Ministry of Higher Education and Scientific Research
University of Karbala
College of Science

Effect of some fruits peel extracts on the virulence of some pathogenic bacteria isolated from urinary tract infection in pregnant women in Karbala

A thesis
Submitted to the council of the
College of science – University of Karbala
In partial fulfillment of the requirements for the degree
of Master of Science
In Biology/ microbiology

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July 2013 1434 Shaaban

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رَبِّكَ حِينَ تَقُومُ ۞

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الطور :48

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Muhammad ……

(sallaallah alaihi wa ala alehi wa sallem)

Who guide the world to the light

(Peace be upon him)

Iraq …..

My wounded country

Mother …….

My love

Father …..

My power

Sisters & Brother…

My support

I dedicate this work

Researcher

B. S. H.

Acknowledgments

First of all, I would like to thank God for his blessing and mercy that without them, this study have not been achieved.

I would like to thank the dean of the College of Science and the head of department of Biology for his continuous cooperation and assistance.

I wish to express my sincere gratitude and appreciation to all who have helped me to complete this study. The first one I would like to express my deepest gratitude to my supervisor Asst. Prof. Dr. Wafaa Sadeq Al-Wazni for her supervision, encouragement, guidance, helpful, her advice and infinite support throughout all stages of this work.

Also I would thank the entire staff member in the Department of Biology/ College of Science /Karbala University, especially Asst. prof. Dr. Thikra Adnan Jawad, Sind AL-Dorri and Hussain Ali for their indefinite help.

Special thanks go to the staff of Laboratory in Maternity and Women's Hospital in Karbala, especially Ftimma and Nuras.

I am grateful to Dr. Thamer Kareem AL-Janabi for his assist in the statistical analysis of the data.

I would like to express my sincere thanks to Alaa, Maitham and Haider, students of the master at the College of Education of Karbala University for their help experimental work.

Also, I am indebted to my colleagues of master for their help during my experimental study.

Finally, special thanks to members of my family for their patience, encouragements and never ending assistance. To them I own a great gratitude for helping me to achieve this work.

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LIST OF ABBREVIATIONS

Abbreviation	Key
BHIb	Brain Heart Infusion broth
Cnf1	Cytotoxic necrotizing factor 1
DNA	Deoxyribonucleic acid
EMB	Eosin methylene blue
EPS	Extracellular polymeric substance
FIC	Fractional inhibitory concentration
H_2S	Hydrogen sulfate
KOH	Potassium hydroxide
LPS	Lipopolysaccharides
LSD	Less significant difference
mμ	Millimicron
MDR	Multi-drug resistance
MIC	Minimum inhibitory concentration
MRVP	Methyl Red Voges Proskauer
MSCRAM	Microbial surface components recognizing adhesive matrix molecules
OD	Optical density
PBS	Phosphate buffered saline
RBCs	Red blood cells
RNA	Ribonucleic acid
TCP	Tissue culture plate
TM	Tube method
TSI	Triple sugar iron
UTI	Urinary tract infection

SUMMERY

The present study included the isolation and identification of pathogenic bacteria responsible for urinary tract infection in pregnant women, attending Maternity and Women's Hospital in Karbala province during the period from December 2011 till March 2012.

A total of 100 morning mid-stream urine samples from pregnant aged from 16 to 40 years were streaked on general and selective media, then the growing bacteria were diagnosed biochemically and by used Api kits.

The results revealed the occurrence of 52 isolates of pathogenic bacteria which is divided to Gram– positive bacteria 43 (83%) which represented by coagulase positive *Staphylococcus aureus* 20 (39%)*;* coagulase negative *Staphylococcus* spp. 15 (29%), *Streptococcus* spp. 8(15%) and Gram – negative bacteria 9 (17%) which is divided between *Escherichia coli* 6 (11%) and *Klebsiella* spp. 3 (6%). Then the ability of *S. aureus* and *E. coli* bacteria which are responsible for the highest percentage of urinary tract infection to form biofilm were tested by using tube methods (TM), in order to select one isolate from *S. aureus* and other one from *E. coli* which had the higher ability to form the biofilm for continue the other steps of these study. Then the ability of these two isolates to form some virulence factors such as hemolysin; protease; β – lactamase and adherence are tested.

After screening 11types of the fruits peel extracts including (apple, banana, cucumber, lemon, muskmelon, peach, pummel, pomegranate, sour orange, sweet orange and watermelon) which had been extracted by three solvents (alcoholic 96%, aqueous and the acetone 70%), the results revealed that only the pomegranate had the ability to be as the antibacterial on the studied *S. aureus* and *E. coli* bacteria. The inhibition

zones diameters of the pomegranate peel extracts against studied bacteria increase significantly with concentrations increase to reach for *S. aureus* to 1.88 cm in 0.1 g/ml from aqueous extract and 2.26 cm for alcohol extract, but the inhibition zone diameter for the acetone extract was 2.5 cm at the same concentration. While the 1.78 cm, 2.36 cm and 2.33 cm were considered the inhibition zones diameters against *E. coli* when used 0.1 g/ml from aqueous, acetone and alcohol extracts respectively.

The three extracts of the pomegranate peel contain alkaloid, tannins, phenolic, glycosides and resins, while the flavonoids and saponins were absent in aqueous extract but ethanol extract did not have saponins only.

The MIC of the extracts was determined against studied bacteria which reached to 0.04 g/ml when used both the ethanol and the acetone extracts, while reached to 0.06 g/ml in the aqueous extract. The ability of two isolates to produce virulence factors were compared by using MIC before and after treated with extracts which revealed there is not effect of the extracts on the ability of bacteria to produce hemolysin and protease enzymes, while both *S. aureus* and *E. coli* bacteria loss its ability to produce β – lactamase enzyme after treated with MIC of the pomegranate peel extracts, in contrast with bacteria which were not treated with the extracts.

The ability of *S. aureus* and *E. coli* bacteria to form biofilm after treated with MIC of the extracts. The results were 0.76, 0.58 and 0.35 when *E. coli* treated with the aqueous, the acetone and the ethanol pomegranate peel extracts respectively (In comparison with *E. coli* without extracts which reach to 1.5). While *S. aureus* results were 1.7 without extracts, but reached to 0.86, 0.85 and 0.12 in the present of the aqueous, the acetone and the ethanol pomegranate peel extracts respectively.

The three extracts were vary largely in their effect on adherence activity for these bacteria, *S. aureus* adhered cell declined after treated with the extracts to be with the aqueous extract10 bacteria /cell only but reach to 3 and 1 bacteria /cell in the presence of the acetone and the alcohol extracts respectively, while *S. aureus* adhered cell was 40 bacteria/cell when the extracts did not find. The adherence of the *E. coli* bacteria reached to one bacteria /cell when the acetone extract added to bacterial suspension, but only three bacterial cell seen attached to epithelial cells after the bacterial suspension incubated with the alcohol extract, while only 5 bacteria / cell remain attached after the aqueous extract was added, in contrast with the control *E. coli* without extracts which was 20 bacteria /cell.

The used of the ethanol extract together with chloramphenicol and ciprofloxacin antibiotics against *S. aureus* was synergy, while in the studied of *E .coli* bacteria, found that the synergy appear only in the interaction between the Tetracycline and the acetone extracts, but at the case of the ethanol extracts and the chloramphenicol which was additivity.

Introduction

INTRODUCTION

Urinary Tract Infection (UTIs) is an infection caused by the presence and the growth of microorganism anywhere in the urinary tract and is perhaps the single commonest bacterial infection of mankind (Haider *et al*., 2010) so it could be defined as the colonization of and invasion of the structures in the urinary tract by micro-organisms, incomplete bladder emptying, fluid intake, stone in the urinary tract, sex, diabetes and pregnancy are considered factors increasing the risk of acquiring UTI (Al-Dulaymi, 2005).

The pathogen able to cause infection due to their virulence factors, which either responsible for the resistance to the antibiotics, by the capacity to produce β- lactamase enzyme or increasing their adhesion and colonize the inner surface of human body. When pathologic bacteria enter the host body, they must first adhere to the surfaces of the cells. Mucus or other fluids of the normal defense mechanism carried the bacterial out if they did not adhere. Bacterial adhesion to the host tissue was an important initiating step in many types of infection by helping the bacteria to resist the defense mechanism in the body, enable colonization and growth, and might be the first phase in bacterial invasion into mammalian cells (Juuti, 2004; Al-Dulaymi, 2005; Atabek, 2006).

The initial adhesion and aggregation of microbes' onto multiple layers is followed by biofilm formation which plays a vital role in pathogenesis and plays a significant part in morbidity and mortality. The biofilm development requires adhesion forces for both the colonization of the surfaces and cell to cell interactions so the primary adhesion depend on the chemistry of the material used for the implanted devices, which also

affect other processes including biofilm formation and its thickness (Hussain, 2011).

Many of plants are used in medicine for treatment of disease such as pomegranate which was one of the oldest fruits that have not changed much through the history of man (Dahham *et al*., 2010). The pomegranate (*Punica granatum*) has been used to treat several diseases. The fruit is consumed mainly fresh or in beverages and is a rich source of phenolic compounds, including hydrolyzable tannins, which possess high antioxidant activity. Ellagitannins are the major polyphenols found in pomegranate fruit (Endo *et al*., 2012).

Some resistant pathogens which responsible for the infection of urinary tract are routinely complicated to be dealt with due to thier virulence factors and because of a relatively limited choice of antimicrobial agents. Thus, it is extremely important to find novel antimicrobials or new techniques that are effective for the treatment of infectious diseases caused by drug-resistant microorganisms. Many studies have demonstrated that plants either contain antimicrobials that can operate in synergy with antibiotics or possess compounds that have no intrinsic antibacterial activity but are able to sensitize the pathogen to a previously ineffective antibiotic (Aiyegoro *et al.*, 2011).

Aim of study

The present project was designated to found if the fruits peel extracts that give antibacterial activity could be used as inhibiter for the virulence factors production from pathogenic bacteria. The aim was achieved by these objects:

1. Isolation and identify the pathogenic bacteria isolated from urinary tract infection in the pregnant women and determination the most common isolated bacteria.

2. Detection of the virulence factors of the most common isolates bacteria, such as adhesion, biofilm formation and β- lactamase enzyme.

3. Determination the antibacterial activity of some extracts of fruits peels.

4. Search for the phytochemical screening for the fruit peel extracts which gives antibacterial activity.

5. Make a correlation between the active fruit peel extracts and a suitable antibiotic *in vitro*.

1

Chapter one

Literature Review

1.1 Urinary tract system

The blood was filtered by regulatory organ which located behind the peritoneum situated on the posterior wall of the abdomen on each side of the vertebral column, at about the level of the twelfth rib which is the kidney. The kidney reabsorption the filtered fluid and excreted urine (liquid waste).Urine composed of various chemical substances dissolved in the excess water filtered from the blood by the kidneys. Urine formed in the kidneys passed down the fibro-muscular ureters and collected in the bladder .Then excreted outside the body via the uretha these parts represented the parts of urinary system as shown in figure (1-1) (Al Run, 2008; Okonko *et al*, 2010).

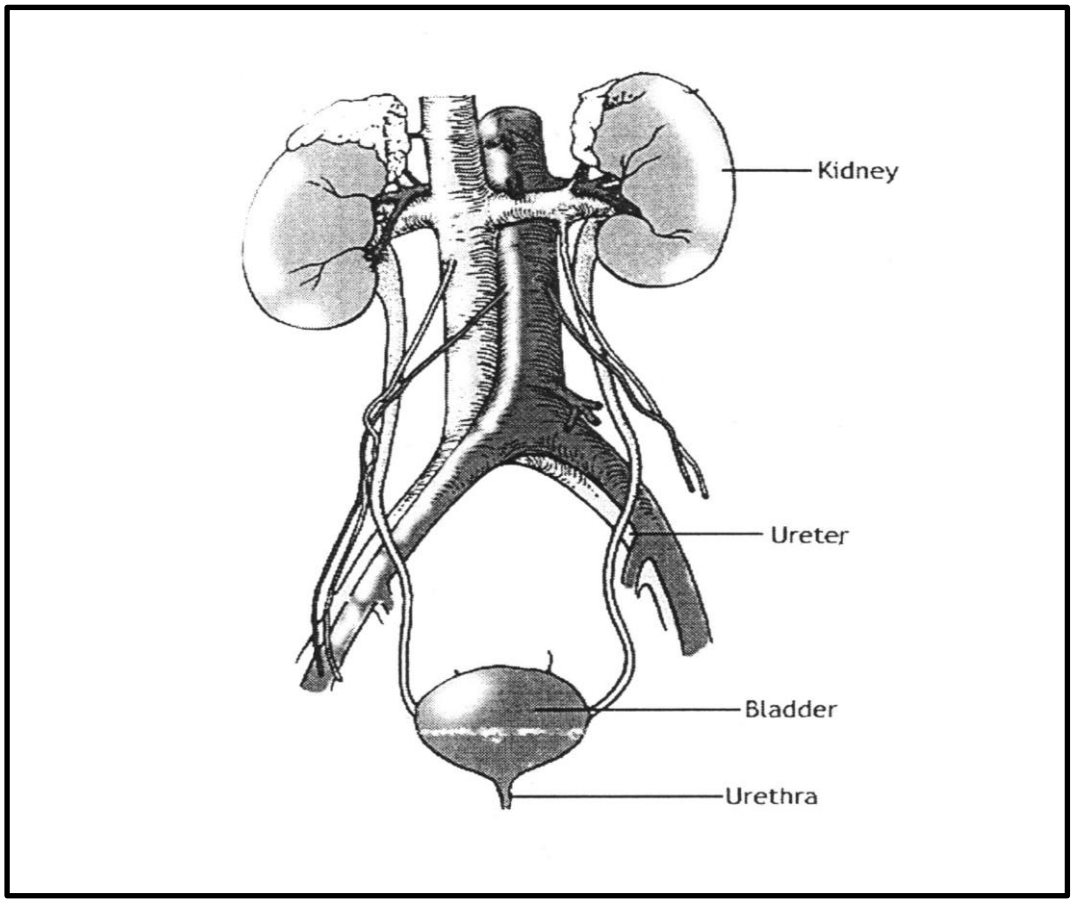

Figure (1-1): Urinary tract system (AL-Karhhi, 2006)

1.2 Urinary Tract Infection

When there was an infection in urinary tract the urine was not sterile, these infections usually caused by microorganism that present and growth in urinary tract causing infection that knew as urinary tract infection. Therefore these infection could be defined as the microorganism were colonized and invasion one structure of urinary tract. In human the urinary tract was one of the most common sites of bacterial infection (Nahar *et al*., 2010; Nielubowicz and Mobley, 2010; Okonko *et al*., 2010).

Wilson and Gaido, (2004) reported that the urinary tract infections (UTIs) are among the most common bacterial infections and account for a significant part of the workload in clinical microbiology laboratories, and UTIs are challenging case because of the large number of infections that occur each year.

Chang and Shortliffe, (2006) showed that the urinary tract is a common site of infection in the population, urinary tract infection in the pediatric population is well recognized as a cause of acute morbidity and chronic medical conditions, such as hypertension and renal insufficiency in adulthood.

1.3 Classification

1.3.1 Anatomically urinary tract infection could be divided to:

1 – Lower urinary tract infection which is divided into:

A. Cystitis: term used to refer to the bladder infection, cystitis is caused by uropathogenic bacteria in the faecal flora that colonize the vaginal and periurethral openings, and ascend the urethra into the bladder typically characterized by symptoms including frequency, urgency and dysuria. (Larcombe, 2011)

B. Urethritis: this term is used to refer to urethra infection and that happened when bacteria grow and colonize in urethra so it would be infected. (Larcombe, 2011)

2 – Upper urinary tract infection: when lower urinary tract infection was left without treatment, the infection could be developed to reach the upper part of urinary system, such as this infection called pyelonephritis and it is associated with symptoms such as fevers, nausea, and vomiting and flank pain. Pyelonephritis is an inflammatory process of the kidneys and adjacent structures. It is always happens when bacteria that exist in the bladder ascend the ureters and invades the kidneys. In some cases, bacteria enter and multiply in the bloodstream. The uncomplicated pyelonephritis is similar to acute cystitis (Nielubowicz and Mobley, 2010; Larcombe, 2011).

1.3.2 Based on the host condition urinary tract infection could be:

Complicated and uncomplicated infections:

Most urinary infections occur in person who is healthy, and this is known as uncomplicated infections. When upper or lower UTIs are associated with urinary tract abnormalities, this causes long-term inflammation. They include anatomical or functional defects of the urinary tract (Larcombe, 2011).

1 – Complicated: infection occurred in individuals with predisposing lesion of urinary tract that interferes with the normal flow of urine and urinary tract defiance (Nielubowicz and Mobley, 2010).

2- Uncomplicated: infection occurred in individuals who are healthy with normal flow of urine or voiding mechanism (Nielubowicz and Mobley, 2010).

1.3.3 Based on finding or not the symptom UTI could be divided to:

1. Symptomatic UTI

Urinary tract infection could occur with either the classical symptoms of frequency, such as urgency, pain and fever (Nawaz, 2005).

2. Asymptomatic UTI

Asymptomatic bacteriuria was condition characterized by the absence of symptoms of acute urinary infection at a time when true bacteriuria exists (Nawaz, 2005).

1.4 Routes of infection

Most uropathogens intervention the bladder from rectal flora passes through urethra. When bacteria enter to urethra initially adhesive and colonized to urethelium of distal urethra and this knew as a ceding route. Most cases of pyelonephritis were caused to the ascent of bacteria from the bladder through the ureters and into the renal parenchyma. Most case of urinary tract infection was caused to bacteria which ascend from the perineum, and the reason of the ascent of bacteria was raised by conditions like pregnancy (Sawalha, 2009).

1.5 Risk Factors

In normal condition urine was pure. The urinary tract in human body out word flow of urine, prevent growth 99% of microorganism. The bacteria growth would be discouraged by the acidic (pH 5.5) and the low osmolarity of urine. These were considered defense mechanisms which prevent bacterial infection (Al Run, 2008).

Ramzan *et al*., (2004) showed that there are many intrinsic and extrinsic risk factors which are main causes urinary tract infection. So to investigate urinary tract infection we need to know the relationship between various risk factors and urinary tract infection, there are several factors which increase the infection of urinary tract, such as:

A. In complete bladder emptying

In normal situation the bladder is completely empty each time it passes water. This was the most important defense mechanism against urinary tract infection which 99% of bacteria remove, usually incomplete emptiness of the bladder is regarded factor which allow pathogenic

bacteria to grow in urine as a result of not fully empty of the bladder (Al Run, 2008).

B. Fluid intake

A good fluid intake keeps the bladder free from urinary tract infection. Excessive fluid intake would lead to produce largeness in the size of urine. Urine (the filtered product containing waste materials and water) excreted from the kidneys passes down the fibro-muscular ureters and collects in the bladder and normally is sterile (Al Run, 2008).

The bladder has two functions, a storing one and an expelling one. Initially it receives the urine from the kidneys and stores it. As the bladder fills the muscle of the bladder wall (the detrusor muscle) relaxes, this is extremely important as it allows the urine to be stored at low pressure, and the bladder muscle (the detrusor muscle) is capable of distending to accept urine without increasing the pressure inside; this means that large volumes can be collected (500-700ml) and when the collected fluid volume more than that it causing stretch to bladder muscle and this would weak the muscle so not all urine was pushed out, and without high-pressure damage to the renal system would be occurred (Al Run, 2008).

So the bladder stretches to hold a large quantity, once sufficient urine has been collected, a person will become aware of the need to pass urine and the bladder then contracts to empty itself. When urine is passed, the urethral sphincter at the base of the bladder relaxes, the detrusor contracts, and urine is voided via the urethra. Urine is then discharged via the urethra (Al Run, 2008).

