Antimicrobial activity and bio-active compounds analysis in ethanolic plant extract of *Punica granatum* (Pomegranate) using GC-MS

**ABSTRACT:**
Medicinal plants are considered a bountiful origin of antimicrobial compounds. *Punica granatum* commonly known as pomegranate has developed as a medicinal plant with a possibility of antimicrobial activity. So, this study aimed to First: - Extract the antimicrobial components from pomegranate peel by 95% ethanol and then determine their in vitro effects against some clinical pathogenic bacteria and fungi. The clinical isolates were obtained from the National Research Centre in Dokki, and Intensive Care Units (Ismailia), Suez Canal University Hospital. Second: - Study the chemical composition of the peel extract by GC-MS chromatogram (Phytochemical analysis). By using the diffusion agar method, *Punica granatum* ethanolic extract used with different concentrations (10, 20, 30, and 40) mg/well against the studied pathogenic bacteria and fungi. These different concentrations were highly active against pathogenic bacteria like *Staphylococcus aureus* ATCC29213 and *Klebsiella pneumonia* ATCC13883. However, not active against the pathogenic fungi *Aspergillus niger*, *Candida albicans* ATCC10231, and *Candida pelliculosa* MH248066. Different concentrations of *Punica granatum* ethanolic extract (200, 400, 600, and 800) mg/well gave marked inhibition against all the tested fungal species. The zone of inhibition was compared with different standard antimicrobial agents as streptomycin and rifampin for bacteria, Amphotericin B and fluconazole for fungi as a positive control. Forty bioactive phytochemical compounds were identified in the Ethanolic extract of *Punica granatum* by GC-MS method based on the peak area, molecular weight and retention time. Among forty compounds identified, only fourteen were reported to have biological activities. The present results indicate that *Punica granatum* contains various bioactive components.

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**INTRODUCTION:**
Pomegranate (*Punica granatum*) was examined as one of the oldest trees in the world. known to treat many diseases like Diabetes, Inflammation, Ischemia, Cardiac disease, Aids, and Cancer.  

It contains polyphenols and anthocyanidins thus became a potent free-radical scavenger and more effective against disease than are those in green tea (Kaur *et al.*, 2006; Ghaidaa *et al.*, 2016).

*In vitro* and *in vivo* biological activity against bacteria and fungi were done in the Pomegranate (*Punica granatum*) extract and showed that it had an antimicrobial effect, antioxidant, and anti-cancer (Lansky and Robert, 2007; Ponnusamy *et al.*, 2010; Endo *et al.*, 2010; Saad *et al.*, 2010; Nuamsetti *et al.*, 2012).

As a result of increasing resistance of some pathogenic microorganisms to the synthetic drugs, it was necessary to find new natural components have potent and low toxic effects (Akerele, 1988; Pai *et al.*, 2004; Papadopoulou *et al.*, 2005; Seneviratne *et al.*, 2008; Viuda-Martos *et al.*, 2010). Some fungi as *C. albicans* and non *C. albicans* species live as normal micro-flora in many human organs especially in the oral cavity and vagina, but in some cases like diabetes...
mellitus (DM), antibiotic therapy, patients chemotherapy, and HIV patients, transplanted patients can cause life-threatening disease as an opportunistic flora as oral Candidiasis (Lanchares and Hernández, 2000; Citak et al., 2005).

Synthetic drugs have low potency, toxic effects, and a lot of side effects besides that a lot of microorganisms as Candida albicans become more resistant to it (Devkatte et al., 2005). So, it is essential to use various plant extracts as an anti-Candida. Several studies on different plant extract showed that they had an antimicrobial effect against some pathogenic fungi. And had anti-inflammatory properties (Saikia et al., 2001; Taweechaisupapong et al., 2005; Pawar and Thaker, 2006; Chaeib et al., 2007).

It was very important to separate the mixture in a plant extract to its components to be able to know the function of each one, known as phytoconstituents analyses.

This type of analysis can be done by Gas chromatography (GC), which used in many branches of science and technology (Proestos et al., 2006; Priya et al., 2011; Nehad and Abdulrahman, 2015).

The objectives of the present study were to assess the antimicrobial activity of the Ethanolic extract of Punica granatum (Pomegranate) peel against some pathogenic bacteria and fungi then Study the chemical composition of the peel extract by GC –MS chromatogram (Phytochemical analysis).

MATERIAL AND METHODS:

Collection and preparation of plant extract:

Pomegranate fruits were attained from local markets in Ismailia. Collected peels were dried at room temperature for six days after completely dried, the peels powdered by using clean pestle and mortar, and then sieving it to obtain a very small fine powder.

