EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 5
Issue: 12
436-442
Year: 2018

# *IN VITRO* ANTIMICROBIAL ACTIVITY OF *PUNICA GRANATUM, HIBISCUS SABDARIFFA* AND *ANNONA SQUAMOSA* EXTRACTS AGAINST SOME MICROORGANISMS ISOLATED FROM WOMEN WITH HIGH VAGINAL INFECTIONS

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**Article Received on 21/10/2018** **Article Revised on 11/11/2018** **Article Accepted on 03/12/2018**

**ABSTRACT**
Through a period extended for three months (October to December, 2017), 25 fungal and bacterial isolates were isolated from Fattima-AL-Zahra hospital for pediatric and obstetric in Baghdad from women with high vaginal infections. Out of these 25 isolates, 16 (64%) were Candida albicans isolates, 4 (16%) were Escherichia coli isolates, 3 (12%) were Enterococcus faecalis isolates and 2 (8%) were Staphylococcus saprophyticus isolates. Escherichia coli isolates showed the highest resistant toward Ampicillin and Cefazolin which gave 4(100%) resistance rate. On the other hand the most effective antibiotic toward Escherichia coli isolates under test was Amikacin which it gave the lowest resistance rate 0(0%). Enterococcus faecalis and Staphylococcus saprophyticus isolates showed a high level resistance to most of the antibiotics under test and the highest resistant antibiotics were Ampicillin and Erythromycin which gave 5(100%) resistance rate, while the most effective antibiotic was Vancomycin which it gave 0 (0%) resistance rate. Punica granatum (peel), Hibiscus sabdariffa and Annona squamosa (peel and seed) extracts were prepared in different concentrations (100, 50, 25 and 12. 5 mg/ml). The result of antimicrobial activity test of Punica granatum (peel) extract showed that the Punica granatum extract was the most effective extract on fungal and bacterial isolates in all concentrations compared with the other plant extracts under study. On other hand Hibiscus sabdariffa extract was effective on fungal and bacterial isolates only in 100, 50 and 25 mg/ml concentrations and not gave antimicrobial activity in the last concentration. The result of antimicrobial activity test of Annona squamosa (peel and seed) extracts showed that Annona squamosa (peel and seed) extracts don't gave antimicrobial activity against all fungal and bacterial isolates under test (G-ve and G+ve bacteria) in all concentrations (100, 50, 25 and 12.5 mg/ml concentrations).

**KEYWORDS:** Antimicrobial activity, High vaginal swabs, Punica granatum, Hibiscus sabdariffa and Annona squamosal.

## INTRODUCTION

Vaginal infections with bacterial vaginosis, candidiasis and trichomoniasis are a global health problem for women.[1] Vaginitis is the inflammation and infection of vagina commonly encountered in clinical medicine. Diverse spectrums of pathogenic agents were observed in the vaginal micro flora. Of these, bacterial vaginosis, candidiasis and trichomoniasis are responsible for majority of vaginal infections in women of reproductive age.[2]

Abnormal vaginal discharge, itching, burning sensation, irritation and discomfort are frequent complaints among patients attending obstetrics and gynecology clinics. However, a number of vaginal infections present with few or no symptoms.[3]

Candida vaginitis (CV) is one of the most frequent infections in women of reproductive age. Approximately 75 % of adult women will have at least one episode of vaginitis by candida during their life time (2 & 3). Vaginal infections are associated with a significant risk of morbidity in women. If untreated they can lead to pelvic inflammatory disease.[2]

Antibiotics can be purchased without prescription in less development countries, which leads to misuse of antibiotics. This may led to the emergence and spread of antimicrobial resistance.[4] The global emergence of multi-drug resistant (MDR) bacteria is increasingly limiting the effectiveness of current drugs and significantly causing treatment failure. Bacterial resistance to chemically unrelated antimicrobial agents is public health concern.[5]

Due to the increase of resistance to antibiotics, there is a pressing need to develop new and innovative antimicrobial agents. Among the potential sources of new agents, plants have long been investigated. So, they contain many bioactive compounds that can be of interest in therapeutic.[5] Plant-derived compounds such as tannins, phenolic compounds and so on, are usually found in various parts of the plants like roots, leaves, shoots and bark.

Many plants have therefore gained widespread interest in the search to identify the alternatives for microbial control and treatment of various infections and diseases as some chemically synthesized drugs have undesirable side effects.[6] So this study aimed to isolation of isolates that cause high vaginal infections, determine the antibiotic susceptibility pattern of identified bacterial isolates and determine *in vitro* the antimicrobial activity of watery plant extracts against candida and bacterial isolates under the study.

## MATERIALS AND METHODS

### Collection of Samples

Thirty four high vaginal swabs were collected from women suffering from high vaginal infections who were attending to Fattima-AL-Zahra hospital for pediatric and obstetric in Baghdad during a period extended for three months (from October to December, 2017).

### Isolation and identification of microorganisms

All high vaginal swabs were inoculated on culture media (MacConkey agar, Sabouraud agar, Blood agar and Chocolate agar plates) (Hi media /India), and incubated for 24 h at 37ºC *Candida albicans*, *Escherichia coli*, *Enterococcus faecalis* and *Staphylococcus saprophyticus* isolates were isolated from infected women with high vaginal infection during a period extended for three months. All bacterial and fungal isolates under study were identified in the laboratories of the hospital mentioned above depending on the morphological characteristic of the colonies color on medium. Then the isolates were identified at species level by using VITEK 2 system (Bio-Merieux, France), according to the manufacturer's instructions.

### Antimicrobial susceptibility test

Kirby-Bauer method was done as described by[7] to carry out the antimicrobial susceptibility test for different antimicrobial as show in table-1.

Microbial suspension prepared by picked (4-5) isolated colonies of bacterial isolates from the original culture and introduced into a test tube containing 4 ml of normal saline to produce a microbial suspension of moderate turbidity which compared with the standard turbidity solution (McFarland standard no. 0.5) this approximately equals to $1.5 \times 10^8$ CFU/ml.

**Table. 1: Antimicrobial discs used in this work.**

| Antimicrobial discs | Code | Disc potency (µg/disc) | Manufacturing Company/ Origin |
|---|---|---|---|
| Ampicillin | AMP | 10 | Bioanalyse/ Turkey |
| Amikacin | AK | 30 | Bioanalyse/ Turkey |
| Amoxicillin-Clavulanic acid | AMC | 20/10 | Bioanalyse/ Turkey |
| Cefazolin | CZ | 30 | Bioanalyse/ Turkey |
| Cefotaxime | CTX | 30 | Bioanalyse/ Turkey |
| Ceftazidime | CAZ | 30 | Bioanalyse/ Turkey |
| Chloramphinical | C | 30 | Bioanalyse/ Turkey |
| Ciprofloxacin | CIP | 5 | Bioanalyse/ Turkey |
| Clindamycin | CL | 2 | Bioanalyse/ Turkey |
| Erythromycin | E | 15 | Bioanalyse/ Turkey |
| Gentamicin | CN | 10 | Bioanalyse/ Turkey |
| Tetracycline | TE | 30 | Bioanalyse/ Turkey |
| Vancomycin | VA | 30 | Bioanalyse/ Turkey |

By a sterile cotton swab a portion of bacterial and fungal suspension was transferred and carefully and evenly spread on Mueller- Hinton agar medium, after that it was left for 10 min. Thereafter the antimicrobial discs were placed on the agar with a sterile forceps pressed firmly to ensure contact with the agar. Later than the plates were inverted and incubated at 37ºC for 18-24 hours. Inhibition zones developed around the discs were measured by millimeter (mm) using a metric ruler according to Clinical and Laboratory Standards Institute.[8]

### Preparation of hot watery plant extracts

Some plant species including: *Punica granatum* (peel), *Hibiscus sabdariffa* and *Annona squamosa* (peel and seed) (figure -1), were selected and obtained from Baghdad markets for study the antimicrobial activity of them on different microbial species isolated from vaginal infections.

Purchased dry each plant species were ground into powder in an electric blender. 10g of each plant powder was dissolved in 100ml of distilled water in a glass bottles, heated to 85ºC in a water bath and kept at this temperature with shaking for 8hr. After cooling, the liquid was filtered through the Whatman No. 1 filter paper.

The filtrates were then condensed and dried in smaller glass bottles at 37ºC for 48hr or more. Then, 1g of dried extracts was dissolved in 5mL of distilled water to obtain concentration l00mg/ml.[9] Different concentrations were prepared by diluted with distal water to obtain final concentrations (100, 50, 25 and 12.5 mg/ml).


