Evaluation of antimicrobial activity of Algerian Lemon (Citrus limon v. Eureka) peels and juice extracts

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ABSTRACT

This work explores underutilized peels and juice lemon (Citrus limon v. Eureka) extracts for their phenolic contents and in vitro antimicrobial activities. In this study, phenolics and flavonoids contents were found to be more present in the peel extract (30.10 ±2.98 mg of GAE/g) and (19.78 ±0.10 mg of QE/g) respectively compared to the juice extract (2.78 ±0.06 mg of GAE/g) and (0.13±0.001 mg of QE/g) respectively. Phytochemical study showed the presence of quercetin and gallic acid in both extracts as vanillin was identified only in the juice extract. The antimicrobial activity of peels and juice extracts was tested against six pathogen bacteria and one fungal strains using disk diffusion method. Results of this research indicated that peel extract presented an important activity on all tested strains, that gram-positive bacteria were more susceptible than gram-negative bacteria. The most susceptible gram-positive bacteria was Staphylococcus epidermedis with 32mm diameter of inhibition zone. Exceptionally Staphylococcus epidermedis that showed a higher zone of inhibition against juice extract (32mm) compared with its inhibition zone against peels extract (31mm). So, the peel extract had an inhibitory effect more than juice extract. It could be concluded that peels extract of this plant can be explored as an economically viable source of naturel antimicrobials which can be used as an alternative for antibiotics.

Keywords: Citrus limon, Phenolics, flavonoids, antibiotics, antimicrobial activity.

INTRODUCTION

Even though pharmacological industries have produced a number of new antibiotics in the last three decades, resistance to these drugs by microorganisms has increased. In general, bacteria have the genetic ability to transmit and acquire resistance to drugs, which are utilized as therapeutic agents [1]. For a long period of time, plants have been a valuable source of natural products for maintaining human health. Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant. These products are known by their active substances (the phenolic compounds) [2].

Lemon is an important medicinal plant of the family Rutaceae, which are having anticancer activities and the antimicrobial potential in crude extracts of different parts (leaves, peels, seeds and flower) [3]. Citrus fruits are mainly used by juice processing industries while the peels are generally wasted. During the processing of citrus fruit for juice, peels are the primary byproduct, the highest amount of flavonoids (a major group of citrus secondary metabolites) occurs in the peel which are very rare in other plants [4, 5, 6].

Since there is an increase in the number of antibiotic resistance pathogens, there is always a search of an alternative drug that is regarded as safe. Citrus peels if proved to have antimicrobial activity, they can also be used in some food industry which generates large peel wastes as a food preservative. The aim of this study was to evaluate the potential of plant extracts and phytochemicals on standard microorganism strains by using disk diffusion method. Herein we
have developed a comparative study between peels and juice crude extracts in order to understand which of them are preferable for antimicrobial activity.

**MATERIALS AND METHODS**

**Plant material**

*Citrus limon* v. Eureka peels and juice were collected locally from Chlef region in Algeria in 2013. They were identified by National Institute of Vegetal Protection. After drying in a shadow at room temperature, the peels were grinded into powdered form.

**Preparation of peels extract (PE)**

3g of the dried sample were weighed and extracted by stirring with 50 mL of methanol at 25°C at 150 rpm for 12 h and filtered through Whatman N°4 paper. The residue was then extracted with one additional 50 mL portion of the methanol. The extract was evaporated to dryness and redissolved in methanol at a concentration of 20 mg/mL, and stored at 4°C for further use [7]. The extraction yield was 14.97%.

**Preparation of juice extract (JE)**

One mL of citrus juice was extracted with 9 mL of 80% methanol for 30 min at room temperature. After centrifugation at 5000 rpm for 10 min, the supernatant was taken and filtered through a Whatman N°4 filter paper, evaporated under vacuum to dryness and stored at 4 °C until analyzed [8]. The extraction yield was 17%.

**Determination of total phenolic content**

Total phenolic contents (TP) were assayed using the Folin-Ciocalteu (FC) reagent, following the method which was described by [9]. 40 µl of properly diluted fruit extract solution were mixed with 1.8 ml of FC reagent. The reagent was pre-diluted, 10 times, with distilled water. After standing for 5 min at room temperature, 1.2 ml of (7.5% w/v) sodium carbonate solution were added. The final mixture was shaken and then incubated for 1h in the darkness at room temperature. The absorbance was measured spectrophotometrically at 765 nm. A calibration curve was prepared, using a standard solution of gallic acid (GA). Results were expressed as milligrams of GA equivalents per gram of plant powder. Samples were prepared in triplicate for each analysis, and the mean value of absorbance was obtained.

**Determination of total flavonoids content**

The flavonoids content in extracts was determined Spectrophotometrically using an aluminum chloride method involving the formation of flavonoid-aluminum complex having the absorptivity maximum at 430 nm [10]. 1 ml of diluted sample was separately mixed with 1 ml of 2% aluminum chloride methanolic solution. After incubation at room temperature for 15 min, the absorbance of the reaction mixture was measured at 430 nm. A calibration curve was prepared, using a standard solution of quercetin (QE). Results were expressed as milligrams of QE equivalents per gram of plant powder. Samples were prepared in triplicate for each analysis, and the mean value of absorbance was obtained.

**Phytochemical study**

A high performance liquid chromatography system (HPLC) was used to determine the contents of phenolic of peels and juice extracts. Chromatograph which was used is RP-HPLC-C18 reserved phase, equipped with following:

- Column (125 x 4.6 mm) packed closely by the apolar stationary phase (consisting of silica grafted by residue to C18);
- The mobile phase is constant composition: methanol/water (60:40 v/v) [11];
- A pumping system for moving the mobile phase with a high pressure (flow rate 1ml/min);
- The injector is used to introduce the sample into the system (Injection volume = 20µl);
- A UV detector at a wavelength of 254 nm;
- Finally, computer software used to view the signals recorded by the detector;
- Temperature setting at 25°C.

**Test Microorganisms and Preparation of standard culture inoculums of test organism**

Microorganisms used in the present study were obtained from the laboratory of microbiology (Antibiotic group – Medea, Algeria). Six species of bacteria were tested: *Escherichia coli* (ATCC 10536), *Pseudomonas aeruginosa* (ATCC 27853), *Sarcina lutea* (Pasteur Institute, Algeria), *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6633), *Staphylococcus epidermedis* (ATCC 12228) and one fungal strain: *Candidas albicans* (ATCC 10231).
Microbial strains tested were cultured in nutrient agar. After 18 hours of incubation at 37°C, microbial suspensions with an optical density of 0.5 McFarland (1.5 x 10^8 CFU/ml) were prepared for each microorganism in 10 ml of sterile distilled water [12].

**Antimicrobial activity assay using disk diffusion method**

The antimicrobial activity of peels and juice extracts was determined through the agar disk diffusion [13], briefly, Muller Hinton (MH) agar poured in sterilized petri dishes was cultured with a standardized inoculum (1.5 x 10^8 CFU/ml) of each bacterial strains while the standardized inoculum of fungal strain was cultured in Sabouroud agar. Then the filter paper disks (6mm in diameter) contain specific amount of extracts were placed onto the agar plates. Before incubation, all petri dishes were kept in refrigerator (4°C) for 2h and incubated after at 37°C for 24 h for bacteria growth and for 48h for fungal growth. The diameter of inhibition zones were measured in mm and the results were recorded. Inhibition zone ