed by *Trichophyton tonsurans* (32.73 %), *Trichophyton verrucosum* (15.45%), *Microsporum gypseum* (8.18%), and *Microsporum fulvum* (2.73 %) (Najem *et al.,* 2016).

*Trichophyton interdigitale* is usually one variant of *Trichophyton mentagrophytes* that cause dermatophytoses in the human as anthropophilic fungi (Oyeka, 2000). *Trichophyton mentagrophytes* (33.6%) was the most predominate species in Diala city followed by *T. rubrum* (21.5), *T.verrucosum* (16.8%), *T. tonsurans* (11.2%), *T. soudanense* (9.3%) and *T.violaceum* (7.5%) (Abdul Hussan *et al.,* 2014). Whereas, it associated in the presence with *E. floccos*um in Al- Hindya (Karbala) (Mohammed and Al-Daamy, 2012). This also noted in Saudi Arabia when the *T. mentagrophytes* was higher common than *M. canis* and *E. floccosum* (Al Sheikh, 2009). On the other hand, *T. mentagrophytes* showed to be a second most common species after *T. rubrum* in many of Iraqi cities such as in Baghdad with 32.5% and 50%, respectively, in Bursa (Turkey), and in Belgaum (India) (Akcaglar *et al.,* 2011;Mohammed *et al.*,2015;Anupama,2017).However, most of *Trichophyton*

spp. considers more prevalence dermatophytic group found in Iraq as recorded in Erbil (Ismael, 2011).

During the study, the failed to get a dermatophytic species after direct examination and culturing of collected specimens from patients with suspected dermatophytoses were found at high percentage than the specimens which gave positive results. Whereas, most of studies found that positive obtaining of dermatophytes from clinical specimens are higher than the negative results. This is mostly differed from other studies in Iraq. About 87.7% of specimens gave positive results for isolating of dermatophytes in Al-Najaf city (Al-Hmadani *et al.*, 2014). From 180 cases of dermatophytes, positive results were about 52.2 %, while false negative results were recorded in 8.9 % of specimens (Najem *et al.*, 2016). In Diala City, direct examine and culture of collected specimens of dermatophytoses showed that 50.3% was positive and 22.5% was negative (Abdul Hussan *et al.*, 2014), while 73% of cases was positive from patients in Al- Hindya (Karbala) (Mohammed and Al-Daamy, 2012). Moreover, positive results in Erbil city recorded to be about 63% (Ismael, 2011). The high percentage of positive results also found in other countries such as in Saudi Arabia (71.54%), Bursa (Turkey) (57.30%), and in Belgaum (India) in which 63 samples were KOH positive and 37 samples were KOH negative (Al Sheikh,2009; Akcaglar *et al.*, 2011; Anupama, 2017).

## 5.2. Pyocyanin (PCN) production:

*Pseudomonas aeruginosa* is the main producing bacteria of PCN. Our strain was successfully diagnosed by using the sequences of 16S rRNA gene. It has now a registration code (as mentioned in results) in GenBank as an isolate diagnosed in Karbala-Iraq. Its ability to produce PCN was good enough to do the antimicrobial tests.

The PCN is a blue to green soluble pigment mainly produces by *P. aeruginosa* which is a Gram negative, motile, and rod bacteria (El-Fouly *et al.*, 2015). It is immediately dissemination in solid culture media leading to blue color, while in liquid media is appeared with a deep green color (Priyaja, 2012;Gahlout *et al.*, 2017). The highest rate of PCN production could be obtained when culturing of *P. aeruginosa* on nutrient agar at 37° C and pH 7.2 for 92 h (Vipin *et al.*, 2017) or on King´s B agar medium at 35° C for 48-72 h (Gahlout *et al.*, 2017). The differences in strain of *P. aeruginosa* could also be affected on the production amount of PCN. *P. aeruginosa* A10 strain isolated from urine specimen produced highest amount of PCN compared with the lowest production by *P. aeruginosa* A1 strain isolated from abdomen (Özyürek *et al.*, 2016). Incubation periods with strain differences is also effected on PCN production when most of bacterial strains began production of PCN after 24 h and reach maximum level after 48 h, while some strains produce high amount after 72 h (El-Shouny *et al.*, 2011). Otherwise, chemical components of medium have an important role in production of PCN. Culturing of *P. aeruginosa* on a peptone-containing media increase PCN synthesis due to the role of such compound as a main source of nitrogen through its content of peptides (Das and Das, 2015). Also the production of PCN is affected by pH value when its concentration increased at pH 7.0, while it decreased at higher pH (Mathew *et al.*, 2011; Agrawal and Chauhan, 2016). Moreover, the variation in PCN production from different *Pseudomonas* strains may be related to quorum threshold expression gene QteE (QteE) when over expression of this gene will significantly reduced the accumulation of homoserine lactone signals and affected the QS-controlled phenotypes such as the production of PCN (Liang *et al.*, 2008).