**Figure. 1: Plants used in this study.**

**Determination of antimicrobial activity of plant extracts against fungal and bacterial isolates *in vitro***

Agar well diffusion method was employed to determine the antimicrobial activity of plant extracts as described by[10], in which isolates suspension of turbidity equals to 1.5x $10^8$ CFU/ml was prepared by picked (4-5) isolated colonies of bacterial and yeast isolates from the original culture and introduced into a test tube containing 4 ml of normal saline to produce a bacterial suspension of moderate turbidity which compared with the standard turbidity solution (McFarland standard no. 0.5).

By a sterile cotton swab a portion of bacterial suspension was transferred and carefully and evenly spread on Mueller- Hinton agar medium, after that it was left for 10 min.

A well of 6mm diameter was made using a sterile Cork borer. Separately, 100µl of each plant extract dilution was placed in 6mm diameter well. Also in one 6mm diameter well, 100µl of D.W. was placed as a negative control for antibacterial activity of plant extract then the plates were incubated at 37ºC for 18-24 hours. The zone diameter of inhibition has been measured by millimeter (mm) using a metric ruler.

**RESULTS**

**Isolation and Identification of microorganisms**

From 34 high vaginal swabs were taken ferom women suffering from high vaginal infections who were attending to the clinic of patients in Fattima-AL-Zahra hospital for pediatric and obstetric in Baghdad during a period extended for three months (from October to December, 2017), 23 (67.65%) women with high vaginal infections (growth of pathogenic microorganisms) and 11 (32.35%) women without high vaginal infections (growth of normal flora).

Also from those 23 women with high vaginal infections, 2 women were with mix growth of microorganisms, so the numbers of isolates collected during this study were 25 isolates. Out of 25 isolates, 16 (64%) were *Candida albicans* isolates, 4 (16%) were *Escherichia coli* isolates, 3 (12%) were *Enterococcus faecalis* isolates and 2 (8%) were *Staphylococcus saprophyticus* isolates as in figure -2.

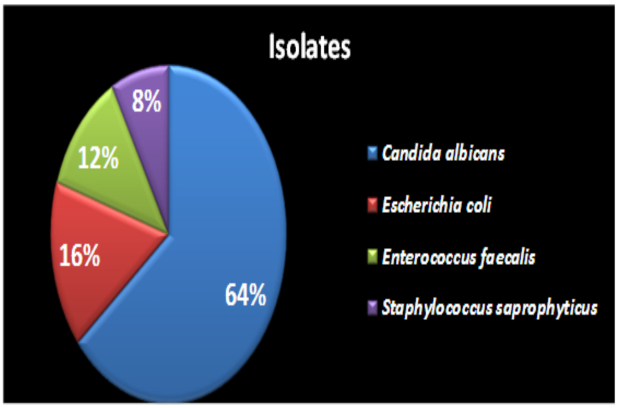


**Figure. 2: Percentage of pathogenic isolates isolated from high vaginal infections.**

According to Figure -3, out of 25 isolates, 16(64%) isolates were fungal isolates while 9 (36%) were bacterial isolates. During this study we noticed that the majority of isolates were fungal isolates (yeasts).

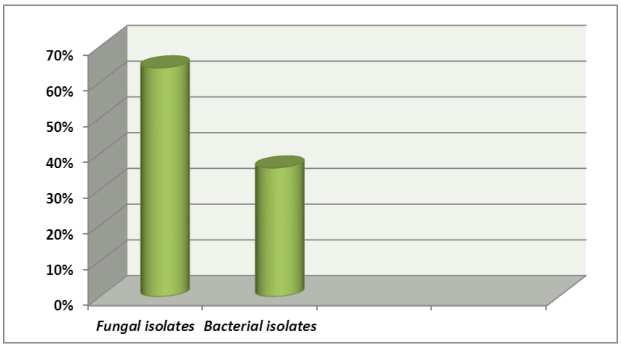


**Figure -3: Percentage of fungal isolates and bacterial isolates isolated during the study**

On the other hand out of 9 bacterial isolates, 4 (44.44%) isolates were Gram negative bacteria (G-ve) while 5 (55.56%) isolates were belong to Gram positive bacteria (G+ve) as shown in figure -4.

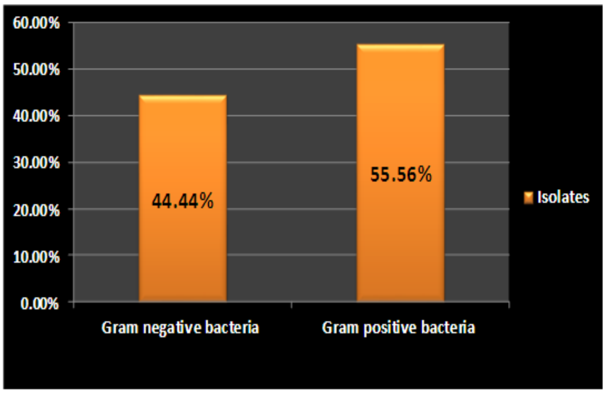


**Figure. 4: Percentage of Gram negative bacteria and Gram positive bacteria isolated during study**

**Antibiotics Susceptibility test**

Susceptibility of all bacterial isolates under study to different antibiotics was investigated.