C. Stone in the urinary tract

There are two types of stone; one contains calcium in combination with either oxalate or phosphate, and outer kidneys uric acid or crystal, this stone is found in both male and female but in male more than in female. Immobility and loss the muscle tone after injury can cause the bones to lose a lot of calcium and other minerals that pass via the blood into the urine. They can then form stone in the kidneys or bladder. Other factors encourage stone formation such as the pH of the urine, if the urine remained alkaline long time and over saturate with ions that helped stone formation (Al Run, 2008; Jan *et al*., 2008).

D. Sex

Urinary tract infection could occur in both male and female at any age. The urethra in women short and wide therefor the bacteria from the rectum can easily travel up to urethra and cause infection. But in men there was distance between the anus and the urethra, urethral meatus and the urethra were long in male and these decrease bacterial infection (Okonko *et al.*, 2010; Haider *et al.*, 2010).

So female was more susceptible than male to have urinary tract infection, in female there is no prostatic gland which resistant to bacterial infection with several mechanism defense such as defense barriers, gently tract muscular, the acidity of prostatic fluid (pH: 6.1- 6.7) and zinc – associated prostatic antibacterial factor that secreted by it, therefor this gland might be normal defense mechanism in male (Parry and Mrcvs, 2006).

Hooton, (2000) said urinary tract infection occurred as a result of dynamic interaction between the host and the uropathogens, and the

large difference between man and women's' urinary system's design is thought to result from a variety of factors.

E. Diabetes

Diabetes mellitus is disease characterized by increasing blood glucose level (hyperglycemia) resulting from, defects in insulin secretion, insulin action or both. Host defense system became very weak in diabetes presence, in this case the risk of urinary tract infection increases and the modification of chemical composition of urine (high glucose concentration urine) with diabetes which increases the ability of microorganism to grow in urine because it creates culture media for bacteria (Baloch *et al*., 2011).

Shill *et al*., (2010) found that the incidence of diabetes mellitus throughout the world is increasing strikingly and in the long run, it has some major effects on the genitourinary system which makes diabetic patients more liable to urinary tract infection.

F. Pregnancy

Urinary tract infections are relatively common problems during pregnancy. The Physiologic changes which are related to pregnancy make otherwise healthy women susceptible to serious infectious complications such as urinary tract infections. The combination of mechanical, hormonal and physiologic changes during pregnancy contributes to significant changes in the urinary tract, during pregnancy (Sescon *et al*., 2003).

In pregnant women there are different anatomical and physiological changes which took place in urinary tract such as the dilation occurred in

urethra, increasing of secretion the progesterone and estrogen hormones, that lead to increase in their effects, and as a result of these hormones effects the size of bladder, decrease because dilution of the utera and decrease in bladder tone. Additionally, the physiologic change increase in plasma volume during pregnancy, so urine concentration would be increased. These factors affect to the urine state which has a profound impact on the acquisition of bacteriuria because it contain high concentration of several nutrients which are related with the urine during pregnancy, like glucose , B – complex , vitamins ,even amino acid and lactose (Nawaz, 2005; Nicolle, 2008; Obiogbolu *et al.*, 2009).

During pregnancy, the pH of urine is raised and that encourages bacteria growth; also there is great increase in the moistness and in the bacterial population of the vagina and reflex from the bladder into the ureters may occur (Nawaz, 2005).

Dimetry *et al*., (2007) told that urinary tract infection "UTIs" are one of the most problems facing the family. UTIs during Pregnancy are among the commonest health problems worldwide, especially in developing countries.

Cheriachan *el at*., (2008) showed that in most pregnant women, there are physiological changes due to hormonal changes and these are responsible for urinary tract infections (UTI). Hydronephrosis and hydroureter are physiological changes that often occur in pregnancy. Hydronephrosis causes urinary stasis, which predisposes urinary tract infections (UTI).

1.6 Causative agents of UTI

Most of urinary tract infections are caused by gram-negative bacteria like *Escherichia coli, Klebsiella* species, *Proteus mirabilis*, *Pseudomonas aeruginosa, Acinetobacter*, and *Serratia.* 90% of UTI cases are caused by gram-negative bacteria while only 10% of the cases are caused by gram positive bacteria .Gram-positive bacteria include *Enterococcus, Staphylococcus, and Streptococcus agalactiae* (Sawalha, 2009).

AL-Karkhi, (2006) isolated the bacteria which caused the urinary tract infection , these bacterial included the following 27.5 % *E. coli*, 15 % *S. aureus*, 7.5 % *Proteus* spp. , 6 % *Klebsiella* spp. , 3% *Pseud.* spp. , 5.5 % *S. saprophyticus* and 1.5 % simultaneous growth of both *E. coli* and *Proteus* spp.

Hamdan *et al*., (2011) studied urinary tract infection in pregnant women, and isolated gram negative and positive bacteria, respectively, the most predominant isolated organism was *E. coli* [(42.4%)], and other isolates were S. *aureus* [(39.3%)], *K. pneumoniae* [(9%)], group B *streptococcus* [(6%)] and *P. aeruginosa* [(3%)].

Okonko *et al.*, (2010) found that urinary tract infection (UTI) is challenging because of the large number of infections that occur each year, and the pregnancy is considered one of the risk factor which could increase the infection, and *E. coli* is the first reason of these infection , *S. aureus* the second pathogen causing infection.

1.6.1 *Escherichia coli*

The major etiological agent of urinary tract infection was *E. coli* this is confirmed by many studies .*E. coli* is responsible for two types of urinary tract infection which are asymptom and symptom infection, and they are regard major etiological agent for this infection (Abraham, 2011).

E. coli possess specific adherence factors that allow this pathogen to colonize in specific position. Adhesion to the cells could be function of physiochemical surface properties of bacteria as determined by a specific composition of lipopolysaccharides (LPS) and capsule. In addition, *E .coli* possesses definite morphological structure called fimbriae or pili that play important role in adhesion these bacteria mucous layers. (Chromek, 2006)

Fimbriae are complex surface structures that, in general, mediate adherence of bacteria to host epithelial receptors, and it is a definitive virulence factor helps the ascending urinary tract infection (UTI), facilitates adherence to the bladder cell surface glycoprotein uroplakin and other host proteins that contain mannosides. Although fimbriae is always present in *E. coli* strains *E. coli* which isolated from human patients. *E. coli* Fimbriae bind to different receptors and are therefore speculated to aid in colonization of different areas in the host urinary tract by formation of biofilms and mediates adhesion to human bladder epithelial cells (Nielubowicz and Mobley, 2010).

Some *E. coli* strains have been shown to express flagella, which contribute to bacterial mobility. In contrast, the role of flagella in colonization of urinary tract seems to be of a subordinate importance.

E. coli produce number of toxin which either associated with the membrane or secreted, and this toxin participate of urinary tract infection by different mechanism. LPS, bacterial endotoxin, a principal component of the bacterial cell membrane is recognized by the immune system as a pathogen associated molecular pattern and initiates local and systemic response, its toxicity to the side effects of triggered immune reaction (Chromek, 2006; Nielubowicz and Mobley, 2010).

Three main types of toxin are produced by uropathogenic *E. coli*: hemolysin, cytotoxic necrotizing factor (CnF1), and secreted autotransporter toxins. CnF1 has been implicated in adherence to and invasion of host cells. This toxin causes apoptosis of bladder cells, which might stimulate their exfoliation *in vivo*, thereby exposing underlying tissue, CnF1 is not required for infection, but might be a fitness factor. In addition, CnF1positive strains cause more inflammation than strains lacking this toxin. Autotransporter toxins have been characterized in uropathogenic *E. coli*; effects on bladder and kidney cell lines *in vitro* and elicit tissue damage and an immune response in infected (Chromek, 2006; Nielubowicz and Mobley, 2010).

Al- Hemidaei, (2005) found that gram negative bacteria of urinary tract infection were prevalent (76.3%) and *E. coli* was the most prevalent (42.1%).

Rahimkhani *et al*., (2012) reported that *E. coli* caused 20%, of urinary tract infection in pregnant women, and showed *E. coli* was responsible for 1/3 of the infections.

Obirikorang *et al.*, (2012) showed *E. coli* (36.8%) was the common bacteria isolate from pregnant women who apparent decline in the immunity which was responsible of urinary tract infection. While (Boye *et al*., 2012) investigated the urinary tract infections during pregnancy; *E. coli* was the most implicated organism (48.7 %).

1.6.2 *Staphylococcus aureus*

The infection of these pathogens bacteria caused by their virulence factors which help them to cross the barrier immune system created by the epiderm or the mucosa layer (Kumar, 2010).

S .aureus has a wide range of potential virulence factors, either surface associated or recreated, *S .aureus* produce a wide array of extracellular enzyme such as hyaluronate lyase, thermo stable nuclease and different proteases. Exotoxin is involved in many diseases caused by *S .aureus* (Atabek , 2006).

Coagulase is an enzyme that functions like thrombin which converts fibrinogen into fibrin. Coagulase is a thermo stable enzyme found primarily in *S. aureus*. There are two forms of coagulase, one bound to the cell wall and the other free coagulase because it liberated from the cell wall. The bound coagulase detected by slide coagulase method, the fibrinogen plasma caused clumping to bacteria directly. Tube coagulase test detect free coagulase that act on the prothrombin to produce a thrombin, when acts as fibrinogen to form a fibrin (Kayser, 2005; Tang and Stratton, 2006).

Coagulase has been suspected to assist infection via its procoagulant and fibrinogen-binding activity; it did not appear to promote either adherence to platelet-fibrin clots. By triggering coagulation, vegetation

adherent bacteria might promote additional deposits of platelets and fibrin on top of the infection nidus and thus become protected from further mechanical detachment and/or cellular host defense mechanisms. During host infection, the crystal structure of the coagulase in *S .aureus* conformationally activates the central coagulation zymogen, prothrombin, thereby triggering the cleavage of fibrinogen to fibrin. The association of the tetrameric complex enables fibrinogen binding at a new site with high infinity. This model can explain the coagulant properties and efficient fibrinogen by coagulase (Chromek, 2006).

Moyo *et al*., *(*2010) showed urinary tract infection (UTI) during pregnancy may cause complications such as pyelonephritis, hypertensive disease of pregnancy, and one of the most commonly isolated bacteria was *S. aureus*.

Muder *et al*., (2012) reported that *S .aureus* is frequently isolated from urine samples which are obtained from long-term care patients. The significance of Staphylococcal bacteriuria is uncertain.
S. aureus is a urinary pathogen and that colonized in urine could be a source of future staphylococcal infection.

1.7 Virulence factors of the pathogenic bacteria

1.7.1 Hemolysin

Hemolysin is an important virulence factor; this term refers to a protein toxin capable of lysine either human, rabbit and hours erythrocytes. Culturing on blood agar plates recognizes hemolysin production. *S .aureus* usually produced β. Hemolysis on blood agar which surrounded by a large yellowish hemolytic zone which no more

erythrocytes are present. Hemolysin attached with especial receptors on the erythrocytes wall then making pours in the cell wall so the erythrocytes will lyses (Al-Dulaymi, 2005; Kayser *et al*, 2005; Tang and Stratton, 2006).

Hemolysin is a prototypical calcium-dependent repeats in toxin secreted protein that is found more commonly in uropathogenic strains of *E. coli* than fecal strains. This toxin inserts into host cell membranes and is active against many cell types, including uroepithelial cells. Hemolysin, which is also implicated in invasion, stimulates cytokine production and leads to an inflammatory response. Although hemolysin is not required for colonization in experimental urinary tract infection (UTI), but it contributes as virulence because it is responsible for epithelial damage and bladder hemorrhage *in vivo* and has been implicated in causing renal damage and increasing risk for septicemia (Nielubowicz and Mobley, 2010).

1.7.2 β- lactamase

One of the reasons for the success of human pathogen is its great variability, occurring at different periods and places with diverse clonal types and antibiotic resistance patterns within regions and countries.Although infections caused by antibiotic- resistance (Akindele *et al*., 2010).

The best known mechanism of bacterial resistance is resistance to β - lactam, some bacteria resistance to β - lactem antibiotic family (cephamycins, oxyiminocephalo-sporins, carbapenems, monobactams, and clavam and penicillanic acid sulfone inhibitors). Because they are capable to produce β- lactamase enzyme many of pathological bacteria

threat public health by causing change to medical chemical structure of the antibiotics when developing new and more effective to β- lactam antibiotics. β -lactamase (also known as penicillinase) is enzyme that cleaves the ß-lactam ring and inactivates the antibiotic. β- lactamase enzyme contain serine amino acid in their hydroxlic group which represented the active site of the enzyme (Al-Dulaymi, 2005; Akindele *et al*., 2010; Issa, *et al.*, 2010; Medeiros, 2012).

This enzyme is able to activate β- lactam antibiotics by covalently binding to the carbonyl moiety of β- lactam ring and hydrolyzing its amide bond, one β- lactamase can stop the effect of more than one antibiotic molecular by breaking the β- lactam ring, this enzyme attacks β- lactam antibiotic before arriving to the target site in the bacterial cell wall (Al-Dulaymi, 2005; Issa *et al*., 2010; Medeiros, 2012).

β- Lactamase enzyme is produced from gram positive plus gram negative bacteria but there is an important different point between its. The enzymes in gram positive is an extra cellular enzyme secrete to the culture media while β- Lactam antibiotic find inside the gram negative bacteria because this enzyme is cell bound enzyme (Husein, 2012).

Many studies investigated the relationship between that *S. aureus* and *E. coli* and their ability of producing β -lactamase. (Olowe *et al.*, 2007) studied the susceptibility of *E. coli* to produc β –lactamases by recognizing mechanism of resistance to β-lactam antibiotics, such as penicillin and ampicillin in *E. coli*.

Akindele *et al.*, (2010) and Mamza *et al*., (2010) studied the antimicrobial susceptibility patterns of both *S. aureus* and *E. coli* to produce β -lactamase enzyme.

1.7.3 Bacterial adhesion

The physiochemical interaction between the bacteria and the surface is known as a bacterial adhesion. The adhesion nature of bacteria due to various outer membrane feature such as pili, flagella, proteins, and lipopolysaccharides. When bacteria enter the host body, they must first adhere to the surface of the cells. Mucus or other fluids of the normal defense mechanism carried the bacterial out if they did not adhere. Bacteria adhesion to the host tissue was an important initiating step in many types of infection by helping the bacteria to resist the defense mechanism in the body, enable colonization and growth, and might be the first phase in bacterial invasion into mammalian cells (Juuti, 2004; Al-Dulaymi, 2005; Atabek, 2006).

Adhesion of bacteria was governed not only by long range forces such as steric and electrostatic interaction but also by short range forces such as hydrogen bonding , acid base and bio specific interaction . Hydrogen, ionic and covalent bonding which knew as short range molecular interaction made bacterial adhesion occurred, bacteria cell surface which contain a variety of functional groups (extracellular structures), exhibit repulsive and attractive interaction with the surface and mediate bacterial adhesion (Atabek, 2006).

Ethel, (2006) studied the adherence of *E .coli* to the vaginal epithelial cells and showed that the greater receptivity of epithelial cells to bacteria may increase the susceptibility of urinary tract infection.

1.7.4 Biofilm formation

Most species of bacteria prefer biofilm as the most common means of growth in the environment. This kind of bacterial socialization has recently been described as a very successful form of life on earth. Although they can have considerable advantages in terms of self-protection for the microbial community involved or to develop in situ bioremediation systems, biofilms have great negative impacts on the world's economy and pose serious problems to industry, marine transportation, public health and medicine due to increase resistance to antibiotics and chemical biocides, increase rates of genetic exchange, altered biodegradability and increase production of secondary metabolites (Sayem *et al*., 2011).

Biofilms groups of microorganism attached to a surface and covered by an exopolysaccharide matrix vaxous chanche occur during their transition from planktonic to a surface attached community. In response to certain environment signals, new phenotypic characteristics develop in such bacteria (Bose *et al*., 2009).

Bacteria living in the outer environment as well as the pathogens bacteria special the human pathogens, which have the ability to form biofilm, a slimy layer with embedded micro colonies and this is the most important and most widespread mode for increasing pathogen of the microorganism and the biofilm formation helps bacteria to resist the surrounding environment condition (Hola *et al*., 2006).

The environmental condition plays an important role for biofilm development. Planktonic bacteria initially form a reversible attachment on the surface within minutes. Other chemical and physical interactions transform the reversible attachment to ending irreversible adsorption.

Then the bacteria produced extracellular polymeric substance [EPS] containing sugar, such as glucose and fructose which create a protective environment and help the bacteria to develop antibiotic resistance.

EPS is mainly composed of polysaccharides and can be considered as the primary compound of biofilms. It can link to metal ions and other macromolecular such as proteins, DNA and hunic substance due to the composition of [EPS]. Matrix [EPS] may also have an important role on antibiotic resistance by showing down the diffusion of antibiotic into the biofilm; figure (1-2) shows biofilm formation (Atabek, 2006).

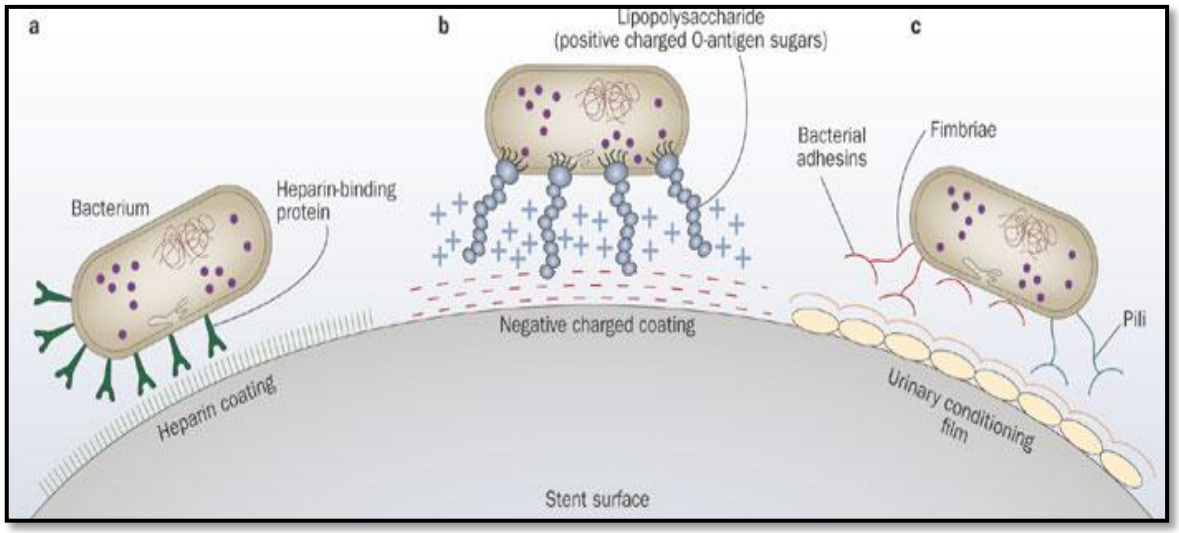

Figure (1-2): Elements responsible for biofilm formation (Chew and Lang, 2009)

1.8 Antibiotic agents

Infection has been one of the greatest blights in the history of humanity. There are many studies which find materials that could control the diseases and diseases agents, and this is done to be the discovery of antibiotics and their use as the chemotherapeutic agents, these substances have played a significant role in improving public health by helping to reduce the number of deaths from diseases, antibiotic can be defined as

microbial agents used to destroy or prevent the growth of bacteria, (Antimicrobial agents are which react against bacteria, viruses and other micro-organisms, Antimicrobial agents could be synthetic or natural substances), so the natural antibiotics is produced and isolated from living organisms, when antibiotic is modified chemically from original compounds found in nature, this antibiotic is semisynthetic one. The selective toxicity of antibiotics lies in the in cellular structures between eukaryotic and prokaryotic cells. However, differences in cellular structure among bacterial species can lead to resistance to certain antibiotics (Gangle, 2005; Sibanda and Okoh, 2007; Brown, 2010).