## 5.3. Antibacterial effects of PCN:

The PCN as one of virulence factors of *P. aeruginosa* can be used by this bacteria to eliminate other competition microorganisms (Mathew *et al.,* 2011; Hameed *et al*., 2017). Thus, PCN have variable antimicrobial effects on different M.O., including Gram positive bacteria, Gram negative bacteria and fungi (Saha *et al.,* 2008; El-Shouny *et al.,* 2011; Gharieb *et al.,* 2013; Sudhakar *et al*., 2015; Abdul-Hussein and Atia, 2016).

From this study, PCN showed antimicrobial effects at MIC 4 µg/ml on 11 bacterial strains, including 4 Gram positive and 7 Gram negative from 14 isolated bacteria, while other 3 strains revealed sensitivity to lower concentrations of PCN with less MIC value. Most of studies found that PCN have more antimicrobial activity against Gram positive bacteria than Gram negative bacteria (El-Shouny *et al.,* 2011; Gharieb *et al*., 2013; Jameel *et al.,* 2017). The Gram positive bacteria found to affected by PCN at MIC 0.06 mg/ml compared with the resistance of most of Gram negative group (Gharieb *et al.,* 2013). *Staphylococcus aureus* as one of Gram positive bacteria is more sensitive to PCN than two species of Gram negative (*E. coli* and *Klebsiella pneumoniae*) (Al-Jaff *et al*., 2016). This bacterium needed less MIC value (20 µg/ml) of PCN than *E. coli* (50 µg/ml) (El-Fouly *et al.,* 2015). *Bacillus* spp. was also more inhibited than *E. coli* after exposure to PCN (Özyürek *et al.,* 2016). The growth of all Gram positive bacteria inhibited in the presence of 10-20 µg/ml of PCN, while Gram negative showed variable results between resistance to the pigment as with *Klebsiella pneumoniae* and moderate sensitive as with *Salmonella typhi* and *Proteus mirabilis* (El-Shouny *et al.,* 2011). At 4 µg/ml of PCN, *Staphylococcus epidermidis* showed high sensitivity than other strains of bacteria. This result was also noted by Jameel *et al* (2017) who found that this bacterium also more affected by PCN, but at high concentration 167 mg/ml, while most of Gram negative bacteria were

resistance to such concentration of PCN as with *Klebsiella pneumonia*, *Acinetobacter* spp., *Proteus* spp. and *E. coli*. The antibacterial activity of PCN could be elevated by increasing the concentrations as showed by Saha *et al.* (2008) when zone of inhibition against *Citrobacter* spp. increased from 1.4 cm at concentration 5 mg/ml of PCN to 1.7 cm at 25 mg/ml.

Among all of tested bacteria, *Streptococcus agalactiae* was the most bacterial strains susceptible to PCN with lower MIC value (3.5 µg/ml). This bacteria is known to be more susceptible to different antibacterial agents as noted with the strains isolated from pregnant women which revealed more sensitivity toward eight various antibiotic with high susceptibility percentage (de Melo *et al*., 2016). Meanwhile, all of 1,166 isolates of *Streptococcus agalactiae* from pregnant women in Kuwait showed susceptibility to penicillin, ampicillin and cephalothin, while 0.7% of them were resistance to erythromycin and 1.7% resistance to clindamycin (Al-Sweih *et al*., 2005). Moreover, the 87 isolates of this bacterium from UTI cases revealed more susceptibility to penicillin, cefuroxime, cefaclor, and ceftriaxone, while high percentage of them resistance to tetracycline (80%) (Piccinelli *et al.*, 2015).

## 5.4. Antifungal effects of PCN:

In addition to antibacterial effects of PCN, it has antifungal activity against most of isolated fungi in this study. Among these isolated strains, *M.canis*(MH383044) was the most affected species by PCN (4 µg/ml), followed by *E.floccosum* and two other strains, while *Cryptococcus terreus* and *Candida albicanis* of isolated yeasts were susceptibility to PCN. Dermatophytes in another study needed a high concentration of PCN to prevent their growth as observed with *Trichophyton mentagrophytes* and *T. rubrum* which inhibited at concentration 167 mg/ml of PCN (Jameel *et al.*, 2017).

*Candida albicans* exhibited high sensitivity toward PCN at 4 µg/ml than other type of tested yeast followed by *Cryptococcus terreus*. Its activity in another study found to inhibited at high concentrations of PCN (167 mg/ml) (Jameel *et al.*, 2017) and the growth rate decrease when PCN concentration increased (Özyürek *et al*., 2016). El-Fouly *et al*. (2015) showed that *Candida albicans* inhibited at MIC 35 µg/ml of PCN. In some cases, *Candida albicans* needed very high concentration of PCN to inhibit which may reach to twenty fold of the original concentration (Gharieb *et al*., 2013) or it will not effected by such high concentration of PCN (100%) (Mohammed and Almahde, 2017). However, the susceptibility of yeast to PCN could be more than mold as noted with high sensitivity of *Candida* spp. compared with resistance of *Aspergillus niger* (Özyürek *et al.*, 2016). On the other hand, Sudhakar *et. al.* (2013) found that the growth of *Candida albicans* was inhibited at high MIC value (128 µg /ml) of PCN than molds (64 µg/ml). The antifungal activity of PCN on fungi is mainly related to the ability of PCN to arrest the electron transport chain of fungi (Karpagam *et al*., 2013).

## 5.5. Mechanism of action of PCN:

PCN is a redox-active compound by shuttling electrons between donors and acceptors leading to the catalyzing redox reactions in the host cell (Ran *et al.*, 2003; Lau *et al*., 2004; Wang and Newman, 2008; El-Fouly *et al*., 2015). PCN have a non-enzymatic NAD(P)H oxidase activity under aerobic conditions when oxygen molecular is the primary electron acceptor and NADPH is the main electron donor (Rada *et al*., 2008). Thus, the inhibitory effect of PCN on microorganism is through alteration of the normal electron transport in respiratory chain and formation of free oxygen radicals (Ran *et al*., 2003). PCN exists in an oxidized blue form and can easily cross host cell membrane and reacts with NADH and NADPH leading to convert it into

reduction form (O'Malley *et al*., 2004). The reduced form can react with molecular oxygen forming superoxide (O) and hydrogen peroxide ($H_2O_2$) as first introduced by Hassan and Fridovich in 1980. Therefore, PCN considers a key in oxidative stress due to its ability to increase intracellular levels of reactive oxygen species (ROS) (Muller, 2002; O'Malley *et al*., 2004; Reszka *et al*., 2004; Muller, 2006; Rada *et al*., 2008). This process will give the *P.aeruginosa* an ability to survive through the competition between the host and pathogen (Rada *et al*., 2008). The diffusible nature of PCN means that it can easily pass through the host cell membrane and undergo redox reactions with other molecules (Schwarzer *et al*.,2008). In general, PCN induce $H_2O_2$ production and subsequently trigger cell death in mammalian host or any other competing microorganism such as fungi and bacteria (Das and Manefield, 2012).

## 5.6. Effect of PCN on antibacterial agents

Combination of PCN with other chemical agents could be shown a synergistic or antagonistic antimicrobial effect on PCN itself or on the combined agent. Silver nanoparticles revealed synergistic antimicrobial effects with PCN on three species of bacteria (*Staphylococcus aureus*, *E. coli*, and *Pseudomonas aeruginosa*) and one yeast (*Candida albicans*) (Nowroozi *et al.*, 2012). Antibacterial action of PCN on *Staphylococcus aureus* increased after equal volume combination with bacteriocin (50 µl/50 µl) at 72 h contact time, while the action decreased against *E. coli* after mixing 30 µl of PCN with 70 µl of bacteriocin at 24 h contact time (Markraphael *et al.*, 2017). Kumer *et al.* (2014) found that the antifungal activity of three phenazines (phenazine-1-ol, phenazine-1-carboxylic acid, and phenazine-1-carboxamide)

revealed synergistic action against *Candida albicans* after combined with three azole agents (fluconazole, itraconazole, and clotrimazole).