The antibiotic susceptibility of four *Escherichia coli* isolates under study toward different antibiotics was showed in table -2. From this table, we found that *Escherichia coli* isolates shows a high level resistance to most of the antibiotics under test and the highest resistant antibiotics were Ampicillin and Cefazolin which gave 4(100%) resistance.

On the other hand the most effective antibiotic toward *Escherichia coli* isolates under the test was Amikacin which it gave the lowest resistance rate 0(0%). Isolates showing intermediate levels of susceptibility were classified as resistant.[8]

The bacterial isolates were revealed different degrees of resistance towards remaining antibiotics under study.

**Table. 2: Antibiotic resistance of 4 *Escherichia coli* isolates.**

| Isolate No. | AMP | CIP | AK | CN | AMC | CZ | CTX | CAZ | C |
|---|---|---|---|---|---|---|---|---|---|
| E1 | R | S | S | R | S | R | I | S | S |
| E2 | R | S | S | R | R | R | R | R | R |
| E3 | R | S | S | S | R | R | R | I | S |
| E4 | R | I | S | R | R | R | S | R | I |

***E= Escherichia coli* isolates**
**S= Sensitive I= Intermediate R= Resistant**
**AMP= Ampicillin, CIP=Ciprofloxacin, AK= Amikacin, CN= Gentamicin, AMC= Amoxicillin-Clavulanic acid, CZ= Cefazolin, CTX= Cefotaxime, CAZ= Ceftazidime and C= Chloramphinical**

The antibiotic susceptibility test also done for Gram positive bacteria (3 *Enterococcus faecalis* isolates and 2 *Staphylococcus saprophyticus* isolates) under the study toward different antibiotics and the results showed in table-3. From this table, we found that all *Enterococcus faecalis* and *Staphylococcus saprophyticus* isolates showed different resistance level to all the antibiotics under test and the highest resistant antibiotics were Ampicillin and Erythromycin which gave 5(100%) resistance rate. On the other hand the most effective antibiotic toward all Gram positive bacteria isolates under test was Vancomycin which it gave the lowest resistance rate 0(0%). Isolates showing intermediate levels of susceptibility were classified as resistant (34). The bacterial isolates were revealed different degrees of resistance towards remaining antibiotics under study.

**Table -3: Antibiotic resistance of 5 Gram positive bacteria isolates**

| Isolate No. | AMP | CAZ | CL | E | CN | AK | TE | CTX | VA |
|---|---|---|---|---|---|---|---|---|---|
| Ent.1 | R | R | S | R | I | S | R | R | S |
| Ent.2 | R | R | R | R | S | R | S | R | S |
| Ent.3 | R | I | R | R | I | S | S | S | S |
| St.1 | R | R | I | I | S | R | R | R | S |
| St.2 | R | S | R | R | S | I | S | I | S |

**Ent.= *Enterococcus faecalis* isolates**
**St.= *Staphylococcus saprophyticus* isolates**
**S= Sensitive I= Intermediate R= Resistant**
**AMP= Ampicillin, AK= Amikacin, CN= Gentamicin, CTX= Cefotaxime, CAZ= Ceftazidime, CL = Clindamycin, E = Erythromycin, TE = Tetracycline, and VA = Vancomycin.**

**Preparation of hot watery plat extracts**

*Punica granatum* (peel), *Hibiscus sabdariffa* and *Annona squamosa* (peel and seed) extracts were prepared (figure -5) as previously mentioned above.

All plat extracts left to dry and then different concentrations were prepared by diluted with distal water to obtain a final concentrations (100, 50, 25 and 12. 5 mg/ml).

After that all extracts were used for determine the antimicrobial activity against bacterial and fungal isolates from high vaginal infections.

**Figure. 5: Preparation of hot watery plat extracts.**

**Determination of antimicrobial activity of plant extracts against fungal and bacterial isolates *in vitro***

In this test, we chose three *Candida albicans* isolates and also we chose three of the most resistant bacterial isolates by antibiotics susceptibility test in this study (1 isolates of *Escherichia coli*, 1 isolates of *Enterococcus faecalis* and 1 isolates of *Staphylococcus saprophyticus*).

Antimicrobial activity test of *Punica granatum* (peel), *Hibiscus sabdariffa* and *Annona squamosa* (peel and seed) extracts against fungal and bacterial isolates was done in different concentrations (100, 50, 25 and 12.5 mg/ml) for each plant extract alone by using agar well diffusion method.

The result of antimicrobial activity test of *Punica granatum* extract showed that the *Punica granatum* extract was the most effective extract on all isolates in all concentrations compared with the other plant extracts under study. *Punica granatum* extract gave antimicrobial activity against all fungal (*Candida albicans* ) and bacterial isolates under test (G-ve and G+ve bacteria) in all concentrations (100, 50, 25 and 12.5 mg/ml concentrations) as shown in figure -6 and table -4.

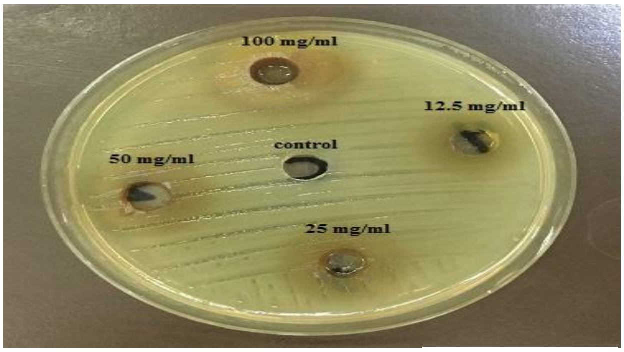


**Figure. 6: Antimicrobial activity of *Punica granatum* (peel)extract (100, 50, 25 and 12.5 mg/ml) and negative control (D.W.) on isolate from high vaginal infection.**

**Table. 4: Zone diameter of inhibition in millimeter (mm) of *Punica granatum* (peel) extract on fungal and bacterial isolates.**

| Isolate No. | Extract Concentrations ( mg/ml) | | | |
|---|---|---|---|---|
| | **100** | **50** | **25** | **12.5** |
| *Candida albicans* (1) | 30 | 28 | 25 | 25 |
| *Candida albicans* (2) | 31 | 30 | 26 | 22 |
| *Candida albicans* (3) | 29 | 22 | 20 | 20 |
| *Escherichia coli* (1) | 29 | 25 | 22 | 20 |
| *Enterococcus faecalis* (1) | 28 | 26 | 26 | 23 |
| *Staphylococcus saprophyticus* (1) | 30 | 25 | 22 | 22 |

On other hand the result of antimicrobial activity test of *Hibiscus sabdariffa* extract showed that the *Hibiscus sabdariffa* extract was effective on fungal and bacterial isolates only in 100, 50 and 25 mg/ml concentrations and not gave antimicrobial activity in last concentration (12.5 mg/ml) (table -5). It gave antibacterial activity against fungal and bacterial isolates under test (G-ve and G+ve bacteria) but in less degree than *Punica granatum* as shown in figure -7.

**Table. 5: Zone diameter of inhibition in millimeter (mm) of *Hibiscus sabdariffa* extract.**

| Isolate No. | Extract Concentrations ( mg/ml) | | | |
|---|---|---|---|---|
| | **100** | **50** | **25** | **12.5** |
| *Candida albicans* (1) | 20 | 15 | 10 | R |
| *Candida albicans* (2) | 21 | 16 | 13 | R |
| *Candida albicans* (3) | 21 | 14 | 12 | R |
| *Escherichia coli* (1) | 19 | 15 | 11 | R |
| *Enterococcus faecalis* (1) | 20 | 15 | 10 | R |
| *Staphylococcus saprophyticus* (1) | 19 | 14 | 10 | R |

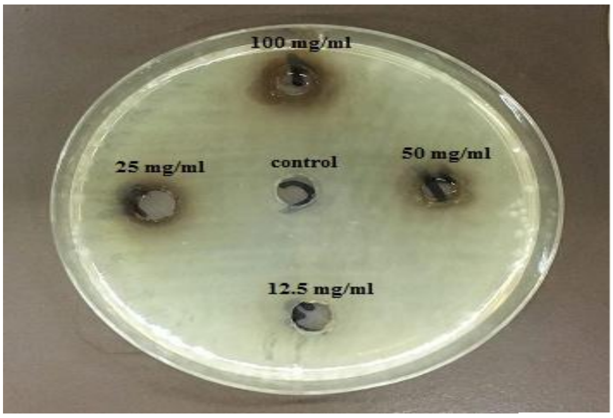


**Figure -7: Antimicrobial activity of *Hibiscus sabdariffa* extract (100, 50, 25 and 12.5 mg/ml) and negative control (D.W.) on isolate from high vaginal infection.**

The result of antimicrobial activity test of *Annona squamosa* (peel and seed) extracts showed there was no antimicrobial activity on fungal and bacterial isolates under study as shown in table -6.