Antibiotics are used to treat bacterial infection in humans and animals, either by inhibiting bacteria cell wall biosynthesis or inhibiting protein biosynthesis but other agents can be used in action on synthesis of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) synthesis. There are many types of antibiotic agents which have been used for treating many bacterial infections, such as:

a. **Tetracyclines**: are another group of broad-spectrum antibiotics that inhibit bacterial protein synthesis. They are brought into the cell by active transport and, once there, bind to the 30S subunit to prevent binding of aminoacyl tRNA by inhibit binding of aminoacyl-tRNA to the mRNA-ribosome thereby inhibiting translation process which is an important step in protein synthesis. Resistance to the tetracyclines occurs via three mechanisms. First, production of a membrane efflux pump removes the drug as rapidly as it enters. Second, several ribosome protection proteins act to prevent tetracycline from binding to the ribosome, thus conferring

resistance. Third, enzymatically inactivates tetracycline, and the third one found only in *Bacteroides* spp. (Gangle, 2005).

b. Ciprofloxacin is consumed than any other antibacterial agent. Ciprofloxacin inhibits bacterial growth by acting on DNA gyrase and DNA replication, which are necessary for correct functioning of supercoiled DNA. This antibiotic acts in both gram negative and gram positive organisms. The resistance to ciprofloxacin occurs with decreased expression of membrane porins Cross-resistance to other drugs requiring these porins for activity which also results from these changes. And mechanism of resistance is expression of efflux pumps in both gram negative and gram positive organisms , and the last is alteration of the target enzymes, these are three main mechanisms of resistance happened in ciprofloxacin (Gangle, 2005).

c. Chloramphenicol is a broad-spectrum antibiotic. Chloramphenicol inhibits peptide bond formation on 70S ribosomes. This drug is especially useful in that it can penetrate eukaryotic cells and cerebrospinal fluid, making it a drug of choice for treatment of meningitis and intracellular bacterial infections such as those caused by chlamydia. It is not widespread use, however, because of potentially fatal side-effects, namely, aplastic anemia .The resistance to chloramphenicol is conferred by the enzyme chloramphenicol acetyl-transferase. , altering happened to the chloramphenicol molecule to prevent binding to the bacterial ribosome. Chloramphenicol resistance in gram negative cells can also arise from alteration in outer membrane permeability that prevents the drug from entering the cell (Gangle, 2005).

1.9 Medicinal plants

The diseases agents' resistance to multi antibiotic therapies and antimicrobial agents would have a short life expectancy that lead to search new drugs or preparations from the natural sources including plants (Sibanda and Okoh, 2007; Zahin *et al*., 2010). Plants have formed the basis of traditional systems of medicine that have been existed for thousands of years and continue. Human uses plants as a source of drays for various therapeutic properties. Medical plants are used as in traditional systems of medicine for their antibacterial activity with special reference to characterized compounds. Medical plants are valuable natural resource and regarded as potentially safe drugs, they have been playing an important role in alleviating human sufferings (Zahin *et al*., 2010; Al-Naimy *et al*., 2012).

Tegos *et al*., (2002) showed that the activity of putative plant antimicrobials against gram positive and gram negative organisms were significantly enhanced by synthetic (MDR) inhibitors of (MDR) efflux proteins. Those findings have provided a basis to believe that plants can be potential sources of natural MDR inhibitors that can potentially improve the performance of antibiotics against resistant strains.

1.9.1 Pomegranate

Pomegranate was one of the oldest fruits that have not changed much through the history of man. It was native in north of Iran, Turkey and Afghanistan and had a wide spread to other regions. It has been found in many ancient cultures as food as well as medical remedy, so I deeply embedded in human history. In ancient Greek mythology it was known as "the fruit of dead", in China it was widely represented in ceramic art

symbolizing, while in Christianity it was a symbol of resurrection and eternal life and in Islam the heavenly paradise of the Quran describes the Pomegranate (Dahham *et al*., 2010; Arjmand, 2011).

The edible part of Pomegranate contains acids, sugars, vitamins, polysaccharides, polyphenols and minerals. However, several factors may contribute to the chemical changes, including cultivars, environmental conditions, ripening, storage and postharvest treatments, which may affect fruit quality and health beneficial compounds (Miguel *et al*., 2010).

The pomegranate: *Punica granatum* (family Punicaceae) is a small tree originating from Asia and cultivated throughout the Mediterranean region, China, India, South Africa, and the Americas. Since ancient times, it has been used to treat several diseases. In recent times, the plant has attracted the interest of researchers in examining its composition and biological properties. The fruit is consumed mainly fresh or in beverages, and is a rich source of phenolic compounds, including hydrolyzable tannins, which possess high antioxidant activity. Ellagitannins are the major polyphenols found in pomegranate fruit (Endo *et al*., 2012**)**.

Pomegranate is a large deciduous shrub or small tree, (6-10) m in high, brak smoth, gray, then often armed with small axillary or terminal thorns. It grew well in warm areas. Studies have shown there were many potential effects which belong to pomegranate including; bactericidal, antifungal, antiviral, immune modulation, vermifuge, and stimulant, refrigerant, astringent, stomachic, styptic, laxative, diuretic and anthelmintic. A recent review reported the chemical constituents of diverse parts of pomegranate as well as their potential for prevention and

treatment of inflammation and cancer (Miguel *et al*., 2010; Vishal, 2010; Abdollahzadeh, *et al.*, 2011).

Furthermore, pomegranate is an amazing source of cyaniding, delphinidin(both are anthocyanidins), caffeic acid, chlorogenic acid (both are phenolic acids), gallic acid, ellagic acid (tannic acids), luteolin, quercetin (flavones), kaempferol (a flavonol), naringenin (a flavanone) as well as 17-alphaestradiol, estrone, estriol, testosterone, betasistosterol, coumesterol, gamma-tocopherol, punicie acid, campesterol and stigmasterol in its juice, peels and seed oil that are chemopreventive and therapeutic potentials of this plant (Abdollahzadeh, *et al*., 2011).

Many researches had focused on antioxidant action *in vitro* and *in vivo*, while other work hard elaborated on the ability of pomegranate,juice ,seed , oil ,peel or flower extraction and their derivative to kill bacteria ,viruses or to fight vascular diseases, diabetes and cancer. Consumers across the world were more interesting to find a link between food and health (Tepsorn, 2009; Arjmand, 2011).

A large number of articles concerning antimicrobial, antioxidant, anti-inflammatory, anticancer and immunesuppressive activities in the pomegranate were found. Protective effects on hepatic function or on the glucose and lipid metabolism were also reported among other biological properties (Miguel *et al*., 2010).

Hydrolyzable tannins are the most abundant polyphenols and antioxidant compounds in pomegranates which include gallotannins, ellagitannins and gallagyl esters such as punicalagin and punicalin . Pomegranate also contains oligomeric ellagitannins with two to five

glucose core molecules crosslinked by dehydrodigalloyl and/or valoneoyl esters (Madrigal-Carballob *et al*., 2009).

Shan *et al*., (2007) suggested that the antibacterial activity of *S. aureus, E. coli* of the pomegranate extracts was closely associated with their phenolic constituents. So there were highly positive relationships between antibacterial activities and phenolic content of the pomegranate.

Khan *et al.*, (2011) made study of antibacterial properties of pomegranate pericarp (peels) ethanolic extracts were evaluated against *E. coli*, and *S. aureus* using agar well diffusion method, and determined MIC of the antimicrobial extracts.

Ardekani *et al*., (2011) analyzed the bioactive phytocomponents of pomegranate peel extracts and studied their activity against antibiotic resistance strains of *E. coli*.

1.9.1.1 Chemical compounds in pomegranate peel

Flavonoids and tannins are more abundant in the peels of wild-crafted compared to cultivated fruits. Complex polysaccharides from the peels have been studied and partially characterized. The main chemical constituents isolated from Pomegranate Skin/Pericarp/Peel are:

- Hydroxybenzoic acids: which include Gallic acid, Ellagic acid. And Hydroxycinnamic acids: Caffeic acid, Chlorogenic acid, p-Coumaric acid Cyclitol carboxylic acids: Qunic acid.
- Flavon-3-ols/Flavonoids and their glycosides: Catechin, Epicatechin, Epigallocatechin-3-gallate, Quercetin, Kaempferol, Luteolin, Rutin, Kaempferol-3-O-glycoside, Kaempferol-3-O-

rhamnoglycoside, Naringin. Anthocyanins: that represent by Cyanidin, Pelarginidin, Delphinidin.

- Ellagitannins: Punicallin, Punicalagin, Corilagin, Casuarinin, Gallagyldilacton, Pedunculagin, Tellimagrandin, Granatin A, Granatin B Alkaloids: Pelleteriene (Chaturvedula and Indra, 2011).

1.10 Interactions between the pomegranates peel extracts and antibiotics

The ample exploit of antibiotics in the management of bacterial infections has led to the emergence and multiplication of resistant bacterial strains (Aiyegoro *et al.,* 2011).

Some resistance pathogens which are responsible for the infection are routinely complicated to deal with due to virulence factors and because of a relatively limited choice of antimicrobial agents. Thus, it is extremely important to find novel antimicrobials or new techniques that are effective for the treatment of infectious diseases caused by drug-resistant microorganisms. Two drugs used in mixture may yield enhanced or reduced end product, combinations of two drugs, that yield visibly similar effects may produce synergistic or antagonistic interactions. Few studies have found that the efficacy of antimicrobial agents can be improved by combining them with crude plant extracts against different pathogens and a number of compounds with an *in vitro* activity of reducing the minimum inhibitory concentrations (MICs) of antibiotics against resistant organisms have been isolated from plants. Many studies have demonstrated that plants either contain antimicrobials that can operate in synergy with antibiotics or possess compounds that

have no intrinsic antibacterial activity but are able to sensitize the pathogen to a previously ineffective antibiotic (Aiyegoro *et al.*, 2011).

The effect of combinations of the pomegranate peel extraction and antibiotics was investigated by means of the fractional inhibitory concentration (FIC) indices (Sibanda, 2007).

When drug was used as treatment some infection would be resistance, different mechanisms of action appeared if combinations of antibiotics have been used in the treatment. Synergy which is one of the well-established indications for combination antimicrobial .Antimicrobial synergism occurs when different types of antibiotics, in combination exert an inhibitory effect of pathogenic bacteria that is greater than the additive effects of the individual antibiotics. Combinations of antimicrobials that demonstrate an *in vitro* synergism against infecting strains are more likely to result in successful therapeutic outcome. Thus, evidence of *in vitro* synergism could be useful in selecting optimal combinations of antimicrobials for the empirical therapy of serious bacterial infections. It has been hypothesized that, in addition to the production of intrinsic antimicrobial compounds, plants also produce (MDR) inhibitors which enhance the activity of the antimicrobial compounds (Sibanda, 2007).

The screening of crude medical plant extracts for synergistic interactions with antibiotics is expected to provide chance for the isolation of (MDR) inhibitors that may be benefits in medicine. Some researchers observed that the crude extracts of plants potentiated the activity of antibiotics and it is anticipated to form the basis for the bioassay directed isolation of potential resistance modulators from plants (Sibanda, 2007).

Diterpenes, triterpenes, alkyl gallates, flavones and pyridines have also been reported to possess resistance modulating abilities in combination with various antibiotics against resistant strains of *S. aureus* (Sibanda and Okoh, 2007), who studied the ability of some chemical compounds of crude extracts of medicinal plants to modify the resistance phenotype in bacteria by working synergistically with antibiotics *in vitro* has since been observed, the compounds which can be combined with antibiotics in the treatment of drug resistant infections may be an alternative to overcome the problem of resistance in bacteria.

Ghaly *et al*., (2009) found that there was synergy effect obtained for the plants extracts and some antibiotics when they are used against tested clinical bacterial isolated from patients with urinary tract infection.

Chung *et al*., (2011) also reported that the combination between plants extracts and antibiotic therapy may produce synergistic effects in the treatment of bacterial infection and has been shown to delay the emergence of antimicrobial resistance, by studying the efficacy of the combination against *S. aureus*.

2

Chapter two

Materials and Methods

2-Materials and methods

2-1 Materials

2-1-1 apparatuses and instruments

Table (2-1) shows apparatus and instruments that were used in the experiments of the study:

Table (2-1): Apparatuses and instruments

No.	Instrument	Company	country of origin
1	Autoclave	YX-280 B	China
2	Balance	Sartorius	Germany
3	Centrifuge	Hettich	Germany
4	Coffee grainder	Maximan	China
5	Incubator	Fisher scientific	Germany
6	Laminar flow cabinet	Jeio-Tech	Korea
7	Loop	Loop Shandon	England
8	Magnetic Stirrer with hotplate	Labtech	Korea
9	Micropipettes	Human	Germany
10	Microscope	Motic	Germany
11	Millipore filter unite (0.45 and 0.22)mμ	Gallenkamp	England
12	Oven	Memmert	Germany
13	pH- meter	Labtach	Korea
14	Refrigerator	LG	Korea
15	Sensitive balance	Sartorious	Germany
16	Shaker incubator	Binder	Germany
17	Spectrophotometer	Tuder	Korea
18	Water bath	Tafesa	Germany

2. 1. 2. Culture media:

The following culture media were used throughout this study:

Table (2.2) culture media used in this study

No.	Media	Company and country of origin	Advantage
1	Blood Agar	Oxoid	Bacterial isolation and Hemolysin production
2	Brain Heart Infusion Broth	HIMIDIA/ India	Biofilm and Adhesive tests
3	Eosin Methylene Blue Agar (EMB)	Oxoid	Bacterial isolation
4	Kliglers Iron Agar	Mast	Bacterial identification
5	MacConkey Agar	HIMIDIA/ India	Bacterial isolation
6	Mannitol Salt Agar	HIMIDIA/ India	Bacterial isolation
7	Muller Hinton Agar	HIMIDIA/ India	Antibiotic sensitivity
8	Muller Hinton Broth	HIMIDIA/ India	Antibiotic sensitivity
9	Nutrient Agar	HIMIDIA/ India	Bacterial inocula
10	Nutrient Broth	HIMIDIA/ India	Bacterial inocula
11	Peptone Water	HIMIDIA/ India	Bacterial identification
12	Triple sugar iron agar (TSI)	Microbiologie	Bacterial identification
13	Skim milk Agar	HIMIDIA/ India	Protease production
14	Simmon Citrate Agar	HIMIDIA/ India	Bacterial identification
15	UreaAgar	Carloerba	Bacterial identification

2.2 Methods

2.2.1 Culture media:

Sterilization of culture media and solutions were achieved by autoclaving at 121 ^{0}C of 15 lb/inch^2 for 15 minutes, after adjusted pH for 7.2, other solutions that destroy by heat were sterilized by millipore filtration through 0.22 and 0.45 mμ filters.

2.2.1.1 Culture media used in bacterial isolation:

1-Blood agar medium

It was prepared by dissolving 40 grams of the medium in 1 liter of distilled water, autoclaved, left to cool for 45-50 C then whole human blood cells was added to the medium and homogenized thoroughly and poured into Petri dishes. This medium was used for primary isolation and to detect the ability of bacteria to hemolysis red blood corpuscles (RBCs).

2- MacConkey agar

It was prepared by dissolving 50g of the medium in 1liter of distilled water, boiled for complete dissolving, autoclaved, and poured into Petri dishes. It was used to isolate gram- negative bacteria and to differentiate lactose fermenters from none lactose fermenters.

3- Mannitol salt agar medium

It was prepared by dissolving 111 g of the medium in 1liter of distilled water, autoclaved and poured into Petri dishes. It was used as selective and differential medium for *S. aureus* bacteria.

4- Eosin methylene blue agar (EMB)

It was prepared by dissolving 36 g of the medium in 1liter of distilled water, autoclaved and poured into Petri dishes. This medium was used to differentiate *E. coli* bacteria from other Enterbacteria. In this medium, *E. coli* colonies appear with green metallic shine coloration.

5- Nutrient agar

It was prepared by dissolving 28 g of the medium in 1liter of distilled water, autoclaved and poured into Petri dishes.

2.2.1.2 Culture media used in bacterial identification:

1- Urea agar

It was prepared by dissolving 24g of the medium in 1liter of distilled water, boiled for complete dissolving and autoclaved, then left to cool for 45-50 ^{0}C and 50 ml of 40 % urea which sterilized by filtration was added to this medium, mixed well, poured into screw-capped test tubes, dispensed in 5 ml volume per tube, and allowed to solidify in a sloped position. This medium was used to detect the ability of bacteria to produce urease enzyme.

2- Kligler sugar iron agar

It was prepared by dissolving 65 g of the medium in 1liter of distilled water, boiled for complete dissolving, poured into screw-capped test tubes, dispensed in 5 ml volume per tube, autoclaved, and allowed to solidify in a sloped position. This medium was used to detect the ability of bacteria to produce H_2S gas and sugar fermentation.

3- Peptone water

It was prepared by dissolving 25.5 g of the medium in 1liter of distilled water, distributed into screw-capped test tubes, dispensed in 5 ml volume per tube , and autoclaved. This medium was used to detect the ability of bacteria to produce indole.

4- Simmon citrate agar

It was prepared by dissolving 24.2 g of the medium in 1liter of distilled water, poured into screw-capped test tubes, dispensed in 4- 5 ml volume per tube, autoclaved, and allowed to solidify in a sloped position. This medium was used to detect the ability of bacteria to used citrate as the only carbon source.

5- Methyl red voges proskauer (MRVP) media

It was prepared by dissolving 7g peptone (Himedia /India) with, 5g K_2HPO_4 (Analar/England) and 5 g of Glucose (AFCO/India) in 1liter of distilled water, poured into screw-capped test tubes, dispensed in 4 - 5 ml volume per tube, autoclaved.

6- Glucose fermentation medium

It was prepared by dissolving 10g peptone and 5g NaCl (Thomas/baker) in 100 ml of distilled water, then 5% of Phenol red 0.2% (BDH) was added, the PH was adjusted to 7.2, autoclaved and then added 1% Glucose solution, distributed into screw-capped test tubes, dispensed in 5 ml volume per tube.

7- Glucose Phosphate medium

This medium was used to detection of asetoin production. It was prepared by dissolving 1g Peptone, 5g Glucose and 5g K_2HPO_4 in 100mL of distilled water, the pH was adjusted to 7.2, distributed into screw-capped test tubes, dispensed in 5 ml volume per tube and autoclaved (Collins *et al*., 1989).

8- Triple sugar iron agar (TSI)

It was prepared by dissolving 24.5 g of the medium in 1liter of distilled water, boiled for complete dissolving, poured into screw-capped test tubes, dispensed in 5 ml volume per tube, autoclaved, and allowed to solidify in a sloped position.

9- Motility medium

It was prepared by dissolving 13g of the medium Nutrient broth in 1liter of distilled water, then 0.4% agar agar (Himedia) was dissolving with the medium, distributed into screw-capped test tubes, dispensed in 5 ml volume per tube and autoclaved.

2.2.1.3 Culture media, solvents and antibiotics used for antibiotic sensitivity:

1- Muller – Hinton agar

It was prepared by dissolving 36 g of the medium in 1liter of distilled water, and then boiled for complete dissolving, autoclaved and poured into Petri dishes.

2- Muller – Hinton broth

It was prepared by dissolving 37g of the medium in 1liter of distilled water, distributed into screw-capped test tubes, dispensed in 5 ml volume per tube and autoclaved.

3- McFarland Tube No. 0.5

Consist of:

A - 1.75 % $BaCl_2$ -$2H_2O$ (BDH) in distilled water.

B - 1% H_2SO_4 (Analar/England) (1ml of H_2SO_4 and the volum completed by added 99 ml of Nutrient broth).

Then 0.5 ml of $BaCl_2$ -$2H_2O$ a solution added to 99.5 ml of H_2SO_4 solution (Cruickshank *et al*., 1975).