The role of PCN on the activity of two antibacterial agents (ampicillin and cefotaxime) was examined on the isolated bacteria. Ampicillin which is a derivative antibiotic from penicillin by adding amino group to the benzyl penicillin molecule have a broad spectrum activity against bacteria by inhibiting microbial cell wall synthesis (Raynor,1997). It found to inhibit 50% to 60% of Gram-negative clinical isolates (Shibl, 1994). Resistance to ampicillin is increased among coliform bacteria (Raynor, 1997).

Combination of ampicillin with PCN at concentration 4 µg/ml decreased the ampicillin activity against most of isolated bacteria with increased the MIC value of such antibacterial agent. Activity of ampicillin found to be decreased after combination with several compounds resulting to antagonistic effects. Chloramphenicol has the ability to limit the bactericidal activity of ampicillin against group B streptococcal meningitis (Weeks *et al*.,1981). This result is related to that chloramphenicol is bacteriostatic agent, while ampicillin is bactericidal agent and combination of these two antibiotic will decrease the activity of each other (Ocampo *et al*., 2014). The hydroalcoholic extract of different parts of *Passiflora cincinnata* plant showed antagonistic effects with ampicillin on *Staphylococcus aureus* and *E. coli* (Siebra *et al*., 2018). Also some extract type of *Agrimonia eupatoria* L. showed antagonistic effects on ampicillin activity when tested against *E. coli* (Muruzović *et al*., 2017). Although *Streptococcus pyogenes* is resistance to ampicillin (Dakhil and Hamim, 2016), it showed more susceptible to the combination of ampicillin with high concentration of PCN.

The inhibitory effects of PCN on ampicillin activity were significantly declined after decrease the PCN concentration into 2 µg/ml. Thus, low concentration of PCN could be considered useful to enhance the antibacterial action of ampicillin. This type of combination will give a promising sign about using of ampicillin against the resistance bacteria. *Acinetobacter baumannii* revealed a high resistance to the combination of low concentration of PCN with ampicillin. This bacterium is well known as a multi-drug resistance to most of antibiotic as with ampicillin (Karumathil *et al*., 2018). Resistance ability of such bacterium is related to its contents of extended spectrum beta-lactamase (ESBL) genes (Ghaima, 2018).

Cefotaxime is a third generation of cephalosporin group with a broad spectrum antibacterial activity against Gram-positive and Gram-negative aerobic and anaerobic bacteria (Carmine *et al*., 1983). It has the ability to penetrate the bacterial cell wall with high β-Lactamase stability and has high affinity to important target enzymes (Schrinner *et al*., 1980). It is also favorable in use than ampicillin or first and second generation of cephalosporin due to its activity against multidrug-resistant Enterobacteriaceae (Carmine *et al.*, 1983).

As noted with ampicillin after combination with PCN, antibacterial activity of cefotaxime was also decreased against most of isolated bacteria. The antibacterial action of cefotaxime proved to be limited after combination with other agents. Chloramphenicol as bacteriostatic agent reduced cefotaxime activity against 26 clinical isolates of Gram-negative rods, group B streptococci, and *Staphylococcus aureus* (Asmar *et al.*, 1988). Cefotaxime activity against 24 strains of *Propionibacterium acnes* was also decrease as result from antagonistic action on either of linezolid, ciprofloxacin or clindamycin (Mory *et al.*, 2005). Aminoglycoside agents could also have

inhibitory effects on cefotaxime action, but such results have not been clearly established (Carmine *et al*., 1983).

On the other hand, PCN could not have any effects on antibiotic as showed by unaffected of some bacterial strains by the combination of PCN with either of ampicillin or cefotaxime in compared with the effect of any of these antibiotics alone. This result was very clear when combined a low concentration of PCN with cefotaxime. The antibacterial activity of cefotaxime on *Klebsiella pneumoniae* didn't affected by the presence of matrine agent compared with the synergistic effects with baicalein and clavulanic acid (Cai *et al.*, 2016). Combination of ampicillin with water extract of *Agrimonia eupatoria* L. showed no antibacterial effects on *P.aeruginosa* (Muruzović *et al*., 2017).

## 5.7. Effect of PCN on griseofulvin:

Griseofulvin is one of the most common antifungal agent used against the growth of dermatophytes via its inhibitory effect on the growing hypha (Dias *et al*., 2013). The main site of action of griseofulvin in fungal cells is preventing the cell division after binding with mitotic spindle and also has inhibitory effect on the synthesis of nucleic acid (Dias *et al*., 2013; Suganthi, 2016).Resistance to griseofulvin is emerged among many species of dermatophytes such as *T. tonsurans*, *M. canis*, and *T. violaceum* (Gupta *et al*., 2009). Thus a combination with other drugs is one choose to increase the antifungal activity of griseofulvin.

In this study, PCN showed inhibitory effects on the antifungal activity of griseofulvin against *M. canis* and *C. albicans*. This antagonistic activity reduces the suitability of griseofulvin to use in the treatment of

dermatophytoses. Thus, antidermatophytic activity of griseofulvin can increase by either converts of its pharmaceutical form or by mixing with other compounds. Application of griseofulvin in nanoparticles form showed a significant curative ability of guinea pigs infected with *Microsporum canis* after used as a gel for 8 days (Aggarwal and Goindi, 2013). Dissolution of griseofulvin could also be increased by mixing with beta-cyclodextrin in pellet form due to the role of beta-cyclodextrin in elevation of the solubilization of griseofulvin (Dhanaraju *et al*., 1998).

Combination of griseofulvin with other antifungal agents could be the successful action to increase its activity against dermatophytes. About 28 strains of *M. canis* revealed most susceptible to griseofulvin after combined with ketoconazole in compared with the effect of each agent alone (Banic and Lunder,1989). Combination of griseofulvin with miconazole exhibited a synergistic activity against 300 isolates of *Candida* spp., including *Candida albicans* (Mahmoudabadi *et al*., 2006). The *in vitro* and *in vivo* fungistatic activity of griseofulvin also increased after combined with amorolfine against *Trichophyton mentagrophytes* (Polak, 1993). The multidrug-resistant isolates of *T. mentagrophytes* (10 strains), *T. rubrum* (11 strains), *M. canis* (8 strains), and *M. gypseum* (12 strains) revealed susceptibility to the combination of griseofulvin with terbinafine (16:2 µg/ml) than antifungals alone (Lana *et al*., 2018).

# *Conclusions and Recommendations*

# Conclusions:

1. Among males, positive culture of dermatophytes was found only in two case of tinea capitis, while two of tinea capitis and three of tinea corporis were given a positive culture of dermatophytes from females.
2. Pyocyanin (PCN) was successively purified from clinical isolate of *Pseudomonas aeruginosa* which is registered in the database of GenBank under the number MH382164.
3. PCN showed approximately an equal antimicrobial effect against both of Gram positive and Gram negative bacteria at 4 µg/ml.
4. *Streptococcus agalactiae* needed less MIC value of PCN (3.5 µg/ml) to inhibit than other bacteria.
5. Antifungal action of PCN was also found against two strains of yeasts and four strains of dermatophytes which were registered with other three in GenBank.
6. *M. canis* (MH383044), *E. floccosum* (MH383041) and *Candida albicans* were revealed more sensitivity toward PCN at 4 µg/ml**.**
7. Antibacterial action of ampicillin decreased by the effects of PCN even at high concentration against *E. coli* and *E. cloacae*
8. Enhancement effect of PCN on ampicillin also showed on *S. pyogenes* at concentrations 4 and 6 µg/ml.
9. *Citrobacter freundii* revealed no differences in the sensitivity to ampicillin after mixed with PCN at concentration 6 µg/ml.
10. Antifungal activity of griseofulvin was decreased after mixing with 2 µg/ml of PCN against *M. canis* and *C. albicans* with elevation of MIC value to 100 µg/ml.

# Recommendations:

1. Antimicrobial effects of PCN need to be tested against other group of organisms.
2. Application of *in vivo* antimicrobial action of PCN against microbial infection in animals is suggested.
3. Determination of toxicity dose of PCN required performing by *in vivo* studies.
4. Activity of PCN on other antimicrobial agents is suggested to do.
5. Study of another pigments as antimicrobial effect.

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# الخلاصة


اصابة السعفة الجلدية تسببها فطريات جلدية تقع في طبقة الجلد و كذلك تصيب الاضافر و الشعر و تعتبر من الامراض الفطرية الواسعة الانتشار في العالم. شخصت اصابات السعفة الجلدية (Dermatophytoses) عند ثمانية عشر مريضا (تسعة ذكور وتسع إناث) من ضمن المشاركين في هذه الدراسة. اذ عزلت فطريات السعفة (Dermatophytes) من سبعة (38.88٪) من هؤلاء المرضى, بينما ظهرت النتائج السالبة عند احد عشر( 61.11٪) منهم. اظهر توزيع هذه الفطريات بين الجنسين وجودها عند اثنين من الذكور وعند خمسه من الإناث وكانت نسبة الاصابة بسعفة الرأس Tinea capitis حوالي اثنان (11.11٪) وهو النوع الوحيد من اصابات السعفة الجلدية الذي اعطى زرع ايجابي من الفطريات الجلدية بين الذكور, في حين ان اثنين من سعفة الرأس Tinea corporis (11.11٪) وثلاثة من سعفة الجسم Tinea pedis (16.66٪) اعطت زرع ايجابي من الفطريات الجلدية في الإناث.

البايوسيانين هي صبغات خضراء - مزرقة تنتج بشكل رئيسي من قبل بكتريا الزائفه الزنجارية خلال طور الثبات من منحنى النمو حدد نشاط البايوسيانين المضاد للميكروبات ضد انواع مختلفة من البكتيريا والفطريات. كما وتم قياس تأثيره على عمل مضادات البكتريا التي تشمل الأمبيسلين و السيفوتكسام وكذلك على تأثير الكريزوفيولفين المضادة للفطريات. عزل البايوسيانين بنجاح من العزلة السريرية *Pseudomonas aeruginosa* التي سجلت في قاعدة بيانات GenBank تحت الرقم (MH382164), كما استخدمت طريقة الكلوروفورم لتنقية البايوسيانين من الزرع البكتيري مع تحديد تركيزه بطريقة الطيف الضوئي.

اختبر نشاط البايوسيانين المضاد للبكتيريا ضد اربعة عشر سلالة من البكتيريا المعزولة والتي تم تشخيصها من خلال الخصائص المظهريه والاختبارات البايوكيميائية ونظام (API-20). اذ توزعت هذه البكتريا بين ثمانية سلالات سالبة لصبغة كرام و ستة سلالات موجبة لصبغة كرام, و كذلك اختبر عمل البايوسيانين المضاد للفطريات ضد ثلاثة سلالات من الخميرة وسبع عزلات من الفطريات الجلدية التي سجلت ايضا في (GenBank) بعد التشخيص الجزيئي لتسلسلات جينات ITS والتي تشمل *Trichophyton interdigitale* (MH383047), *Epidermophyton floccosum* (MH383041) وثلاث سلالات من الفطر *Microsporum canis* (MH383044, MH383045, MH383046) وسلالتين من الفطر *Microsporum ferrugineum*

(MH383042, MH383043), وحددت قيمة اقل تركيز مثبط (MIC) للبايوساينين ضد الكائنات الحية المعزولة عن طريق طرائق (CLSI).

اظهر البايوسيانين عند التركيز 4 مايكروغرام/مل تاثيرا متغايرا على كلا" من البكتريا السالبة والموجبة لصبغة كرام, وكانت السلالات الأكثر حساسية هي *Staphylococcus epidermidis*, *Salmonella* spp., *Streptococcus pneumonia*, *Citrobacter freundii* and, *Eenterobacter cloacae*, والتي أظهرت منطقة تثبيط (7 , 5.6 , 5.3 , 5.1 , 5 ملم على التوالي) في حين اظهرت عزلات اخرى اقل حساسية للبايوسيانين (4 مايكروغرام) مثل *Streptococcus agalactiae*, *Streptococcus pyogenes*, *Acinetobacter baumannii*, *E. coli* التي اظهرت منطقة تثبيط ( 2.2 ,2.3 ,2.3 ,2.4 ملم, على التوالي), في حين احتاجت البكتريا *Streptococcus agalactiae* الى اقل تركيز مثبط MIC من البايوسيانين بمقدار (3.5 مايكروغرام/مل) لتثبط.

اختبر البايوسيانين عند التركيز 4 مايكروغرام/مل ضد الفطريات المعزولة, وكانت الفطريات اكثر *Candida albicans*,(MH383041) *E. floccosum*, (MH383044) *M. canis* حساسية للصبغة مع فرق معنوي عند المقارنة مع بقية الفطريات عند احتمالية اقل من 0.05 ( $p < 0.05$) وكانت منطقة التثبيط 1.2, 4.5, 6.9 ملم على التوالي, في حين كانت حساسية الفطريات *Cryptococcus terreus*, (MH383046) *M. canis*, (MH383043) *M. ferrugineum* متغيرة ولكن مع فرق معنوي عند المقارنة مع بعضها عند احتمالية اقل من 0.05($p <0.05$) وكانت مناطق التثبيط 0.3 , 0.7, 2 ملم على التوالي, كما احتاج الفطر *M. canis* (MH383044) الى تركيز مثبط ادنى 3.8 مايكروغرام/مل بينما احتاجت الخميرة *C. albicans* الى 4 مايكروغرام/مل.

اظهر البايوسيانين(4 مايكروغرام/مل) فعالية متغيرة على الفعل المضاد للبكتريا للامبسلين على بعض العزلات البكتيرية, اذ قلل البايوسيانين من فعالية الأمبسلين حتى وان كان تحت تركيز عالي (8 مايكروغرام/مل) مثلما ظهر ضد البكتريا *E. coli*, *E. cloacae* مقارنة مع الأمبسلين لوحدة, كما رفع البايوسيانين من فعالية الأمبسلين ضد *S. pyogenes* بشكل خفض قيمة MIC من 6 الى 4 مايكروغرام/مل, كما خفض البايوسيانين عند التركيز 2 مايكروغرام/مل فعالية تثبيط الأمبسلين الدنيا (MIC) تجاه جميع البكتريا لتصل الى التركيز 2 مايكروغرام/مل, ما عدا البكتريا *Acinetobacter baumannii* التي قاومت هذا التاثير حتى عند قيمة MIC العالية (8 مايكروغرام/مل) من الأمبسلين مقارنة مع الأمبسلين لوحدة.

ثبط نمو جميع البكتريا عند التعرض الى مزيج السيفوتكسام بتركيز 15 مايكروغرام/مل مع البايوساينين بتركيز 4 مايكروغرام/مل ما عدا البكتريا *Streptococcus pyogenes*, *Acinetobacter baumannii*, *Shigella* spp., في حين لم يؤثر المزيج الحاوي على التركيز الواطيء من السيفوتكسام (10 مايكروغرام/مل) على جميع البكتريا ما عدا العزلات *Staphylococcus aureus*, *Citrobacter freundii*, *E. coli*, *Klebsiella pneumoniae*, كما لم يظهر البايوسيانين اي فعل رافع الى فعالية السيفوتكسام ضد جميع البكتريا عندما كان تركيزه عالي (20 مايكروغرام/مل). عموما فان اكثر فعالية للبايوسيانين عند 2 مايكروغرام/مل على السيفوتكسام كانت بصورة زيادة قيمة اقل تركيز مثبط MIC له من 10 الى 15 مايكروغرام/مل ضد البكتريا *S. aureus*, *Citrobacter freundii*, *E. coli*, وكذلك من 20 الى اكثر من 25 مايكروغرام/مل ضد البكتريا *Acinetobacter baumannii*, في حين لم تتغير قيمة اقل تركيز مثبط MIC للسيفوتكسام ضد العزلات الاخرى.

انخفضت فعالية المضاد الفطري كريزوفيولفين عند مزجه مع البايوسيانين بتركيز 2 مايكروغرام/مل ضد الفطريات *M. canis*, *C. albicans* بدلالة زيادة قيمة اقل تركيز مثبط MIC الى 100 مايكروغرام/مل.

جمهورية العراق
وزاره التعليم العالي والبحث العلمي
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فرع الاحياء المجهرية

# التأثير المضاد للمايكروبات للبايوسيانين المنتج من بكتريا الزائفه الزنجاريه المعزولة سريريا"

رسالة مقدمة الى
مجلس كلية الطب جامعة كربلاء
كجزء من متطلبات نيل شهادة الماجستير في الاحياء المجهرية الطبية

من قبل
جنان كاظم محمد الاسدي
بكالوريوس علوم حياه/جامعه بابل (1999)

بأشراف
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