About fifty grams of blended peels were placed in the 250 ml flask, flooded in 120 ml of solvent (95% ethanol) separately and then left them for 144 hours so that all the components that found will get dissolved, then the extract was filtered. The residue was removed, and then the filtrate leaves to evaporate. Finally, the resulted extract kept at 4°C for further processing (Al-Tameme et al., 2015a; Idan et al., 2015). For antimicrobial activity and GC-MS analysis. (Alaa, 2018).

Bacterial and fungal isolates:

Two isolates of bacteria Staphylococcus aureus ATCC29213 and Klebsiella pneumonia ATCC13883 and three isolates of fungi Aspergillus niger, Candida albicans ATCC10231, and Candida pelliculosa MH248066 were subjected to ethanolic pomegranate extracts, all the isolates treated with except Candida pelliculosa MH248066 have been obtained from National Research Centre in Dokki, but Candida pelliculosa MH248066 was isolated from urine samples, which obtained from Intensive Care Units (Ismailla), Suez Canal University Hospitals, and then identified by routine conventional methods and by molecular sequencing.

Determination of Antimicrobial Activity of Punica granatum (Pomegranate) Peel:

The test pathogens (Staphylococcus aureus ATCC29213, Klebsiella pneumonia ATCC13883, were cultured in nutrient agar media, but Aspergillus niger, Candida albicans ATCC10231, and Candida pelliculosa MH248066 were cultured in sabouraud dextrose agar plates. wells were formed in the agar surface about 0.5 cm in diameter, then the different concentrations (10, 20, 30, and 40) mg/well of the plant extract were added against the studied pathogenic bacteria, (200, 400, 600, and 800) mg/well of the plant extract were added against the studied pathogenic fungi. The tests were carried out in triplicate. The plates of bacteria and yeast fungi were incubated at 37°C for 24 hrs and for 72 hrs for filamentous fungi. After the incubation periods, all the plates were examined for determination of the antimicrobial activity (Hameed et al., 2014; Jasim et al., 2015). The antimicrobial activity was measured as inhibition zone diameter formed around the inoculated well.

Ethanol used as a negative control. Amphotericin B and Fluconazole were used as fungi positive control; However, Streptomycin and Rifampin were used for bacteria. Phytochemical Analysis of Punica granatum (Pomegranate) peel extract was done using GC-MS according to Pongpuntaruk (2010). Hameed et al. (2015), and Al-Tameme et al. (2015b).

Gas chromatography-mass spectrometry analysis:

Instrumentation and Chromatographic conditions:

GC/MS System: Thermo Scientific Trace 1310 Gas Chromatograph attached with ISQ LT single quadrupole Mass Spectrometer.

Column: DB5-MS, 30 m; 0.25 mm ID (J&W Scientific).

Ionization mode: EI.

Ionization voltage: 70ev.

Temperature program: 40°C (3 min) - 280°C (5 min) at 5°C/min. - 290°C (1 min) at 7.5°C/min.

Detector temperature: 300°C

Carrier gas: Helium; Flow rate 1 ml/min.
RESULTS AND DISCUSSION:

Determination of antimicrobial activity of plant extract:

The emergence of drug resistance in some patients who complain from immune defect, drug side effects, high cost of therapy, leads to a search for new components that have low side effects, more effective against pathogenic microbes (Unnisa et al., 2012).

Because Punica granatum has reported in some studies as a source of active antimicrobial components against pathogenic bacteria (Braga et al., 2005; Al-Zoreky, 2009) and little studies were done against pathogenic fungi.

So, in this study, Punica granatum peel ethanolic extract was used against some pathogenic bacteria and fungi. The alcoholic extracts of the tested plant with different concentrations (10, 20, 30, and 40) mg/well had different inhibitory effects against Staphylococcus aureus ATCC29213 and Klebsiella pneumonia ATCC13883. The zone of inhibition was measured by (mm) as shown in Table 1. However, the same concentrations had zero effects on the three tested fungal species. However, the different concentrations (200,400,600,800) mg/well gave different inhibitory effects against Aspergillus niger, Candida albicans ATCC10231, and Candida pelliculosa MH248066. The zone of inhibition was measured by (mm) as shown in Table 2.

Table 1. Antibacterial activity of Punica granatum peel extract (mediated by inhibition zone (mm) against clinical bacterial isolates at different concentrations after 24 hrs of incubation at 37°C

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Streptomycin (10 mg/ well)</th>
<th>Rifampin</th>
<th>Punica granatum extract Different concentrations /well</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 mg</td>
<td>20 mg</td>
<td>30 mg</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>2.11 ± 0.390</td>
<td>1.00 ± 0.110</td>
<td>4.20 ± 0.066</td>
</tr>
<tr>
<td>Klebsiella pneumonia</td>
<td>1.83 ± 0.033</td>
<td>0.83 ± 0.033</td>
<td>4.30 ± 0.011</td>
</tr>
</tbody>
</table>

Table 2. Antifungal activity of Punica granatum peel extract (mediated by inhibition zone (mm) against clinical fungal isolates at different concentrations after 24 hrs for Candida and 72 hrs for filamentous fungi of incubation at 37°C.