4- Antibiotic discs: table (2-3)

Table (2-3): Antibiotic discs and standard inhibition zone (Atlas *et al*., 1995)

No.	Antimicrobial Agent	Code	Disc Concentration µg/disc	Inhibition zone in mm		
				Resistance	Intermediate	Sensitive
1	Amikacin	AK	30	≤14	15-16	≥17
2	Ampicillin	Am	10	≤11	12-13	≥14
3	Cefoxitin	CX	30	≤14	17-15	≥18
4	Ceftazidime	CtX	30	≤14	15-22	≥23
5	Chloramphenicol	C	30	< 12	13-17	18>
6	Ciprofloxacin	CIP	5	< 15	16-20	≥ 21
7	Clindamycin	CD	2	≤ 14	15-20	≥21
8	Erythromycin	E	10	13≤	14-17	≥18
9	Nalidixic	NA	30	≤13	13-16	≥19
10	Neomycin	N	30	≤12	13-16	≥17
11	Nitrofurantion	NIT	300	≤ 13	14 – 22	≥22
12	Oxacillin	OX	1	10<	10-12	>12
13	Tetracycline	TE	30	14≤	15-18	≥19
14	Tobramycin	TOB	10	< 12	13-14	≥ 15
15	Vancomycin	VA	30	-	-	≥ 15

2.2. 2 Biochemical Reagents:

2.2.2.1 Catalase reagent

It consists of hydrogen peroxide 3% H_2O_2 (BDH).

2.2.2.2 Oxidase reagent

Freshly prepared from adding 0.1 g of tetramethyl – phenl – diamine dihydro chloride (BDH) in 0.9 ml of distilled water, then completed to 10 ml in clean, dark and sterile container (Baron *et al.*, 1995).

2.2.2.3 Gram stain (VSI)

It consist of crystal violate stain, iodide solution, ethanol, and safranine stain.

2.2.2.4 Normal saline solution

This solution was prepared by dissolved 0.85g of NaCl in 100 ml of distilled water; pH was adjusted to 7.2, and then sterilized by the autoclave.

2.2.2.5 Kovac's reagent (Indole test)

This solution was prepared by dissolved 5g of P. dimethyl - amino benzyl aldehyde (BDH) in 75 ml of Amylo alcohol (BDH), then 25 ml of the acidic HCL (Analytical Rasayan) was added slowly to the aldehyde alcohol mixture (Collee *et al*., 1996).

2.2.2.6 Methyl –red reagent

This solution was prepared by adding 0.1 g of methyl red powder in 300 ml of 95% ethanol (alcohol) (GCC) then complete the valume to 500 ml by adding distill water (Macfaddin, 2000).

2.2.2.7 Voges Proskauer Reagent

According to (Collee *et al*., 1996) this reagent consists of:

A. Alpha Naphthol solution: It was prepared by dissolving 5 g of alpha naphthol in 100 ml of absolute ethanol.

B. Potassium Hydroxide solution: It was prepared by dissolving 40 g of KOH in 100 ml distill water.

2.2.3 Api Systems (bioMerieux)

Api 20 E and Api staph are identification system for Enteriobacteriaceae and staphylococcus respectively. Each system contains 20 standardized and miniaturized biochemical tests and database. These Api systems include a strip consist of 20 microtubes containing dehydrated substrates. These tubes are inoculated with a bacterial suspension that re-constitutes the media.

During incubation, metabolism produces color changes that are either spontaneous or revealed by the addition of reagents. The reactions are read according to the reading table and the identification is obtained by referring to the analytical profile index.

2.2.4 Detection of bacterial virulant factors

2.2.4.1 β - lactamase detecting solutions.

1. Starch solution.

Freshly prepared by adding 1 g of soluble starch in 100ml distilled water, boiled in water bath till completely dissolved then kept in dark bottle in 4 °C.

2. Iodine solution.

Prepared by dissolving 2.03g from crystal iodine (Analar /England) and 5.32 g from potassium iodine (Griffin/England) in 90 ml distilled water and completed to 100ml.

3. Penicillin G solution.

First phosphate buffer was prepared from two solutions as follows.

Solution (A): -

Prepared by dissolving 0.907g KH_2PO_4 (Fluka-Switzerland) in 100ml of distilled water, then completed to 100ml.

Solution (B): -

Prepared by dissolving 0.946g Na_2HPO_4 (BDH) and 1.19g from $Na_2PO_4.2H_2O$ (BDH) in 90ml of distilled water, and then completed to 100ml.

From solution A, 87.7ml was added to 12.3ml of solution B, pH was fixed at 6 and then 0.5693g of penicillin G (MUNCHEN/ Germany) was added to phosphate buffer, sterilized by filtration and kept in20°C.

2.2.4.2 Protease detection solution

Skim milk agar (Collee *et al*., 1996)

This contains:

Nutrient agar, sterile 87.5 ml

Skimmed milk (Himedia /India), sterile 12.5 ml

The nutrient agar was autoclaved, cooled to 50°C, and then the skim milk was added and poured in sterile plates.

2.2.4.3 Adhesive detection solutions

1. Giemsa stain

This stain was used to color uroepithelial cells. It was prepared according to (Who Bulletin, 1975) by dissolving 0.5 g of powdered Giemsa stain (BDH) in 33 ml of glycerol (BDH) at 55-60 C^0 for 1-1.5 h; thereafter 33 ml of absolute methanol (GCC- VK) was added. After mixing well was filtered then stored at room temperature as a stock solution of Giemsa stain. Experimental solution was prepared by diluting the stock solution in hydrated water as flowing:

Solution A: It was prepared by dissolving 9.5g of Na_2HPO_4 (BDH) in 1L of distilled water.

Solution B: Prepared by dissolved 9.2 g of NaH_2PO_4 in 1liter of distilled water. Hydrated water was prepared at pH 7.2 by mixing 72 ml of solution A with 28 ml of solution B, and then 900 ml of distilled water was added. Experimental solution (prepared immediately before use) was prepared by mixing 1 part of stain stock with 40 or 50 parts of hydrated water.

2. Phosphate buffered saline (PBS) pH 7.2

It was prepared according to (Cruickshank *et al*, 1975), by dissolving 8g NaCl, 0.1g KCL, 1.15g Na_2HPO_4 and 0.2g K_2HPO_4 in 1liter of distilled water, autoclaved and stored in refrigerator.

2.2.4.4 Biofilm formation

1. Brain Heart Infusion broth (BHI) (Himedia/India)

It was prepared by dissolving 37 g of the medium in in 1liter of distilled water, distributed into screw-capped test tubes, dispensed in 5 ml volume per tube and autoclaved.

The (BHISuc) was prepared by added 2ml sterile sucrose sugar (BDH) to 100 of autoclaved brain heart infusion broth (BHI) after cooled to 40^0C.

2. Phosphate buffered saline (PBS) pH 7.2

It was prepared by dissolving 8g NaCl, 0.3g NaH_2pO_4 and 1.12g Na_2HPO_4 in 1liter of distilled water, autoclaved and stored at refrigerator.

3. Crystal Violate solution

It was prepared by dissolving 0.1 g from crystal violate stain (BDH) in 100 ml distill water.

2.2.5 Solution used to detect the plant active material

2.2.5.1 Dragendroff reagent

This reagent is prepared as:

Solution (1)

It was prepared by adding 2 ml of concentrated HCl to 0.6g of Bismuth Subnitrate (BDH), then 10 ml of distill water was added to dilute the solution.

Solution (2)

It was prepared by adding 10 ml of distill water to 0.6g of KI.

Solution (1) mixed with solution (2) and 7 ml of concentrated HCl and 15 ml of distill water was added , then the mixture was diluted by added 400 ml of distill water.

2.2.5.2 Mayer reagent

This reagent is prepared as:

Solution (1) 1.36 g of $HgCl_2$ (Carloerba) is added to 60 ml of distills water.

Solution (2) 5g of KI is dissolved in 10 ml of distill water.

Then solution (1) mixed with solution (2) and the volumes is completed to 100 ml by adding distill water.

2.3.1 Collection of samples:

Hundred morning midstream urine were collected from pregnant women aged from 16 to 40 years attending Maternity and Women's Hospital in Karbala Province during December 2011 to March 2012.

Urine specimens were collected according to Vandepitte *et al*., (2003) Urine was collected into sterile screw capped test tubes and cultured immediately after collection by streaking 0.01 ml of urine (using calibrated loop) on Blood and MacConkey agar.

2.3.2 Isolation of bacteria

The clinical specimens were cultured immediately after collection on Blood and MacConkey agar and incubated at 37^0C for 24 hours. Single colony from bacteria that grow on media was recultured on nutrient agar for obtain pure culture.

2.3.3 Preparation of bacterial inocula

Colonies taken from 24 hour old cultures grown on nutrient agar were used to make suspension of the test organisms in saline solution that gave an optical density of approximately 0.5-1 at 600 nm. The suspension was then diluted by used saline solution before use.

2.3.4 Diagnostic of the isolates bacteria

Isolated bacteria were diagnosed according to cultural and morphological properties and by using biochemical tests depending on, (Baron *et al*., 1995; Collee *et al.*, 1996):

2.3.4.1 Morphological and cultural characteristic

The properties of the isolated bacteria were studied by observation the shape and color of the colonies on the selective media like Mannitol salt agar for *S .aureus* that conversion red color for this media to yellow and E.M.B for *E .coli* that gave colonies with green metallic shine.

2.3.4.2 Biochemical and Physiological Test

Biochemical test was done according to (Collins *et al*., 1989; Baron *et al*., 1995; Collee *et al.*, 1996) fresh bacterial growth (18hr.) was used.

1. Catalase test

A drop of 3% hydrogen peroxide was added to a colony or loopful of bacteria transported previously on a slide, positive result was demonstrated when bubbles of oxygen were released from the bacteria.

2. Oxidase test

A part of colony was transported to a wet piece of filter paper with few drops of freshly prepared 1% redox solution. The development of a violet or purple color within 10 seconds indicates as a positive test.

3. Coagulase test:

A- Bound coagulase (slide method):

One drop of normal saline solution was placed on a clean dry slide, and a loop full of suspected bacteria was emulsified on the saline drop, then a drop of human plasma was placed immediately adjacent to the drop of the bacterial suspension and the two drops were thoroughly

mixed. The appearance of white clumps within 3 seconds indicates a positive test.

B- Free coagulase test (Tube method):

Two tubes containing brain heart infusion a loop full of suspected fresh sample of *S. aureus* were added and incubated at 37 ^{0}C for 24 hours. In a small test tube 0.5 ml of human diluted plasma was added to 0.5 ml of fresh broth culture of the strain to be tested and incubated at 37 ^{0}C and examined for coagulation of plasma after 1, 3 and 4 hours (by tilting the tube gently) for coagulase.

Negative tubes were left over night at room temperature before discarding the growth. Those forming a clot after 24 hours were also counted as positive result.

4. Urease test

Pure culture of 18-24 h was streaked onto urea agar slants (that was prepared as in step 2.2.1.2.1) and the tubes were inoculated at 37^{o}C for 24 hours pinkish or red tubes were interpreted as positive. Other they were negative.

5. Hydrogen sulfate (H_2S) production test

This test was done by culturing, the tubes of Kligler sugar iron agar (that was prepared as in step 2.2.1.2.2), then the tubes were incubated for 24 - 48 h at 37^{0}C, the positive was result the black color.

6. Indole test

The test was done by adding a loopfull of the inoculum to peptone water (that was prepared as in step 2.2.1.2.3), incubated at 37^0C for 24-28 h. Occurrence of red –violet ring after addition of Kovac's reagent (5 drops) (that was prepared as in step 2.2.2.5) was indicative of iodole production. It was performed to determine the ability of isolates to split indole from tryptophan.

7. Citrate utilization test

Pure culture of 18-24 h was streaked into simmon citrate agar slants (that was prepared as in step 2.2.1.2.4), the tubes were incubated for 24-48 h at 37^0C. Growth and conversion of medium color from green to blue was indicative of citrate utilization.

8. Methyl red and vogues proskauer test

This test was done by adding a loopfull of the inoculum to two tube of MR.VP. Media (that was prepared as in step 2.2.1.2.5), the tubes were incubated for 24 -48 h at 37^0C. The positive result is the occurrence of red color after 15 min of the adding of the MR reagent (that was prepared as in step 2.2.2.6).While 0.3ml drops of α- naphthol and 0.1 ml drops of KOH solution that was prepared in step 2.2.2.7 were added to the second tube, with shacked gently for 1min, and allow to stand for 10-15 min. The positive result was indicated by the development of a pinkish red color.

9. Acetoin production test

Acetoin production was detected by Barrits method, the tubes of Glucose Phosphate broth (that was prepared as in step 2.2.1.2.7) were cultured, then the tubes were incubated for 5 days at 30^0C, and 3ml of alcoholic α – nephthol solution (that was prepared in step 2.2.2.7) with 3 ml of potassium hydroxides solution (that was prepared in step 2.2.2.7) were added after 5 min, if there was red color it was positive.

10. An aerobic glucose fermentation test

To diagnoses *S. aureus* from other Micrococcus, Glucose fermentation tubes (that was prepared as in step 2.2.1.2.6) cluttered, the tubes covered by hot sterilized parveen wax (BDH) to 2cm high, and incubated for 5 days at 37^0C .The yellow color torn it was positive.

11. TSI test

Inoculates were stabbed into the bottom and streaked on the media slant (that was prepared as in step 2.2.1.2.8) and the tubes were incubated for 24 -48 h at 37^0C. The positive result of this test is:

A/A GAS $^{+/-}$ H_2S $^{+/-}$: all sugars were fermented with or without carbon dioxide and / hydrogen sulfide production.

12. Motility test

It was performed by inoculating the motility media (that was prepared as in step 2.2.1.2.9) to a depth of 1/2 inch. Tubes were incubated for 24 - 48 h at 37^0C. Motility organisms migrate from the stab line into the media, causing turbidity.

13. Api systems test

It was carried out according to the manufacturers (bioMerieux) instructions. The bacteria inoculum was prepared by suspending a single colony on nutrient agar (that was prepared as in step 2.2.1.1.5) in 5 ml of PBS (that was prepared as in step 2.2.4.3.2), then the wells were inoculated with bacterial suspension by using sterile pasteur pipettes, then the strips were incubated at 37^{o}C for 24h.

2.3.5 Antibiotic sensitivity test

Bacterial sensitivity to antibiotic was detected according to Harley and Prescott *et al*., (1996):

1. With a sterile wire loop, the tips of 4-5 isolated colonies of the organism to be tested were picked from the original culture and introduced into a test tube containing 10 ml Mueller Hinton broth (that was prepared as in step 2.2.1.3.2), then incubated at 37^{0}C for about 2 to 5 hours to produce a bacterial suspension of moderate turbidity. Its turbidity was compared to McFarland tube No. 0.5 (that was prepared as in step 2.2.1.3.3).

2. Within 15 minutes of adjusting the density of the inoculum, a sterile cotton swab was dipped into the standardized bacterial suspension. The excess fluid was removed by rotating the swab firmly against the inside of the tube above fluid level. The swab was then streaked onto the dried surface of a Muller – Hinton plate (that was prepared as in step 2.2.1.3.1) in 2 different planes to obtain an even distribution of the inoculum.

3. The plate lids were replaced and the inoculated plates were allowed to remain on a flat and level surface undisturbed for 3-5 minutes to allow absorption of excess moisture.

4. With the sterile forceps, the selected discs were placed on the inoculated plate and pressed gently into the agar. Within 15 minutes the inoculated plates were incubated at 37 ^{0}C for 18 -24 hours in an inverted position.

5. After incubation, the diameters of the complete inhibition zone were noted and measured using reflected light and a ruler. The end point, measured to the nearest millimeter, was taken as the area showing no visible growth.

6. The diameter of inhibition zone for individual antimicrobial agent was translated in terms of sensitive, resistant categories by referring to an interpretative chart of national committee for clinical laboratory standards subcommittee on antimicrobial susceptibility testing (Atlas *et al*., 1995) as is shown in table (2-2)

2.3.6Ability of isolated bacteria to form biofilm layer

Tube method (TM)

A qualitative assessment of biofilm formation was determined as previously described by (Mathur *et al*., 2006) as in the flowing steps:

1. 10 ml of brain heart infusion broth (BHI) (that was prepared as in step 2.2.4.4.1) with 2% sucrose (BHISuc) (10ml) was inoculated with loopful of microorganism from overnight culture plates and incubated for 24 hours at 37°C.

2. The tubes were decanted and washed with PBS (pH 7.2) (that was prepared as in step 2.2.4.4.2) and dried.

3. Dried tubes were stained with crystal violet stain (0.1%) (that was prepared as in step 2.2.4.4.3) for 10 min then excess stain was removed and tubes were washed with deionized water.

4. Tubes were than dried in inverted position and observed for biofilm formation.

5. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation. The result reported dependence on the degree of tubes stained and the amount of biofilm formation was scored as 0- absent, 1- moderate or 2- strong (experiments were performed in triplicate and repeated three times).

2.3.7 Diagnosis of bacterial isolates for the study

The isolated bacteria used in the study were chosen according to the result in step (2.3.6), and then they are used in the late experiments.

2.3.8 Ability of isolated bacteria to produce virulence factors

2.3.8.1 Hemolysin production

Blood agar plates were inoculated with the tested organism and the plates incubated at 37ºC for 48 hours. Then the plates were examined for observing the type of hemolysis.

2.3.8.2 Protease production

Skim milk agar plates (that was prepared as in step 2.2.4.2) were inoculated by streaking and incubated at 37ºC for 48 hours. The clear

zone exists adjacent the positive result. That indicated the production of protease enzyme (Collee *et al*., 1996; Benson, 2001).

2.3.8.3 β – lactamase production

Prepared according to WHO (1978), twenty-four hours bacterial growth on nutrient agar was prepared for each isolates, then 4-5 colonies were transported to test tubes containing 100 µl of penicillin G solution (that was prepared as in step 2.2.4.1.3), incubated at 37°C for 30 minutes.

A 50 µl of starch solution (that was prepared as in step 2.2.4.1.1) was added to each tube and mixed with the other content .After that 20 µl of iodine solution (that was prepared as in step 2.2.4.1.2) was added; a dark blue color will appear immediately due to starch – iodine interaction.

The positive result was recorded if the blue color changes to white within one minute.

2.3.8.4 Adherence activity

This activity was carried out according to Svanborg *et al.*, (1977):

A fresh morning, mid-stream urine samples of healthy female were collected and centrifuged at 3000 rpm for 15 minutes. The pellet was washed four times with phosphate buffered saline (PBS) pH 7.2 (that was prepared as in step 2.2.4.3.2) mixed by vortex and suspended in phosphate buffer saline. Then 0.5 ml of bacterial culture of each isolates was added to a same volume of epithelial cells suspension, mixed well and incubated at 37°C with slowly moving every ten minutes. After one

hour, the tubes were centrifuged and washed four times with phosphate buffer saline to discard unattached bacteria.

Bacterial adhesion uroepithelial cells were examined by Giemsa staining of cells according to Godaly *et al*., (1998). After centrifugation, the supernatant was decanted and the sediment cells were mounted on glass microscope slide, and separated using the margin of other slide.

The slides were air-dried, fixed with absolute methanol for 5 min, and stained with Giemsa stain (that was prepared as in step 2.2.4.3.1); this stain was used to color uroepithelial cells for 1-1.5 h. The excess stain removed by water wash, and the slides were dried and examined by light microscope under oil immersion to calculate the number of bacteria adhered to cells.

2.3.8.5 Detection of biofilm formation activity

1. Tube method (TM)

Like what prepared in step (2.3.6)

2. Tissue culture plate method (TCP)

In present study, we screwed isolates for their ability to form biofilm by (TCP) as described by (Mathur *et al*., 2006; Maldonado *et al*., 2007)

1. Isolates from fresh agar plates were inoculated in respective media (brain heart infusion with 2% sucrose) and incubated for 18 hour at 37^{o}C.