**Table. 6: Zone diameter of inhibition in millimeter (mm) of *Annona squamosa* (peel and seed) extracts.**

| Isolate No. | Extract Concentrations ( mg/ml) | | | |
|---|---|---|---|---|
| | 100 | 50 | 25 | 12.5 |
| *Candida albicans* (1) | R | R | R | R |
| *Candida albicans* (2) | R | R | R | R |
| *Candida albicans* (3) | R | R | R | R |
| *Escherichia coli* (1) | R | R | R | R |
| *Enterococcus faecalis* (1) | R | R | R | R |
| *Staphylococcus saprophyticus* (1) | R | R | R | R |

*Annona squamosa* (peel and seed) extracts don't gave antimicrobial activity against all fungal and bacterial isolates under test (G-ve and G+ve bacteria) in all concentrations (100, 50, 25 and 12.5 mg/ml concentrations) as shown in figure -8.

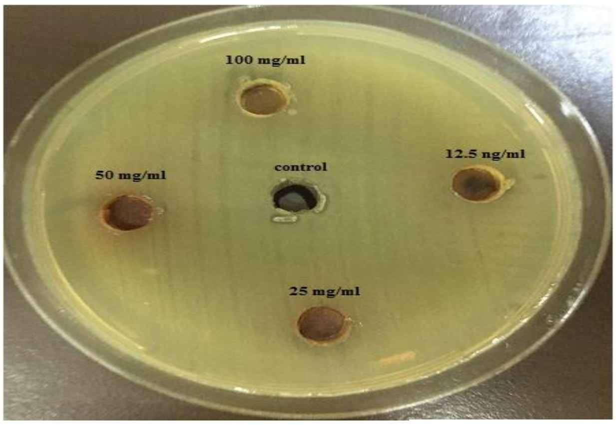


**Figure. 8: Antimicrobial activity of *Annona squamosa* (peel and seed) (100, 50, 25 and 12.5 mg/ml) and negative control (D.W.) on isolate from high vaginal infection.**

## DISCUSSION

Due to the increase of resistance to antibiotics, there is a pressing need to develop new and innovative antimicrobial agents. Among the potential sources of new agents, plants have long been investigated. Because, they contain many bioactive compounds that can be of interest in therapeutic. Because of their low toxicity, there is a long tradition of using dietary plants in the treatment of infectious disease in Cameroonian folk medicine.[11] Plants used in traditional medicine contain a wide range of ingredients that can be used to treat chronic as well as infectious diseases.[12]

Many of these plants have been known to synthesize active secondary metabolites such as phenolic compound found in essential oils with established potent insecticidal and antimicrobial activities, which indeed has formed the basis for their applications in some pharmaceuticals, alternative medicines and natural therapies.[13]

There are many published reports on the effectiveness of traditional herbs against Gram-positive and Gram-negative microorganisms, and as a result plants are still recognized as the bedrock for modern medicine to treat infectious diseases.[14]

Plants and other natural sources can provide a huge range of complex and structurally diverse compounds. Recently, many researchers have focused on the investigation of plant extracts and essential oils as potential antimicrobial agents. Discovery of new antibiotics is an exclusively important objective. Natural products are still one of the major sources of new drug molecules today. Microbial and plant products occupy the major part of the antimicrobial compounds discovered until now.[15] Our study showed that the *Punica granatum* extract was the most effective extract on fungal and bacterial isolates in all concentrations compared with the other plant extracts under study and *Hibiscus sabdariffa* extract was effective on fungal and bacterial isolates only in 100, 50 and 25 mg/ml concentrations.

A study done by Choi *et al*.,[16], showed the antimicrobial activity of *P. granatum* peel extract against *Salmonella*. In another study the results showed good antibacterial effect of pomegranate juice of *Punica granatum* against both Gram negative and Gram positive bacteria, diameter of inhibition zones were significantly higher in pomegranate juice as compared to standard antibiotic disc.[17]

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