<table>
<thead>
<tr>
<th>Fungal isolates</th>
<th>Amphotericin B (200 mg/ well)</th>
<th>Fluconazole</th>
<th>Punica granatum extract Different concentrations /well</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>200 mg</td>
<td>400 mg</td>
<td>600 mg</td>
</tr>
<tr>
<td>Aspergillus niger</td>
<td>1.91 ± 0.180</td>
<td>3.99 ± 0.211</td>
<td>2.8 ± 0.166</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>2.4 ± 0.033</td>
<td>1.8 ± 0.033</td>
<td>3.1 ± 0.088</td>
</tr>
<tr>
<td>Candida pelliculosa</td>
<td>2.4 ± 0.066</td>
<td>1.4 ± 0.033</td>
<td>4.6 ± 0.033</td>
</tr>
</tbody>
</table>

The zone of inhibition was compared with different standard antibiotics as Streptomycin and Rifampin for bacteria, Amphotericin B and Fluconazole for fungi. These results came in agreement with those previously published for example (Melendez and Capriles, 2006) who reported that extracts from pomegranate fruits possess in vitro antibacterial activity against many tested bacteria.

Also, Unnisa et al. (2012) reported that pomegranate fruits ethanolic and aqueous extracts showed high antimicrobial activity when used against S. aureus. Also, it was reported that pomegranate methanolic and ethanolic extracts had antimicrobial activity against S. aureus (Malviya et al., 2014).

Naziri et al. (2012) reported that peel extracts of sour and sweet pomegranate had an inhibition effect against S. aureus. Also, Nozohour et al. (2018) reported that seed and peel extracts of pomegranate showed antibacterial activities against S. aureus and P. aeruginosa.

From the previous work, it was recorded that pomegranate peel extract displayed a strong antifungal activity against C. albicans, which was compared with nystatin as a standard antifungal. The present study agrees with those done by other investigators on the potency of the pomegranate peel extract in inhibiting Candida albicans and Candida pelliculosa growth (Abu-Etteen and Abu-Alteen, 1998; Endo et al., 2010; Anibal et al., 2013; Bassiri et al., 2015).

GC–MS analysis:

Several phytoconstituents analyses have been carried out in many parts of the world by GC–MS technique (Priya et al., 2011).

In the present study, GC–MS analysis of Punica granatum peel ethanolic extract showed the presence of forty major peaks and the components corresponding to the peaks...
were determined. Among these compounds, some have unknown functions, and fourteen biological activities as shown in Table 3.

Table 3. Major phytochemical compounds identified in peel ethanolic extract of *Punica granatum* by GC-MS chromatogram

<table>
<thead>
<tr>
<th>N.</th>
<th>Phytochemical compound</th>
<th>RT mint</th>
<th>Formula</th>
<th>MOL WT</th>
<th>Pharmacological action</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ethanol</td>
<td>11.07</td>
<td>C2H6O</td>
<td>46</td>
<td>Has bactericidal activity and is used often as a topical disinfectant. It is widely used as a solvent and preservative in pharmaceuticals.</td>
</tr>
<tr>
<td>2</td>
<td>4,6-di-tertbutyl-m-cresol</td>
<td>11.07</td>
<td>C15H240</td>
<td>220</td>
<td>Antioxidants, and Anti-inflammatory agents</td>
</tr>
<tr>
<td>3</td>
<td>3-butyl-4nitro-pent4-enolic acid, methyl ester</td>
<td>11.07</td>
<td>C10H17NO4</td>
<td>215</td>
<td>Anticarcinogenic</td>
</tr>
<tr>
<td>4</td>
<td>Dimethylamine</td>
<td>11.14</td>
<td>C2H6DN</td>
<td>45</td>
<td>It is used in veterinary pharmaceuticals as Anthelmintic</td>
</tr>
<tr>
<td>5</td>
<td>α-Tocopheryl acetate</td>
<td>33.03</td>
<td>C14H52O3</td>
<td>472</td>
<td>Anti-inflammatory</td>
</tr>
<tr>
<td>6</td>
<td>Naphthalene, decahydro-1-pentadactyl</td>
<td>33.03</td>
<td>C25H48</td>
<td>165</td>
<td>Antioxidant activity and Antimicrobial activity</td>
</tr>
<tr>
<td>7</td>
<td>1,3-Cyclohexadiene, 5-[(1,5-dimethyl-4-hexenyl)-2methyl-5-[S-(R*,S*)]]</td>
<td>33.14</td>
<td>C15H24</td>
<td>204</td>
<td>Antioxidant, and Anti-inflammatory</td>
</tr>
<tr>
<td>8</td>
<td>5 Hydroxy methyl furfurals</td>
<td>33.53</td>
<td>C6H6O3</td>
<td>126</td>
<td>Antioxidant</td>
</tr>
<tr>
<td>9</td>
<td>Benzene,1- (1,5-dimethyl-4-hexenyl)-4-methy</td>
<td>35.07</td>
<td>C15H22</td>
<td>202</td>
<td>Anti-inflammatory and Antimicrobial</td>
</tr>
<tr>
<td>10</td>
<td>Butan-2-one, 4-(3-hydroxy-methoxyphenyl)-</td>
<td>37.29</td>
<td>C11H14O3</td>
<td>194</td>
<td>Anti-inflammatory Anti-diabetic</td>
</tr>
<tr>
<td>11</td>
<td>Propanoic acid, 2-(3-acetoxy-4,14,17trimethyla ndrost-8-en-17-yi)</td>
<td>49.16</td>
<td>C27H42O4</td>
<td>430</td>
<td>Antihyperglycemic, hypolipidemic and Antimicrobial</td>
</tr>
<tr>
<td>12</td>
<td>Lucenin 2</td>
<td>49.41</td>
<td>C27H30O16</td>
<td>610</td>
<td>Flavonoid possesses Antibacterial activity.</td>
</tr>
<tr>
<td>13</td>
<td>1h-purin-6-amine, <a href="methyl">2-fluorophenyl</a>(gas)</td>
<td>51.91</td>
<td>C12H10FN5</td>
<td>243</td>
<td>Have Antimicrobial activity. Anti-inflammatory, Antitumor, Anti-ulcer</td>
</tr>
<tr>
<td>14</td>
<td>Dotriacontane (CAS)</td>
<td>57.62</td>
<td>C32H66</td>
<td>450</td>
<td>Have Antimicrobial activity, antioxidant, antispasmodic</td>
</tr>
</tbody>
</table>

The identification of the resultant photochemical compounds was proved based on the concentration (peak area %), retention time and molecular formula, as shown in figure 1.

Fig. 1. GC-MS chromatogram of peel ethanolic extract of *Punica granatum*.

Among these compounds, some have unknown function, and some reported to have some biological activities as Ethanol has a bactericidal activity and is used often as a topical disinfectant, these results were proven by many recherche. Ethyl alcohol is normally employed as disinfectants due to their high germicidal activity (Spaulding, 1964). It's not active against bacterial spores, but they have fast bactericidal and bacteriostatic activity against vegetative forms of bacteria. Protein denaturation is the most likely cause of the alcohol action.
Naphthalene, decahydro-1-pentadactyl have antioxidant and antimicrobial activity, Benzene, 1- (1,5-dimethyl-4-hexenyl)-4-methy have Anti-inflammatory and Antimicrobial and Propanoic acid, 2-(3-acetoxy-4,4,14-trimethylndrost-8-en-17-yl) have Antimicrobial as reported by Shareef et al. (2016). Dotriacontane (CAS) has antimicrobial, antioxidant, and antispasmodic activity as reported by Soosairaj and Dons (2016).

Also, Lucenin 2 was identified and considered as a flavonoid which possesses antibacterial activity (Basile et al., 1999). Most of the flavonoids have a role in the inhibition of bacterial respiration and reproduction (Abu Raihan, 2014). It also can overcome bacterial virulence in numerous in vitro studies. 4,6-di-tetbutyl-d cresol and 1,3-Cyclohexadiene, 5-(1,5-dimethyl-4-hexenyl)-2methyl-[S-(R*,S*)] used as antioxidants, and anti-inflammatory agents. 3-butyl-4nitro-pent4-enolic acid, methyl ester used as Anticarcinogenic, a Tocopheryl acetate used as anti-inflammatory agents and 1h-purin-6-amine, [(2-fluorophenyl)methyl] (gas) used as antimicrobial. P. granatum contains chemical constituents like ellagitannins, phenols, tannins, punicic acid, flavonoids, anthocyanins, estrogenic flavonoids, and flavones (Heber et al., 2006; Fateh et al., 2013).

This study proved that the pomegranate extract has more effective antimicrobial agent than the antibiotics currently in us.

CONCLUSION: -

Due to the toxic effects of the antifungal and antibacterial agents, their high side effects, and their low potency, combined with the increase of microbial resistance it is urgently to use therapeutic alternative to treat various infections caused by pathogenic microbes. By GC mass the phytochemical analysis of P. granatum peel extract contains chemical constitutions that may be useful as anti-inflammatory, antimicrobial, antidiabetic, antioxidant, and anticancer. These findings also support the traditional usage of the P. granatum and could be used as powerful antimicrobial agents for the prevention of many diseases. Therefore, further studies have been proposed to elucidate the possible action mechanisms involved and to find new bioactive compounds in P. granatum, cell toxicity assay for a better understanding of their safety and potency.

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