2. Sterile, polystyrene, 96 well-flat bottom tissue culture plates' wells were filled with 0.2 ml aliquots of the cultures, only broth served as control to check sterility and non-specific binding of media.

3. The tissue culture plates were incubated for 24 hours at 37°C. After incubation 25µl of 0.1 % crystal violet was added to each well, shaking the plate three times to help the colorant to get the bottom of the wells. After 15 minutes at room temperature, each well was washed with 200µl sterile PBS (that was prepared as in step 2.2.4.4.2) to remove the planktonic cells and stain not adhered to the wells. This process was repeated three times. Only adhered Bacteria forming biofilm were kept on the surface of the wells.

4 . The crystal violet bound to the biofilm was extracted later with two washes of 200µl of ethyl alcohol. The liquid washing alcohol was transferred, to a tube containing 1.2 ml of alcohol and agitated. To determine the degree of biofilm formation, the absorbance was measured at 540 nm in an UV spectrophotometer. (Mathur *et al.*, 2006)

[The data obtained were used to classify the strains as high producers (OD higher than 0.500), producers (OD between 0.500 and 0.100) or poor producers (OD lower than 0.100)]. (Maldonado *et al* 2007)

2.4.1 Plants

Eleven different fruits ' peels were used in this study as shows in table (2-4).

Table (2-4) The fruits ' peel used in this study

No.	Local Name	Scientific Name	Source
1	Apple	*Malus domestica*	Local market
2	Banana	*Musa Paradisiaca*	Local market
3	Cucumber	*Cucumis sativus*	Local market
4	Lemon	*Citrus limon*	Local market
5	Muskmelon	*Cucumis melo*	Local market
6	Peach	*Prunus persica*	Local market
7	Pomegranate	*Punica granatum*	Local market
8	Pummel	*Citrus grandis*	Local market
9	Sour orange	*Citrus aurantium*	Local market
10	Sweet orange	*Citrus sinensis*	Local market
11	Watermelon	*Citrullus lanatus*	Local market

2.4.2 Determination the plants for study

The plants parts in table (2-3) cleaned by tap water, dried in air, and then milled several times while getting affine powder for each peel fruit.

2.4.3 Plants extracts

2.4.3.1 Water extract

Water extracts were prepared according to (Ahmed *et al*,. 1998; Al-Jboriy *et al.*, 2010) by blinding 10 g of fruits peel powder with 100 ml distal water in glass flask (size 250 ml) and let the suspension with movement in shaker for 24h in 37^0C, after that extract was filtered by using multi-layer of medical gauze first and then by filter paper (kind of Whatman NO.1). The filtered liquid was put in clean petri dishes and left to dry in room temperature. The dry extract was scratched by sterilized clean knife. After that it has been weighted to be kept in refrigerator until it has been used. This operation was repeated many times until getting enough weight from the extract.

2.4.3.2 Alcohol extract

It was carried out as mention in water extract with substitution distal water by absolute ethanol (Schorlau/European United) (Ahmed *et al*,. 1998; Al-Jboriy *et al.*, 2010).

2.4.3.3 Acetone extract

The same steps of prepared water extract have been done with substituting distal water by 70% acetone (Schorlau/European United) (Ahmed *et al*,. 1998; Al-Jboriy *et al.*, 2010).

2.4.4 Concentration of plants extracts

Stock solution was prepared for each extract by dissolving 1g of dry extract with 10 ml of distal water, so the final concentration of extract would be 0.1 g/ml, from this stock solution other concentration were prepared (0.01-0.1) g/ml which was used against bacteria.

2.4.5 Antibacterial activity of peel extracts

Agar well diffusion method was used, and this method included: steps that was prepared in step 2.3.6 with replacing antibiotic disc by 5 holes with 6 mm diameter which did by corky bore and then 100 µl from each concentration of the extract was putting in each hole by using micropipette, and the addition must be on the surface of the culture media carefully, distill water was added to one hole in the cultured media to be as control, then the petri dishes were incubated in 37^0C for 24h. The inhibitions zone was measured by ruler; this was repeated two times (Egharevba *et al*., 2010).

2.4.6 Minimum inhibitory concentration (MIC)

Agar dilution method was used to detect MIC of the plant extraction according to (NCCLS, 1993), the dilutions of the extract ranging from (0.01-0.1) g/ml prepared by mixed the each one of the extract concentration with 100 ml of sterile and cooled Muller Hinton medium. The petri dishes were cultured as spot with 100 µl of bacterial suspension, and the control plate contains the extract only. Then the petri dishes lived 30 min to dry, incubated in 37^oC for 18-24 h. Final results were recorded as positive one when growth presented, and negative result if there was no growth. The MIC represented by the last concentration with little or no visible growth.

2.4.7 Phytochemical analysis

2.4.7.1 Saponins (Bubble test)

Five milliliter each extract was shaken vigorously for 2 min. The appearance from that persisted for at least 15 min or the forming of an emulsion when olive oil was added confirmed the presence of saponins (Ling *et al*., 2011).

2.4.7.2 Tannins

5ml of the extractions was took and 5ml distilled water added then heated at 80-100 ^{0}C for 10 min in water bath, then filtered it after that 1% Ferric chloride (5-6 drops) added , the dark green color indicates the presence of tannins (Pandey *et al*., 2011).

2.4.7.3 Alkloids

Detection by many reagents according to (Harborne, 1984)

A – Dragendroff reagent

Orang precipitation was positive result.

B – Marqus reagent

This reagent was prepared by mixing 1 ml formaldehyde with 10 ml of concentrated H_2SO_4, the turbidity was positive result.

C – Mayer reagent

White precipitation or turbidity was a positive result.

2.4.7.4 Glycosides

0.5 g in 5 ml of water, for each extract was added to boiling fehling's solution (A and B) in a test tube. The solution was observed for a color reaction (Ayoola *et al*., 2008).

2.4.7.5 Resins

1 g of dried powder plants mixed with 10 ml of 95 % ethanol, left for one min at 100 ^{0}C in water bath, the solution filtered then10 ml of 4% HCL was added. Turbidity was a positive result (Shihata, 1951).

2.4.7.6 Flavonoids

A – Alcoholic potassium hydroxide detection

2 ml of the extraction mixed with 1 ml of alcoholic potassium hydroxide (KOH). Yellow color was positive result (Al-Khazragi, 1991).

B – Filavonaad and Flavonols

1 ml of the extract dissolved with concentrated H_2SO_4, dark yellowish was the positive result (Al-Khazragi, 1991).

2.5 The effect of pomegranate peels extracts on the bacterial virulence factors:

2.5.1 Influence on Hemolysin, Protease and β – lactamase production

The nutrient broth (that was prepared as in step 2.3.3) was incubated at 37^{0}C for about 2 to 5 hours to produce a bacterial suspension of moderate turbidity; its turbidity was compared to McFarland tube No. 0.5 (that was prepared as in step 2.2.1.3.3). Then the MIC of the extraction was added to the bacterial suspension, and then made hemolysin production tested as description in step (2.3.8.1), protease production tested as description in step (2.3.8.2) and β– lactamase production tested as description in step (2.3.8.3).

2.5.2 Influence on adherence

The nutrient broth (that was prepared as in step 2.3.3) was incubated at 37^0C for about 2 to 5 hours to produce a bacterial suspension of moderate turbidity; its turbidity was compared to McFarland tube No. 0.5 (that was prepared as in step 2.2.1.3.3). Then MIC of the extraction was added to the bacterial suspension and then made Adherence detection tested as description in step (2.3.8.4)

2.5.3 Influence on biofilm formation

Tissue culture plate method (TCP)

MIC of the extraction was added to the brain heart infusion with 2% sucrose and incubated for 18 hour at 37^oC, and biofilm tested as in step (2.3.8.5.2)

2.6 Interactions between the extracts and antibiotics

2.6.1 Preparation of bacterial inocula

That was prepared as in step 2.3.2

2.6.2 Antibiotics used in this study

The following antibiotics were used in this study: Chloramphenicol (SDI), Tetracycline (SDI) and Ciprofloxacin (new pharmag).

2.6.3 Determination of the minimum inhibitory concentrations (MIC)

The minimum inhibitory concentrations of the antibiotics and plant extracts were determined (as in step 2.4.6).

2.6.4 Combination studies

The combined antimicrobial activity of the plant extracts and antibiotics was done by using the agar dilution method as was described by Mandal *et al*., (2004). Each concentration of extract and antibiotics were combined, incorporation into nutrient agar (that was prepared as in step 2.2.1.1.5), then all plates were incubated for 24h at37^0C.

The fractional inhibitory concentrations (FIC) were derived from the lowest concentration of antibiotic and extract combination permitting no visible growth of the test organisms.

FIC (antibiotic) = MIC of antibiotic in combination / MIC of antibiotic alone

FIC (extract) = MIC of extract in combination / MIC of extract alone

The interactions between the antibiotics and the extracts were evaluated by use of the FIC indices which were calculated by using the formula;

FIC Index = ΣFIC = FIC (antibiotic) + FIC (plant extract)

Combinations were classified as synergistic, if the FIC indices were < 1, additive if the FIC indices were = 1, indifferent if the FIC indices were between 1 and 2 and antagonistic if the FIC indices were > 2 (Kamatou *et al*., 2006). Where more than one combination resulted in a change in the MIC value of the extract or antibiotic, the FIC value was expressed as the average of the individual FIC values as described by Pankey *et al*., (2005).

2.7 Statistical analysis

SAS (2007) was used for statically analysis of data, to study the effect on the studied bacteria and the concentrations (0.01-0.1) g/ml and the three extractions of the pomegranate on the inhibition zone range, and compared the moral differences between the averages by using the less significant difference (LSD) test.

3

Chapter three

Results and Discussions

3.1 The prevalence of infection

A total of 100 urine samples from pregnant women were taken in this study. The result clarified that the frequency of UTIs in pregnant women was 52% (as symptomatic 34 % and asymptomatic18% UTIs), and 48% which were the rest of the samples consider negative results, as shown in figure (3-1)

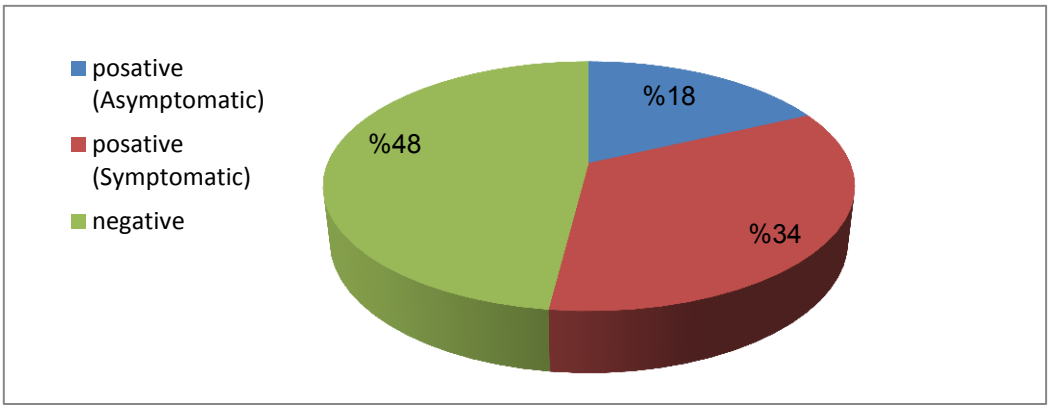

Figure (3-1): percentage of symptomatic and asymptomatic urinary tract infection in pregnant women

The positive results was high percentage, which more than half of the samples and this observation seems to agree with the findings of Dimetry *et al.*, (2007), who reported a substantial risk among pregnant women, especially during the first trimester and gave back the high incidence of UTIs to hormonal effects observed during pregnant, which reduces the tone of the ureteric musculature aided perhaps by mechanical pressure from the gravid uterus leading to urinary stasis, thus encourages bacterial proliferation in urine, which is an excellent media.

Many searchers explained that the urinary tract infection is being one of the most common health problems during pregnancy because of the increment in the sex hormones beside the anatomical and physiological

changes during pregnancy Ali, (2011). These changes beside an already short female urethra (approximately 3 - 4 cm in females) increase the frequency of urinary tract infection in pregnant women. In general, pregnant women are considered immunocompromised host because of the hormone changes that effect on the activity of the immune system; this is regarded as an important factor to increase the risk of urinary tract infections that could be either symptomatic or asymptomatic (Boye *et al*., 2012).

The positive results were divided according to the appearance of the symptom in patients into symptomatic (34%) and asymptomatic (18%), and that was approached with Frank - Peterside *et al*., (2009) who observed asymptomatic bacteriuria was in about 10% of the population and reported that pregnancy causes changes in the urinary tract, so most cases of symptom lower urinary tract were due to pregnancy associated change.

In the present study, the most positive effects belong to symptomatic bacteriuria, this approached with Haider *et al*., (2010) who found that pregnancy related changes in the urinary tract then the ability of this system been infected was increased so these changes were the causative factors for the occurrence of lower urinary tract symptoms.

3.2 The Relationship between infection and gestational age

The highest percentage of infection were appeared in7^{th} (19.2%) and 9^{th} (17.3%) months of pregnancy in the women while women in early months of gestational age had no specific bacterial growth and had shown no signs of UTIs. The positive result divided into two parts, symptomatic and asymptomatic UTI. The asymptomatic outdo

symptomatic in 6th month of pregnancy, but in the 2nd and 3rd months was equal in percentage with symptomatic UTI, which was outdo asymptomatic UTI in last three months of gestation age as figure (3-2) shows.

This result approached with Obiogbolu *et al*., (2009) who found that the highest percentage of UTI can be found in the 6th and 7th months of the gestational age that might be attributed to some factors such as poor housing, poor drainage systems, lack of proper personal and environmental hygiene, low socio-economic status, and sexual intercourse (Okonko *et al.,* 2009).

Ali, (2011) mentioned that there are many factors could be responsible for the high frequency of asymptomatic bacteriuria during pregnancy especially in the first trimester of gestation age, such as women age, history of urinary tract infection, diabetes mellitus and other biological or physiological explanations which may increase infection, one of them is the sexual activity, in addition the large physiological change that occurring in pregnancy which lead to sever course of problem and increase the percentage of asymptomatic urinary tract infection in pregnant women.

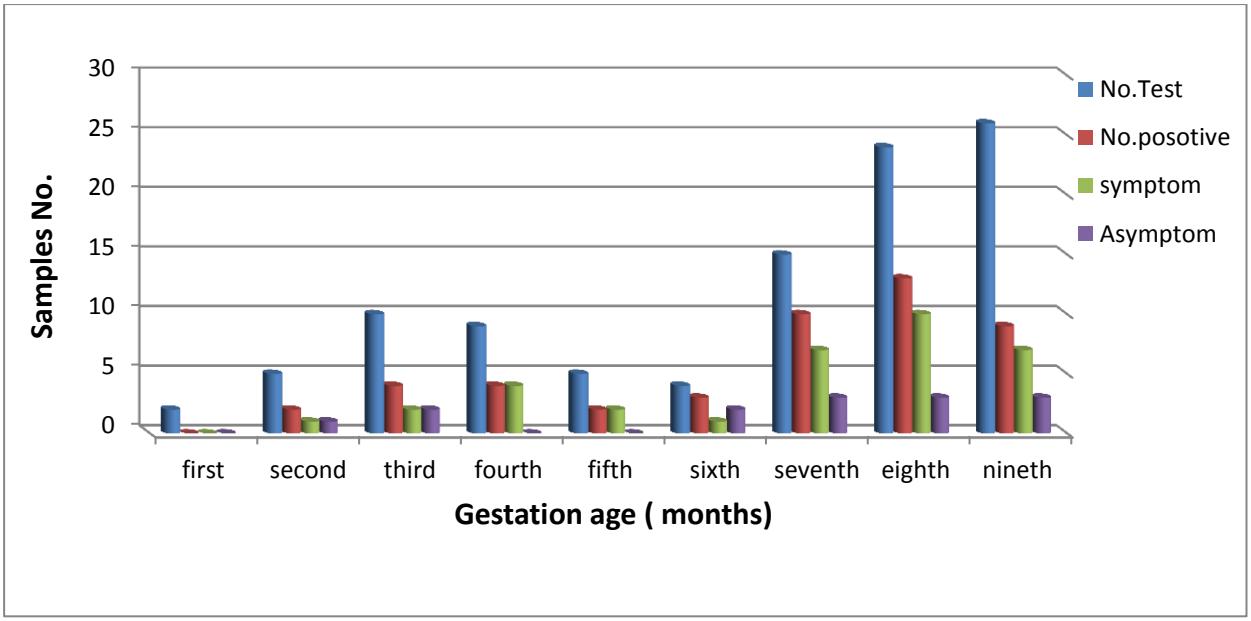

Figure (3-2): Percentage of (symptomatic and asymptomatic) urinary tract infection in gestational age

3.3 Relationship between infection and pregnant age

Figure (3-3) shows that UTIs is related to age of the pregnant women. The highest percentage of infected pregnant women 17 (32.69%) and 16(30.77%) were found within the age brackets of 21-25 years and 26-30 years respectively, followed by 11(21.15%) pregnant women with UTIs were obtained from age groups 16-20 years, while age groups 31 - 35 and 36 - 40 years had the least percentage 5 (9.62%) and 3(5.77%) of infected pregnant women respectively.

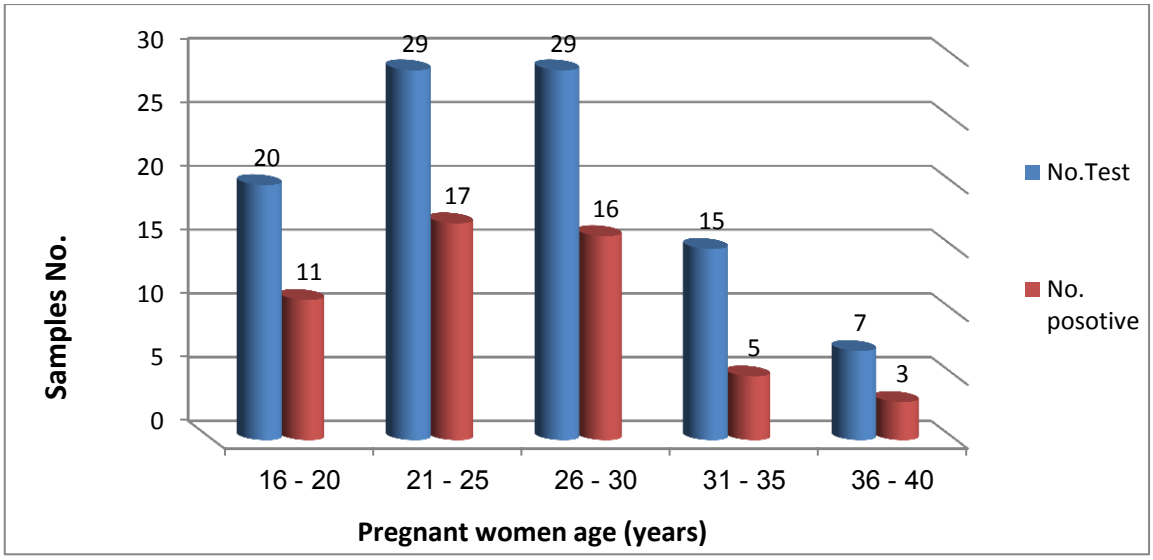

Figure (3-3): The relationship between infection and pregnant age

This study shows results which are different from the result of many previous studies, which may be attributed to many factors such as nearly half of the pregnant women were in age 20 – 30 years this ranged represented the common gestation age in our country, the variation in the environment, social habits of the community, and the standard of personal hygiene and education.

The prevalence of bacteriuria has been shown that it increases with advancing maternal age is likely due to the increasing frequency of co-morbid conditions, which is associated with neurogenic bladder and increased residual urine volume or urinary reflux (Sescon *et al*; 2003).

The results disagree with the studies done by Ali, (2011) who observed that the 20% of pregnant women experienced an episode of UTIs by the age of 30 years, but another searcher proved that the highest rate of symptomatic bacteriuria 13%, was found in the age group 35 - 39 years and the lowest rate was in the age groups 15-19 years and 45-49 years 0 % (Turpin *et al*., 2007).

3.4 Percentage of bacterial isolation

One hundred urine samples were obtained from pregnant women undergoing UTIs. The isolated bacteria were selected directly from urine specimens by culturing the specimen on general and selective media and after incubated for 24 h at 37^0C the growing bacteria were diagnosed biochemically according to methods described by Collee *et al.*, (1996) and Baron *et al*., (1995), as shown in table (3-1) and table (3-2) and the diagnosis of isolated bacteria were confirmed by APi20E and Api Staph system accomplished according to manufacturer's instructions.

Table (3-1): Biochemical tests of gram positive bacteria

Bacteria / Biochemical Tests	*S. aureus*	*Staphylococcus* spp.	*Streptococcus* spp.
An aerobic glucose fermentation test	+	-	-
Catalase	+	+	-
Coagulase	+	-	-
Gram stain	+	+	+
Motility	-	-	-
Oxidase	+	+	+
VP	+	+	-

Key: (+) positive (-) negative

Table (3-2): Biochemical tests of gram negative bacteria

Bacteria / Biochemical Tests	*E. coli*	*Klebsiella* spp.
Catalase	+	+
Citrate utilization	-	+
Gram stain	+	+
H_2S production	-	-
Indol production	+	-
Lactose fermentation	+	+
Motility	+	-
MR	+	-
Oxidase	-	-
TSI	A/A	A/A
Urease production	-	+
VP	-	+

Key: (+) positive (-) negative

Fifty two (52) bacterial isolate were isolated, 43 (83 %) were gram–positive bacteria that was divided into coagulase positive *Staphylococcus aureus* 20 (39%) and coagulase negative which divided between *Staphylococcus* spp. 15 (29%), and *Streptococcus* spp. 8 (15%). But only 9 (17%) was gram – negative bacteria which represented by *Escherichia coli* 6 (11%), and *Klebsiella* spp. 3 (6%), as shown in figure (3-4)

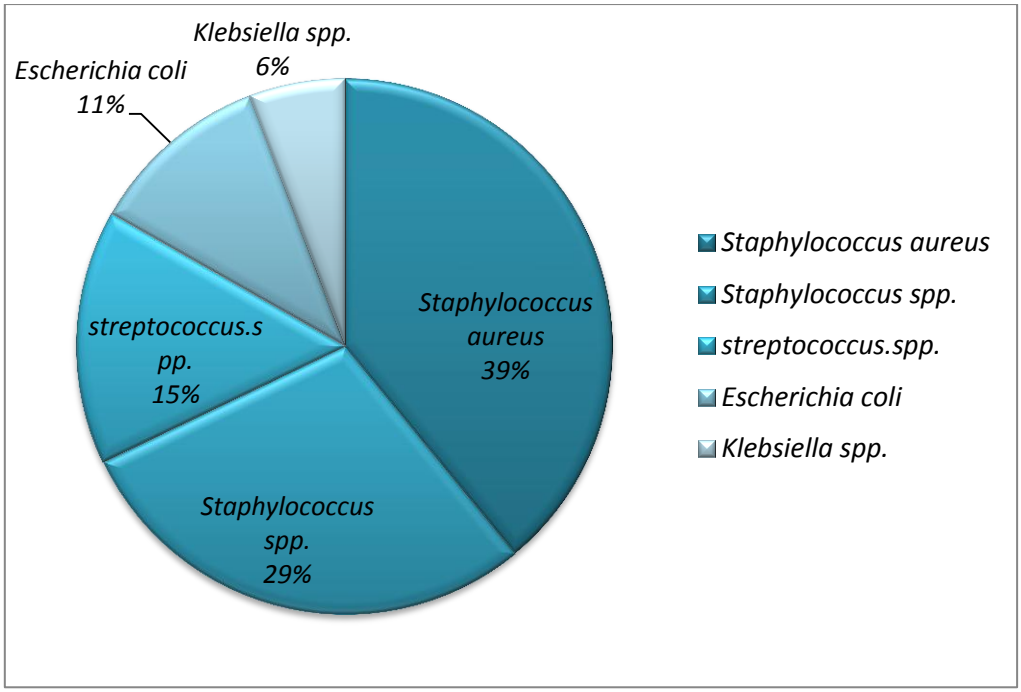

Figure (3-4) Frequency of urinary tract pathogens in pregnant women

In this study gram– positive bacteria occurred more frequently than gram- negative bacteria where *S. aureus* and *E. coli* were the commonest offending isolated bacterial pathogen in gram positive and gram negative frequency; this observation is in agreement with Frank-Peterside and Wokoma, (2009) who reported that *S. aureus* and *E. coli* were most common urinary tract pathogen. This high percentage for these two bacteria have also been observed by another searcher who reported that *E. coli* was the most common bacteria isolated from the urinary tract, it was responsible for urinary tract infection in both male and female in any age specially in pregnant women, and the second pathogen caused urinary tract infection was *S. aureus* (Boye *et al*., 2012).

The reason of such difference might be due to environment first, the socioeconomic conditions of the pregnant and the third cause was the reinfection, when infection was happened in the first trimester of pregnancy then the possibility of the reinfection can be happened in the

third trimester of pregnancy, and that was ensured by the patients' information.

During pregnancy there is an increase in levels of amino acids and lactose which encourages the growth of *E. coli*. There are diversity of the pathogens which are responsible for the urinary tract infection and that would be due to the differences in host susceptibility to pathogens as a result of both biological and environmental factors which encourage biodiversity in host, pathogens, vectors and social factors such as people's efforts in controlling disease (Boye *et al*., 2012).

3.5 Determination of bacterial isolates

In present study *S. aureus* had the highest isolate percentage from gram positive, while from gram negative *E. coli* was the most common isolate bacterial pathogens. The ability of *S. aureus* and *E. coli* bacteria to form biofilm was tested by tube methods (TM). The result illustrated that only four *S. aureus* isolates bacteria and two *E. coli* isolates bacteria showed ability to form biofilm which were determinate by appearing of violet color on the wall and bottom of the test tubes, from these biofilm forming isolates, one isolate *S. aureus* and another from isolate *E. coli* had been chosen to continue and complete other steps of the study. These two isolates bacteria had been chosen depending on their ability to give deep violate color on the wall and bottom of the tubes which used for testing the biofilm formation ability.

The ability of bacterial cell to adhere on living surface is to be considered as the first step of biofilm formation. Some compounds present in the body secretion such as urine play an important role in biofilm formation, this secretion form condition layer act as attachment

surface for the bacteria. In addition the bacteria produce (EPS) containing sugars which considered as the primary component of biofilms. If the bacteria attached to the surface, other chemical and physical interactions transform the reversible attachment to enduring irreversible adsorption. The biofilm formation usually helps the bacteria to cause infection, resist to antibiotic thus, it is explained as a virulence factor (Atabek, 2006; Hussain, 2011).

This result approached with Marques *et al*., (2007) who found that *E. coli* and *S. aureus* were the considerable of pathogenic bacteria which were able to adhere to the surface which leading to biofilm formation. Many researchers depended on the tube method to detect the ability of pathogenic bacteria to form biofilm, so it was considered as one of the biofilm detection methods and to determine the virulence of pathogenic bacteria (Mathur *et al*., 2006; Bose *et al*., 2009; Al-Chalabi *et al*., 2010).

3.6 The virulence factors of bacterial isolate

The result as shown in table (3-3), revealed that *E. coli* and *S. aureus* colonies on the surface of blood agar were surrounded by clear zone, because their ability to produce hemolysin which can induce osmotic lysis of erythrocyte as one of its actions; because of its pore forming activity and cytotoxic to several types of human blood cell (Al-Chalabi *et al*., 2010).

The clear zone on the skim milk agar surrounding the colonies of both *E .coli* and *S. aureus* bacteria was considered the positive result of producing the protease enzyme, which are vital importance to most genes of bacteria and it is one of the bacterial resistances mechanisms. Both *E .coli* and *S. aureus* are protecting themselves against the effects

of toxic peptides by producing some enzymes such as protease (Ulvatne *et al*., 2002).

S .aureus and *E .coli* bacteria were able to produce β -lactamase, enzymes which were detected by the turning of the test media from blue to white as a result of reducing iodine to iodide in the rapid iodometric method. This agrees with Husein *et al*., (2012) who regarded the ability of *S.aureus* and *E .coli* bacteria to produce β -lactamase enzyme effected resistance mechanism against many types of antibiotics, which is play vital role in virulence of these genes.

The results illustrated that *S. aureus* and *E. coli* were able to adhere on surface epithelial cells, and this adherence is usually due to the cell surface-associated to proteins that mediate attachment to the host extracellular matrix termed MSCRAMMs (Microbial surface components recognizing adhesive matrix molecules) which are helping these bacteria to cause infection in the host (Corrigan *et al*., 2007).

Al-Dulaymi, (2005) found that *E. coli* pilli, capsule and some specific surface protein may give bacteria the adherence characteristics which confer the ability to adhere to epithelial cells and mucoid membranes lining the urinary tract.

Test tube method was used to detect the ability of uropathogenic *E. coli* and *S .aureus* bacteria to form biofilm, and there ability was confirmed by the (TCP) test, which was used to measure the biofilm formation from these chosen bacteria. The biofilm is one of the virulence mechanisms that have been represented in *E. coli* pathogenesis and could be responsible for causing UTIs (Al-Chalabi *et al.*, 2010).

Table (3-3): Producing virulant factors by *E. coli* and *S .aureus*

virulence factor	*E. coli*	*S .aureus*
Hemolysin	+	+
Protease	+	+
β – lactamase	+	+
Adherence	+	+
Biofilm	+	+

(+): able to produce

Many studies showed the relationship between the bacterial virulence factors and their pathogenicity such as Al-Deen Fathi, (2007) who found that the bacteria which had the ability to produce the hemolysin and protease enzymes in some way they were showed increase in its invasion activity and ability to resist host immune system. While Al-Chalabi *et al.*, (2010) reported that several virulence factors such as hemolysin, cytotoxic necrotizing factor, aerobactin, biofilm and different types of adhesion have been responsible for *E. coli* pathogenesis. When the same strain of pathogenic bacteria had the ability to produce many virulence factors this increase their pathogenicity, because of the relationship between the bacterial ability to produce virulence factors and their infectivity.

3.7 Antibiotics susceptibility

The results showed that some of routinely available antibiotics used in this study such as Ampicillin, Ceftazidime, Vancomycin, Oxacillin, Erythromycin and Cefoxitin do not have any effect against *E. coli* and *S .aureus* chosen isolates, whereas they were sensitive to Tobramycin, Nalidixic, Clindamycin, Nitrofurantion, Chloramphenicol and Amikacin.

In addition *E coli* was sensitive to Tetracycline and Ciprofloxacin and it was resistant to Neomycin, but *S. aureus* susceptibility against these antibiotics was completely different from *E. coli*, as shown in table (3-4).

Drug resistance is one of the natural process whereby organisms develop a tolerance for environmental conditions, these may be due to preexist factor in the organisms or it may result from acquired some factors, that transfer naturally susceptible strain of bacteria into resistance bacteria. Antibiotic sensitivity *in vitro* is quite different from this obtained *in vivo* because a particular antibiotic is used depending on several factors such as its selective toxicity, drug absorption, metabolism, drug clearance rate, bioavailability and serum attainable level, therefore the risk of increase resistant organisms to antibiotic was developed, on the other hand proper adherence and compliance to drug prescription and dosage on the patients also play a role in the efficacy of the antibiotics in use (Ali, 2011).

The different susceptibility to some antibiotics between *E. coli* and *S. aureus* selected isolates was due to the deference in their cell wall structure, *E. coli* is one of gram –negative while *S. aureus* was considered as gram – positive bacteria and each one shows different mechanism to resist antibiotic agent such as integrin system is a dynamic

force in the evolution of (MDR) and this helps bacteria to acquire novel combinations of resistance genes. Integrin are horizontally transferable genetic elements which play an important role in dissemination and accumulation of resistance genes in bacteria (Muhammad *et al*., 2011).

In addition ribosome protection occurs through ribosome protection proteins that protect the ribosomes from the action of tetracycline which cause an alteration in ribosomal conformation and prevents tetracycline from binding to the ribosome. While the resistance to aminoglycoside group was due to production of the modified enzymes and losing outer membrane pores, which are responsible for permeability of surface cell layer to antimicrobials agents (Sosa et *al*., 2010; Muhammad *et al*., 2011).

E. coli and *S. aureus* bacteria were resistance to ampicillin and that agreed with Karlowsky *et al*., (2002) who found that *E. coli* isolate were resistance to amikacin. The resistance to β -lactamase in many bacteria was usually due to the hydrolysis of the antibiotic by a β -lactamase or the modification of cellular permeability. Also it may be due to minimize the interaction of antimicrobials with target site (Penicillin Binding Proteins) the surface proteins which responsible for cell wall synthesis (Sosa *et al*., 2010; Muhammad *et al*., 2011).

Table (3-4): Susceptibility of bacterial isolates to some antibiotics

Antibiotic	*E. coli*		*S. aureus*	
	Zone inhibition (cm)	Antimicrobial susceptibility	Zone inhibition (cm)	Antimicrobial susceptibility
Amikacin	2.4	S	2	S
Ampicillin	0.0	R	0.0	R
Cefoxitin	0.0	R	0.0	R
Ceftazidime	0.0	R	0.0	R
Chloramphenicol	2	S	2	S
Ciprofloxacin	2.5	S	1.2	R
Clindamycin	2.5	S	2.5	S
Erythromycin	1	R	1	R
Nalidixic	0.0	S	0.0	S
Neomycin	1	R	1.7	S
Nitrofurantion	1.8	S	1.6	S
Oxacillin	0.0	R	0.0	R
Tetracycline	2.5	S	1	R
Tobramycin	2	S	2	S
Vancomycin	1	R	1.4	R

(R=Resistance; S= Sensitive)

3.8 Antibacterial activity of plants extracts

Disk diffusion method was used to find the antibacterial activity for all studied fruits' peel extracts against *E. coli* and *S. aureus* selected isolates. After incubation period results were appeared clearly that all fruits peel extracts such as peel of apple (*Malus domestica*), banana (*Musa Paradisiaca*), cucumber (Cucumis sativus), lemon (*Citrus limon*), muskmelon (*Cucumis melo*), peach (Prunus persica), pummel (*Citrus grandis*), sour orange(*Citrus aurantium*), sweet orange (*Citrus sinensis*), and watermelon (*Citrullus lanatus*) had no detectable effect

against *S .aureus* and *E .coli* bacteria, just pomegranate peel extracts which had efficient against these two selected isolates of bacteria as shown in table (3-5), therefore; pomegranate peel extracts were chosen to complete the study.

Table (3-5): Antibacterial activity of some fruits peel on studied bacteria

No.	Fruits ' peels	*E .coli*	*S .aureus*
1	Apple	-	-
2	Banana	-	-
3	Cucumber	-	-
4	Lemon	-	-
5	Muskmelon	-	-
6	Peach	-	-
7	Pomegranate	+	+
8	Pummel	-	-
9	Sour orange	-	-
10	Sweet orange	-	-
11	Watermelon	-	-

(+): able to be as antibacterial and (-) not able to be as antibacterial

There are some reasons which could be given as explanation for these results, one of them is that the plants used in this study do not contain the active materials which were considered essential for using the plants as antibacterial, and plants parts contain the active materials but in little amounts or these active materials could not affect the bacteria (the select isolates) especially virulant ones.

The antibacterial activity of any plant depends on the extraction conditions such as type and concentration of the solvent, time and temperature for the extraction process, all these factors effect on the type and the amount of the active material that extracted (Kadhim, 2012).

The result of this study approached with Biswas *et al*., (2011) who made investigation about the qualitative phytochemical of some plants and found that *Musa paradisiaca* contained alkaloids, glycosides, steroids, gums, flavonoids, saponins, reducing sugars and tannins therefore; it had high antibacterial activity against gram positive and negative bacteria. While Karadi *et al*., (2011) found that gram positive bacteria are more susceptible than gram negatives to *Musa paradisiaca* peel extracts which contained two new acyl steryl glycosides (Sitoindoside-III and Sitosterol myo-inosityl-beta-D-glucoside) which play important role in antibacterial activity of these extracts. But studies on the antibacterial activity of five different solvent extracts (ethyl acetate, acetone, ethanol, petroleum ether and water) prepared by soxhlet extractor from two citrus fruit peel (*Citrus sinensis* and *Citrus limon*) which were screened against pathogenic bacteria *S .aureus* and *E. coli*, the citrus peel extracts showed significant antibacterial activities against the test organisms (Ashok kumar *et al*., 2011).

The study was quite different from Ambi *et al*., (2007) who used the methanol as a solvent for screening the Phytochemical of fresh seeds of *Colocynthis citrullus* and AL-Jboriiy*et al*., (2010) who studied a comparison between alcoholic and aqueous extracts for *Archis hypogaea* and *Lepidium sativum*, to show bacterial growth inhibition and the study showed that the efficiency of 70% of alcoholic extract against both gram positive and gram negative bacteria such as *S. aureus*.

3.9 The antibacterial activity of pomegranate peel extracts

The ability of pomegranate peel extracts to inhibit the growth of *S. aureus* and *E .coli* bacteria were studied by disk diffusion method. The result appeared clearly that pomegranate peel extracts were active against *S. aureus* and *E .coli* bacteria in comparison to ciprofloxacin as a positive control and the distill water as a negative control.

Table (3-6): Antibacterial Activity of pomegranate peel extracts against *S. aureus* bacteria

Concentration g/ml	Inhibition zone rate (cm)			Concentration Range
	Aqueous pomegranate peel extract	Alcohol pomegranate peel extract	Acetone pomegranate peel extract	
0.01	1.25*	1.65*	1.45*	1.45*
0.025	1.6*	1.73*	1.9*	1.74*
0.05	1.61	1.95*	1.91	1.82
0.075	1.81*	2.21*	2.00	2.01*
0.1	1.88	2.26	2.5*	1.96*
Extraction Range	1.63*	1.96*	2.03*	
Control (Ciprofloxacin) Antibiotic 5µg/disc	1.2			

LSD $_{0.05}$(concentration =0.064, extraction solvent =0.58, interaction = 1.296)
P≤ 0.0001 * = Significant different

The inhibition zone diameter of *S. aureus* and *E .coli* isolates increased as the concentration of extracts increased to reach to 1.88 cm against *S. aureus* in the high concentration of the aqueous extract 0.1 g/ml, but in low concentration 0.01 g/ml was 1.25 cm only. While inhibition zone diameter against *S. aureus* bacteria with the acetone extract come to be between 1.45 and 2.5 cm in the high and low concentration respectively. The alcohol extract was the most effective on both *S. aureus* and *E .coli* bacteria in contrast with the aqueous and the

acetone extracts and its inhibition zones diameters against *S. aureus* ranged from 2.26-1.65 cm in high and low concentration respectively, but it came to be between 2.36-1.5 cm at same concentration against *E .coli* bacteria as shown in tables (3-6) and (3-7). In which the inhibition zone diameter of *E .coli* bacteria reached to 1.78 cm in high concentration as 0.1 g/ml of aqueous extract, but at high concentration 0.1g/ml of the acetone extract gave inhibition zone diameter 2.33 cm only. While the effect at the low concentration 0.01 g/ml in both the aqueous and acetone extracts reached to 1.38 cm and 1.68 cm respectively.

So the alcohol solvent could be considered as the best one from the three solvents which were used in this study. Acetone follow alcohol and distill water could be the last good solvent with respect to their activity against the chosen isolates.

Table (3-7): Antibacterial Activity of pomegranate peel extracts against *E. coli* bacteria

Concentration g/ml	Inhibition zone rate (cm)			Concentration Range
	Aqueous pomegranate peel extract	Alcohol pomegranate peel extract	Acetone pomegranate peel extract	
0.01	1.38*	1.50*	1.68*	1.52*
0.025	1.51*	1.90*	1.76*	1.72*
0.05	1.63*	1.93	1.91*	1.82*
0.075	1.73*	2.08*	2.21*	2.01*
0.1	1.78	2.36*	2.33	2.15*
Extraction Range	1.64*	1.97*	1.98*	
Control (Ciprofloxacin) Antibiotic 5µg/disc	2.5			

LSD $_{0.05}$(concentration = 0.086, extraction solvent = 0.077, interaction = 1.737)
P≤ 0.0001 * = Significant different

The results showed a relationship between the value of inhibition zone diameter and type of solvents used in the extraction process for each isolate of studied bacteria. When the three solvents were used at different concentrations against *S. aureus* bacteria, alcohol solvent gave the widest inhibition zone, when the extracts used at (0.01, 0.05, 0.075 and 0.1) g/ml concentrations, and it was followed by the value of inhibition zone diameter for the acetone extract, but the aqueous extract had the lowest effect when it used at the concentrations above and when 0,025 g/ml concentration of these three types of extracts were used against *S. aureus*, the acetone extract was the best one as the highest effect then followed by the alcohol and the aqueous extracts.

Among the solvent and according to their effects on *E .coli* isolate, acetone extract was considered as the best one when it used at 0.01 g/ml and 0.075 g/ml, but the alcohol extract had the highest effect on *E.coli* bacteria at 0.025 g/ml, 0.05 g/ml and 0.1 g/ml concentration in contrast with the other extracts.

When the pomegranate peel extracts were used as an antibacterial the best solvent chosen as extractor could be the polar solvents especially the ethanol solvent due to the best effect on both selected isolates.

The results agree with Rathinamoorthy *et al*., (2011) who used the disk diffusion method to find the effect of pomegranate peel on some pathogens and attributed the antibacterial activity of pomegranate peel extracts on the presence of metabolic toxins or broad spectrum antimicrobial compounds that act against both gram positive and gram negative bacteria.

The results approached with Dahham *et al*., (2010) who extracted pomegranate peel with different solvents (polar and non-polar) at room temperature and assayed them for antibacterial activity and evaluated the activity of peel extracts against both gram- positive and gram-negative bacteria and found that the acetone extract showed the highest antibacterial activity, followed by water extracts. While the results disagree with Khan and Hanee, (2011) who found that the aqueous and the ethanol pomegranate peel extracts had the same effect.

Vorauthikunchai *et al*., (2005) compared the antibacterial activity of the aqueous and ethanol pomegranate extracts against *E .coli* and showed that both extracts had effect on that bacteria but the ethanol was more effective.

3.10 Phytochemical screening

The active materials of the Pomegranate peel extracts detected by the color change tests. Table (3-8) shows Pomegranate peel screening alkaloid, saponins, flavonoids, tannins, phenolic, glycosides and resins as a phytochemical. The flavonoids and saponins were absent in aqueous extract, but during ethanol extraction, only saponins were not extracted the. While in the acetone extraction this solvent succeeded to extract nearly all the studied active material. The active material in the pomegranate peel needed the polar solvent to be extracted in a good way and that mean that most of these materials were extracted in good amount by the ethanol.

Table (3-8): Preliminary phytochemical screening of pomegranate peel extracts

Pomegranate peel extracts	Alkaloid	Saponins	Flavonoids	Tannins	Phenolic	Glycosides	Resins
Aqueous	+	-	-	+	+	+	+
Ethanol 96%	+	-	+	+	+	+	+
Acetone 70%	+	+	+	+	+	+	+

Key: (+) indicates present and (-) indicates absent

The results approached with Rajan *et al*., (2011) who proved the presence of alkaloids, flavonoids, phenolic compounds and tannins in pomegranate peel extracts but the aqueous and alcohol extracts had different ability to extract the phytochemical compounds. When the two extracts were extracted, the saponins was absent in the both and that agreed with our results. But Qnais *et al*., (2007) found that the flavonoids was exist in the aqueous extract and that disagree with the results of this study which was the flavonoids exist just in the polar extraction. Khan and Hanee, (2011) reported that pomegranate contained large amount of tannins (25%) and that made it as antibacterial due to the presence of these secondary metabolites.

The mode of action of these compounds such as polyphenols is generally attributed to polyphenol-protein interactions, though different mechanisms have been suggested including inhibition of microbial enzymes and action on membranes or deprivation of substrates were required for microbial growth (Janecki and Kolodziej, 2010).

3.11Determination of the minimum inhibition concentration (MIC)

Agar dilution method was used to detect MIC of the extracts and all extracts were diluted to obtain several concentration ranged between 0.01 g/ml and 0.1 g/ml. The results of this study showed that the MIC of the acetone and alcohol extracts was 0.04 g/ml, but for the aqueous extract reached to 0.06 g/ml. This indicates that the polar extracts had the best ability to be an extractor than non-polar solvents, as shown in table (3-9)

Table (3-9): Minimum inhibitory concentration (MIC) of pomegranate peel extracts against *S .aureus* and *E .coli* bacteria

Test organism	Minimum Inhibitory Concentration (MIC)g/ml		
	Distell Water	96% ethanol	70% acetone
S .aureus	0.06	0.04	0.04
E .coli	0.06	0.04	0.04

The effect of all types of extracts seemed to be similar on both *S. aureus* and *E .coli* isolates; this might mean that these extracts did not have any effect on the bacterial cell wall, but the effect could be considered at the other parts of the bacterial cell when the extracts killed them.

The ability of the pomegranate peel extracts to be as antibacterial due to the presence of hydrolysable tannins and polyphenols in addition to the amount of total phenolic in peel which was markedly higher than another parts of the pomegranate fruit. Akbarpour *et al.*, (2009)

Gallotannins which were considered as one of the tannins compounds and consist of about 25% from it in the pomegranate peel and in addition to the alkaloid which was known as punicine which plays an important role in the ability of the peel to effect on bacteria by changing the protein nature then kills the bacteria (Farhan, 2010). It means that the antimicrobial effect of tannins is related to its toxicity and molecular structure. Tannins may act on the cell wall and across the cell membrane because they can precipitate proteins, they may also suppress many enzymes such as glycosyltransferases. Hence, the antibacterial activity of pomegranate may be related to polyphenol structures because polyphenols may affect the bacterial cell wall, inhibit enzymes by oxidized agents, interact with proteins and disturb co-aggregation of microorganisms (Abdollahzadeh *et al*., 2012).

Pomegranate has been used in traditional medicine for different therapies because of its styptic properties. Both pomegranate pulp and peel contain different kinds of antioxidants, including these which have not possibly been well characterized. It has been acknowledged that phenolic compounds such as flavonoids and anthocyanin are the major class of effective antioxidants in many fruits and vegetables (Ardekani *et al*., 2011).

3.12 Effect of the extracts on bacterial virulence factors

The results of this study, explained that Pomegranate peel extracts possess strong antibacterial activity against *S .aureus* and *E. coli* bacteria, therefore to find the exactly effect of that extracts on the studied isolates or to reach the reason of this effect and which part or virulence factors of the bacteria had been more affected by plant extracts. The studied bacteria which had the ability to produce number of the virulence

factors (hemolysin, Protease, β – lactamase) were treated with the (MIC) of each one of the plant extracts, then the capacity of producing hemolysin, Protease, β – lactamase enzymes were assayed after incubation period with these extracts. The results explained that capacity of *S .aureus* and *E. coli* bacteria to produce hemolysin toxin and protease enzyme had not been affected and remained without any alter in compare with the control. While the *S .aureus* and *E. coli* bacteria were completely lost their ability to produce β – lactamase enzymes when treated with the extracts, although these bacteria were active producer for this enzyme before they have been treated with the extracts, as shows in table (3-10)

Table (3-10): The effect of pomegranate peel extracts on bacterial virulence factors

Virulance factors	***S .aureus* with pomegranate peel extracts by**			***E .coli* with pomegranate peel extracts by**		
	Distell Water	**96% ethanol**	**70% acetone**	**Distell Water**	**96% ethanol**	**70% acetone**
Hemolysin	+	+	+	+	+	+
Protease	+	+	+	+	+	+
β – lactamase	-	-	-	-	-	-

(+): able to produce, (-): an able to produce

Pomegranate peel extracts could be as inhibitor by binding with the β –lactamase active site thus prevents the binding of β –lactamase to the antibiotics and then black their action so these antibiotics will not analysis and can effect on the pathogens (Shaokat and Hameed, 2010).

That results disagreed with AL- hmmundu *et al*., (2009) who found that the aqueous and alcoholic pomegranate peel extracts had the ability

to weak hemolysin production from *E .coli*, and the ability of *S .aureus* to produce hemolysin was completely stopped when it treated with aqueous and alcoholic extracts.

3.13 Effect of the extracts on biofilm formation

Figure (3-5) illustrated that *S .aureus* was high producer for biofilm formation; this was shown through its optical density that reaches to 1.7 when it was measured for bacterial suspension without any types of the extracts (regarded as control). But, the optical density came to be reduced largely when the MIC of each one of the extracts was added to *S .aureus* suspension before reading the optical density which was 0.86 in the aqueous extracts and 0.85 in the acetone extracts, while in the alcohol extracts the optical density reached to 0.12.

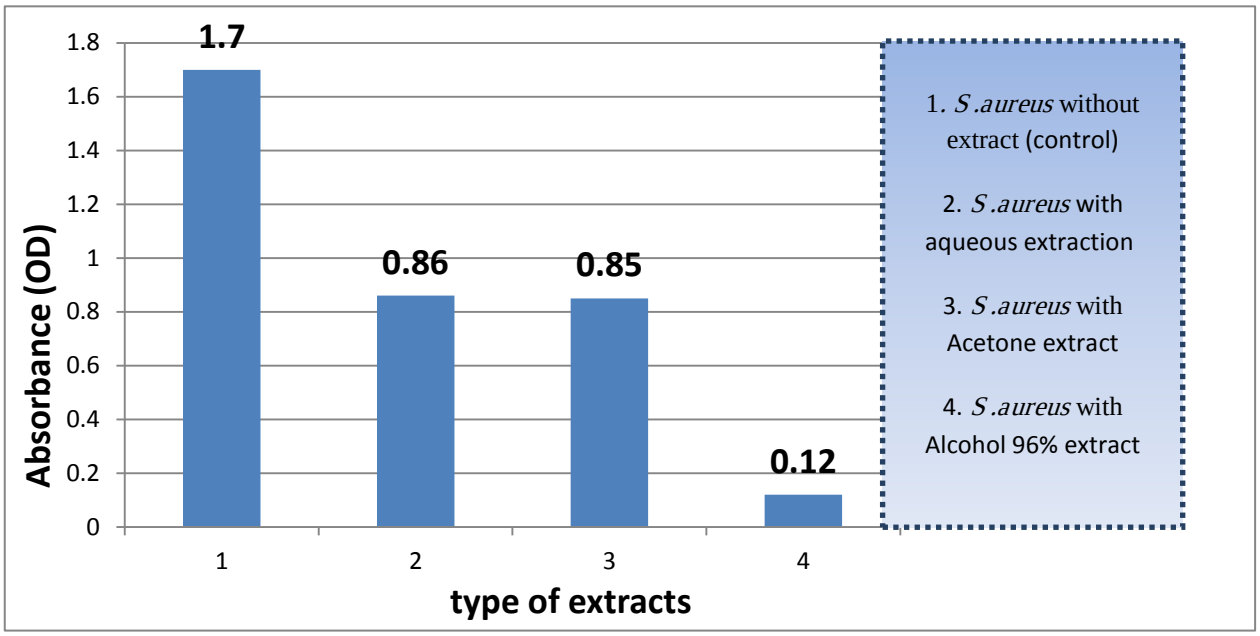

Figure (3-5): Effects of pomegranate peel extracts on biofilm formation activity of *S. aureus* bacteria

The optical density (OD) of *S .aureus* suspension with the MIC of the aqueous and the acetone extracts almost the same, and this result was

different from the results of the other studies which found that the acetone extract had more effect than the aqueous extract. Whereas the alcohol extracts still the best solvent in its effects on *S .aureus* and *E. coli* bacteria which was regarded as high producer biofilm because the OD of the *E. coli* suspension which reached to 1.5 but when the suspension of *E. coli* bacteria treated with the MIC of the aqueous and acetone extracts the OD of then became 0.76 and 0.582 respectively, while reached to 0.35at the presence of the alcohol extract and that mean the biofilm formation activity were declined clearly in compared with the control, as shown in figure (3-6).

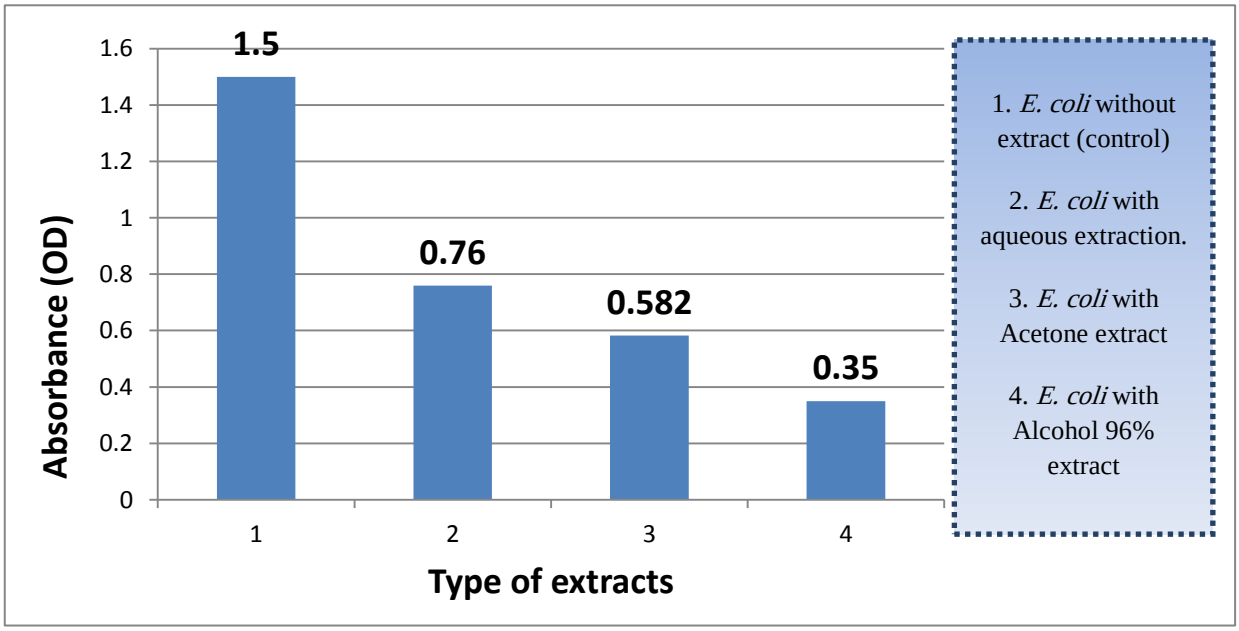

Figure (3-6): Effects of pomegranate peel extracts on biofilm formation activity of *E. coli* bacteria

The pomegranate peel extracts were affected largely on the ability of studied bacteria to form biofilm and there affect difference with the various kinds of the extracts.

The biofilm formation activity of bacteria were declined after treatment with extracts but this decline do not transfer bacteria from high

producer (The control) to the poor producer (OD lower than 0.1) after treatment with extracts. The *S .aureus* and *E. coli* bacteria were treated with the aqueous and the acetone extracts remained as a high producer (OD high than 0.5), but when the bacteria treated with alcohol extract it came to be transferred from high producer to producer only (OD between 0.5 – 0.1). This proved that alcohol solvent represented as the best one in extraction and preserve the activity of active compounds in pomegranate peel extract in compered with the acetone and aqueous extracts and these results may be due to form the variety of biological properties and the activity of the extracts chemical composition when different solvents were used in preparing these extracts.

Janecki and Kolodziej, (2010) explained the activity of plants active compound that could be used as anti-adhesive agents against pathogenic bacteria and showed capabilities of these compounds such as the tannins of interacting with macromolecules, including carbohydrates and proteins, which made these compounds as promising anti-adhesive and antibiofilm.

Many searches were search for any material could be inhibit the bacterial ability of biofilm formation (anti-biofilm), some used antibiotics and found the ciprofloxacin and N.acetylcysteine had the highest ability to inhibit biofilm formation due to their ability to eradicate the pro-formed mature biofilms (El-Feky *et al*., 2009).

Bacterial exopolysaccharides have always been suggested to play crucial roles in the bacterial initial adhesion and development of complex architecture in the later stages of bacterial biofilm formation. Therefore; many searchers were emphasizing by many trials to find

some material that inhibit the activity of these materials (Jiang *et al.*, 2011).

The success of plant extracts in inhibiting cell attachment and reducing microbial colonization on surfaces of epithelial mucosa which lead to infection represented the important steps that achieved the growth of an already established biofilm (Sandasi *et al*., 2009).

3.14 Effect of the plants extracts on adherence activity

The ability of *S .aureus* and *E. coli* isolates to adhere to the uroepithelial cell was detected by calculations of the number of adherence bacterial cells under light microscope.

Forty cells of *S .aureus* bacteria adhered on the assayed epithelial cell, while only 20 cells of *E. coli* adhered to the epithelial cells. These data was regarded as control for detection of the effects of the MIC of pomegranate peel extracts on adhesion ability of studied bacteria.

As shown in figure (3-7) the number of adhered *S. aureus* bacteria cells on the epithelial cell which was clearly declined when the bacteria treated with peel extracts. The aqueous extract reduced the number of the adherence bacterial cell from 40 bacteria /cell to only 10 bacteria /cell, but the number of the adherence cells reach to 3 and 1 bacteria /cell in the presence of the acetone and the alcohol extracts respectively.

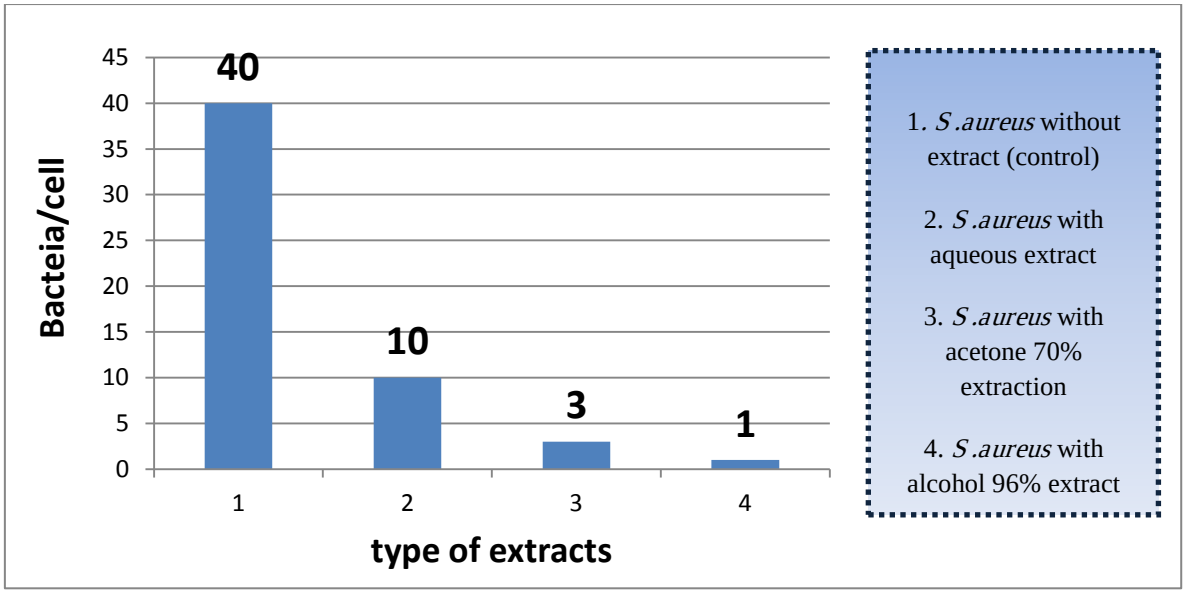

Figure (3-7): Effects of Pomegranate peel extracts on adherence activity of *S. aureus* bacteria

The adherence of the *E. coli* bacteria reached to one bacteria /cell when the acetone extract was added to bacterial suspension, but only three bacterial cell seen to be attached to epithelial cells after the bacterial suspension was incubated with alcohol extract, while only 5 bacteria / cell were attached after treatment with the aqueous extract, in contrast with control (*E. coli* without the extracts), as shown in figure (3-8)

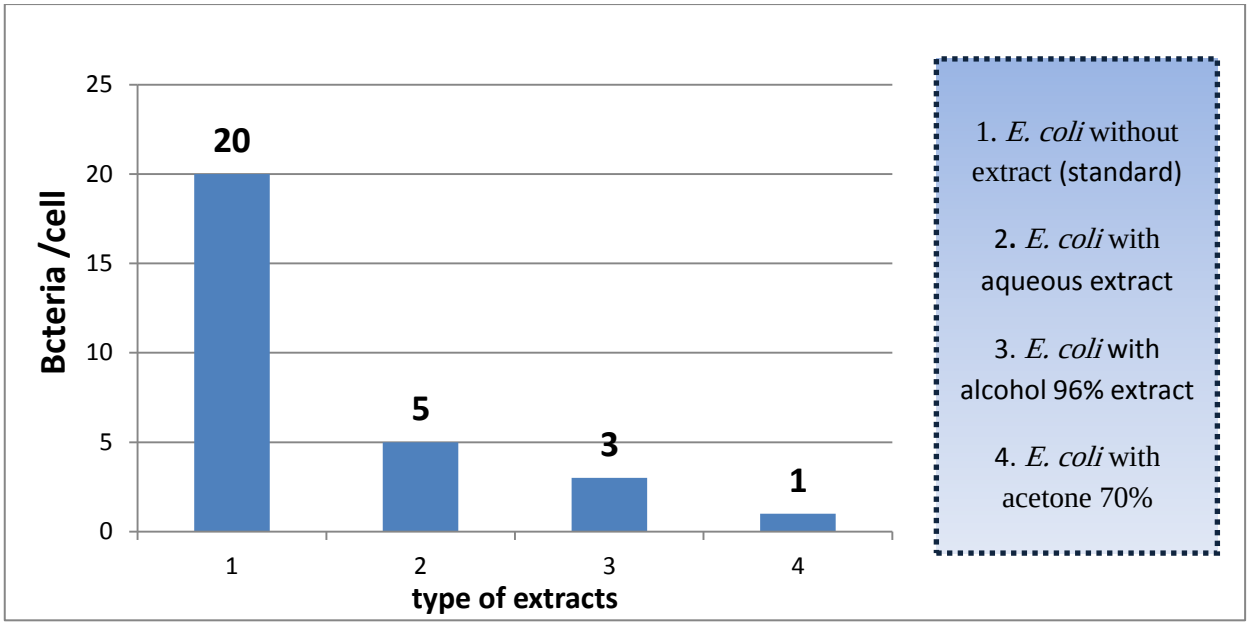

Figure (3-8): Effects of pomegranate peel extracts on adherence activity of *E. coli* bacteria

Like all effects of the extracts the ethanol extract was the best anti adhesive factor, and followed by the acetone, while the aqueous extract had the least effect. This may return to the weak ability of distil water to extract the active materials from plants peel in affected amounts in compare with acetone and alcohol solvents.

The pomegranate peel extracts have been worked as anti –adhesive, because of the large amounts of the saponins, flavonoids, alkaloids, tannins, phenolic, glycosides and resins in the Pomegranate peel, which were directly responsible for the anti-adhesive activity against pathogen (Mahony *et al*., 2005).

The tannins play an important role of this affection and that was approached with Lee *et al*., (2009) who studied the anti-adhesive activity of green tea extracts against Pathogenic bacteria. Green tea extracts contained polyphenols; tannin and caffeine, which have been responsible

for the pharmacological action of green tea, therefore; the presence of tannins and other chemical compounds in pomegranate peel extract explained its antibacterial activity.

The effect of the plant extracts is return to its ability to inhibiting cell attachment. The cell attachment is the initial stage in biofilm formation following surface conditioning which creates a favorable environment for bacterial attachment. Surface conditioning is achieved by the adsorption of substances that include nutrients, organic and inorganic molecules that are important for the growth of the cells, which in turn promotes cell adhesion and biofilm formation, therefore; it can be postulated that pretreatment of the surface with plant extracts produced an unfavourable film that promotes detachment, thereby reduce the surface adhesion (Sandasi *et al*., 2009).

3.15 Interactions between the extracts and antibiotics *in vitro*

The effect of the combination of the peel extracts and the antibiotics on the susceptibility of the *S. aureus* bacteria shown in table (3-11), which was illustrated that the interaction of the ethanol extracts with the chloramphenicol and ciprofloxacin antibiotics were synergy (that occurs when the effect of the two drugs together is greater than the effect of one of the drugs). But with tetracycline it's been indifference (that occurs when the effect of the two drugs together is less than the effect of either it is alone), while at the combination of the acetone extract with the three types of the antibiotics had been antagonism (that occurs when the two drugs together have no effect). The FIC value illustrated that the combination was indifference in the aqueous extracts with the

chloramphenicol and ciprofloxacin antibiotics and come to be antagonism with the tetracycline antibiotic.

Table (3-11): Effect of the combination between pomegranate peel extracts and antibiotics on *S .aureus* bacteria

Antibiotics	pomegranate extract	Mean FIC (Antibiotic)	Mean FIC (Extract)	FIC Index	Interaction
Chlo	Ethanol 96%	0.1	0.1	0.2	Synergy
	Acetone 70%	0.9	1.125	2.025	Antagonism
	D.W.	0.5	0.833	1.33	Indifference
Cip	Ethanol 96%	0.1	0.1	0.2	Synergy
	Acetone 70%	1.8	0.937	2.73	Antagonism
	D.W.	1.2	0.833	1.93	Indifference
Tet	Ethanol 96%	0.375	0.75	1.125	Indifference
	Acetone 70%	0.375	0.937	1.31	Antagonism
	D.W.	0.45	1.5	1.95	Antagonism

Key: **Tet** - Tetracycline; **Chlo** - Chloramphenicol **Cip** - Ciprofloxacin

While in the study of *E .coli* bacteria susceptibility was found that the synergy present only in the interaction between the tetracycline and the acetone extract and the other results were divided between antagonism and indifference effect, but in the case of the ethanol and the chloramphenicol which was additivity (the effect which is less than synergistic but not antagonism), as shown in table (3-12)

Table (3-12): Effect of the combination between pomegranate peel extracts and antibiotics on *E. coli* bacteria

Antibiotics	pomegranate extract	Mean FIC (Antibiotic)	Mean FIC (Extract)	FIC Index	Interaction
Chlo	**Ethanol 96%**	0.5	0.5	1	**Additivity**
	Acetone 70%	0.75	0.937	1.68	**Indifference**
	D.W.	0.75	1.25	2	**Antagonism**
Cip	**Ethanol 96%**	1.125	0.9	2.025	**Antagonism**
	Acetone 70%	0.625	0.625	1.25	**Indifference**
	D.W.	0.937	1.25	2.187	**Antagonism**
Tet	**Ethanol 96%**	0.375	0.75	1.125	**Indifference**
	Acetone 70%	0.25	0.625	0.875	**Synergy**
	D.W.	0.375	1.25	1.625	**Indifference**

Key: **Tet** - Tetracycline; **Chlo** - Chloramphenicol **Cip** – Ciprofloxacin

The effect of the chloramphenicol and ciprofloxacin antibiotics against *S. aureus* bacteria was marginally improved in the presence of the alcohol extract and the effect of the tetracycline antibiotic against *E. coli* was improved in the presence of the acetone extract, while any other combination between studied antibiotic and the extracts had not any beneficial effects against studied *S .aureus* and *E. coli* bacteria.

So the results found that the Pomegranates peels extracts effect alone well than they interact with antibiotics in most of the results. That result was disagreeing with the results done by Hussin and EL-Sayed, (2011) who found that the synergy effect against *S. aureus* and *E. coli* in the interaction between the Pomegranates peels extracts and tetracycline antibiotics. The additive or a synergistic effect due to the double attack of both agents (extracts and antibiotics) on different target sites of the bacteria, and when the extracts have many different phytochemicals which might inhibit bacteria by different mechanisms, these numerous

the compounds within the extracts may have interfered with the actions of one another (Adwan *et al.*, 2008).

While the exploited of antibiotics in the management of bacterial infection has led to the emergence and multiplication of resistant bacterial strains. Infection due to multidrug resistance pathogens are routinely complicated to deal with due to virulence factors and because of a relatively limited choice of antimicrobial agents and some of the antimicrobial agents will have a short life expectancy. Even plant extracts as antimicrobials which are rarely used as systemic antibiotics at present, this may be due to their low level of activity, especially against gram-negative bacteria. Here we are trying to investigate an alternative approached to the treatment of bacterial infections by ability to change the phenotype of a resistant pathogen to certain antibiotics to more susceptible pathogen to those antibiotics. Thus, it is extremely important to find novel antimicrobials or new techniques that are effective for the treatment of infectious diseases (Adwan *et al*., 2008; Aiyegoro *et al*., 2011).

So other studies found that efficacy of antimicrobial agents could be improved by combining antibiotics with crude plant extracts against different pathogens *in vitro* and found that may reducing the (MICs) of antibiotics against resistant organisms. The combined of the plants extracts and the antibiotics could be useful in fight emergency drug resistance pathogens (Adwan *et al.*, 2008).

The plants either contain antimicrobials that can operate in synergy with antibiotics or possess compounds that have no intrinsic antibacterial activity but are able to sensitize the pathogen to a previously ineffective antibiotic (Aiyegoro *et al*., 2011).

The antimicrobial compounds from plants have been found to be synergistic enhancers in that though they may not possess any antimicrobial properties alone, but when used concurrently with standard drugs they enhance the activity of the drug. The synergistic effect of the association of antibiotic and plant extracts against resistant pathogens leads to new choices for the treatment of infectious diseases. Also synergy between bioactive plant product and antibiotics will confront problems of toxicity and overdose since lesser concentrations of two agents in required combination, due to these reasons, there is need, for continuous exploration of multidrug resistance modulating principles from plants sources (Aiyegoro *et al*., 2009).

Conclusions and Recommendations

Conclusions

1. *S .aureus* and *E .coli* bacteria are represented the most common pathogen in urinary tract of pregnant women in Karbala that have many virulence factors which are responsible for pathogenicity.
2. Alcoholic 96% of pomegranate peel extracts have antibacterial activity represented by its effect on the bacterial virulence factors of bacterial infection.
3. The pomegranate peel extracts were high synergism with some antibiotics against pathogenic bacteria.
4. The alcohols extract still the best solvent in its effects on bacterial pathogen and it was effect largely on the ability of the studied bacteria to form biofilm and adhesion on the epithelial cell.

Recommendations

1. Look for more plants have the ability to be anti- virulence factors, and the exactly effect on that factors.
2. Further studied needed to assay the role of combination between the plants extracts and the antibiotics in order to reduce the side effect of using the antibiotics alone by declined the dose of these antibiotics which are used in the treatment of infection.
3. Made more studies about purification of active substances in pomegranate peel extracts.

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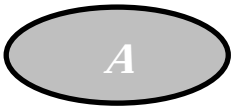

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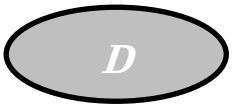

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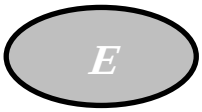

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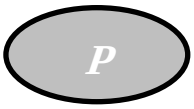

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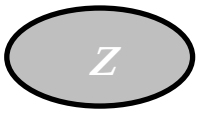

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الخـلاصـــــة

تضمنت هذه الدراسة عزل البكتريا الممرضة المسؤولة عن التهاب المجاري البولية في النساء الحوامل المراجعات لمستشفى النسائية والتوليد في محافظة كربلاء و خلال الفترة من كانون الثاني 2011 ولغاية أذار 2012.

جمعت من 100 أمرأة حامل عينة أدرار وسطي صباحي تراوحت اعمارهن من 16 الى 40 سنة وزرعت على الاوساط الزراعية العامة والانتقائية بعدها شخصت البكتريا النامية بأستخدام الفحوصات البايوكيميائية وبأستخدام اطقم APi.

اظهرت نتائج الدراسة الحالية وجود 52 عزلة بكتيرية ممرضة في عينات الادرار المجموعة والتي توزعت بين البكتريا الموجبة لصبغة غرام بنسبة 83% (43) والتي تمثلت بكل من بكتريا coagulase positive [*Staphylococcus aureus*] بنسبة 39% (20) وبكتريا coagulase negative [*Staphylococcus* spp.] بنسبة 29% (15) وبكتريا [*Streptococcus* spp.] بنسبة 15% (8) والبكتريا السالبة لصبغة غرام والتي كانت مسؤولة عن 17% (9) فقط والتي توزعت بين كل من بكتريا [*Escherichia coli*] بنسبة 11% (6) وبكتريا [*Klebsiella* spp.] بنسبة 6% (3)، وبعدها تم اخضاع كافة العزلات الجرثومية التابعة لكل من بكتريا *S. aureus* و *E. coli* باعتبارهما المسؤولتين عن النسب الاعلى لاصابات المجاري البولية في النساء الحوامل قيد الدراسة الى فحص قابليتهما على تكوين التجمعات الخلوية بواسطة فحص الانابيب (Tube methods) وذلك لاختيار العزلة الاكثر كفاءة لتكوين تلك التجمعات لكل من عزلات بكتريا *S. aureus* ومثيلتها من عزلات بكتريا *E. coli* عندها تم اختيار قابلية تلك العزلتين على انتاج بعض عوامل الضراوة مثل قابليتها على انتاج سم الهيمولايسن(hemolysin)، وانزيمي البروتيز(Protease) والبتا لاكتاميز(β – lactamase) .

تم غربلة قشور(11) نوع من الفواكهه المختلفة (التفاح، الموز، الخيار، الليمون، البطيخ، الخوخ، الرمان ، النارنج، البرتقال والرقي) باستخدام ثلاثة مذيبات (المائي, الكحولي والاسيتوني 70%) حيث وجد ان بعض تراكيز المستخلصات الثلاثة لقشور الرمان هي الوحيدة التي احدثت تأثير تثبيطي واضح في نمو عزلتي بكتريا *S.aureus* و *E. coli* . الاقطار التثبيطية للبكتريا المدروسة تزداد بزيادة تراكيز مستخلصات قشور الرمان لتصل لبكتريا *S.aureus*

الى 1.88 سم عند التركيز 0.1 غم/مل للمستخلص المائي و 2.26 سم للمستخلص الكحولي اما قطر التثبيط بوجود المستخلص الاسيتوني وعند نفس التركيز فوصل الى 2.50سم فقط. بينما 1.78 سم ، 2.36 سم و 2.33 سم فمثلت الاقطار التثبيطية لبكتريا *E .coli* عند استخدام التركيز 0.1 غم/مل لمستخلصات قشور الرمان المائي، الاسيتوني ثم الكحولي على التوالي.

استخدمت الطرق الكيميائية الشائعة لتحديد المكونات الكيميائية لمستخلصات قشور الرمان الثلاثة والتي كانت جميعا حاوية على كل من القلويدات، الراتينجات ،التانينات ، الفينولات و الجلايكوسيدات في حين خلا المستخلص المائي فقط من وجود الفلافونات والصابونيات بينما افتقر المستخلص الكحولي لوجود الصابونيات فقط.

ولمعرفة تأثير مستخلصات قشور الرمان المختلفة على قابلية كل من بكتريا *S .aureus* و*E .coli* على انتاج بعض عوامل الضراوة، فقد تم اولا قياس التركيز المثبط الادنى لتلك المستخلصات تجاه العزلتين البكتيريتين والذي بلغ 0.04 غم/مل لكل من المستخلص الكحولي والاسيتوني في حين كان 0.06 غم/مل للمستخلص المائي. عندها تم مقارنة قابلية تلك العزلتين على انتاج عوامل الضراوة قبل وبعد معاملتهما بالتركيز المثبط الادنى للمستخلصات الثلاثة، حيث ظهر جليا ان تلك المستخلصات ليس لها اي تأثير على قابلية العزلتين على انتاج كل من سم الهيمولايسين وانزيم البروتيز، في حين كان لها الاثر الاكبر في ايقاف قدرتها على انتاج انزيم البتا لاكتميز واما تأثير تلك المستخلصات الثلاثة على قدرة البكتريا على تكوين التجمعات الخلوية (biofilm) فقط درست باتباع طريقة Tissue culture plate method (TCP) حيث كانت القراءات 0.76، 0.582، 0.35 بوجود المستخلص المائي ، الاسيتوني والايثانولي على التوالي مع بكتريا *E. coli*، وبالمقارنة مع السيطرة التي بلغت 1.5 مما يدل على التأثير التثبيطي الكبير لتلك المستخلصات على قابلية تلك الجرثومة على تكوين التجمعات الخلوية . اما في حالة بكتريا *S. aureus* فقد انخفضت القراءة من 1.7 للبكتريا فقط الى 0.86 بوجود المستخلص المائي و 0.85 مع المستخلص الاسيتوني، اما وجود المستخلص الكحولي فأدى الى انخفاض القراءة الى 0.12 فقط.

ولما كان انتاج biofilm يعتمد على قابلية العزلات البكتيريا على الالتصاق فكان لابد من دراسة تأثير المستخلصات الثلاثة لقشور الرمان على هذا العامل، فقد اظهرت تلك المستخلصات تأثير واضح على القابلية الالتصاقية لكلا العزلتين، حيث انخفض عدد خلايا بكتريا *S. aureus* الملتصقة على الخلايا الظهارية من 40 خلية بدون وجود المستخلص الى 10،3،1 خلية ملتصقة

بوجود المستخلص المائي و الكحولي والاسيتوني على التوالي ، كما انخفض عدد خلايا بكتريا *E. coli* الملتصقة من 20 خلية بدون وجود المستخلص الى 5 خلايا فقط بوجود المستخلص المائي و 3 خلايا بوجود المستخلص الكحولي وخلية واحدة فقط عند وجود المستخلص الاسيتوني.

لقد كان لمستخلص قشور الرمان الكحولي تأثير تأزري واضح ضد بكتريا *S .aureus* عند استخدامه مع كل من المضاد الحيوي Chloramphenicol و Ciprofloxacin اما في حالة بكتريا *E .coli* فأن التأثير التأزري وجد عند استخدام المستخلص الكحولي مع المضاد الحيوي Chloramphenicol والمستخلص الاسيتوني مع Tetracycline فقط.

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تأثير مستخلصات قشور بعض الثمار على ضراوة بعض انواع البكتريا الممرضة المعزولة من اصابات المجاري البولية في النساء الحوامل في كربلاء

رسالة مقدمة

إلى مجلس كلية العلوم – جامعة كربلاء

وهي جزء من متطلبات نيل درجة الماجستير

في علوم الحياة/ أحياء مجهرية

مقدمة من

بشائر سامي هادي

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2009

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شعبان 1434هـ **تموز 2013 م**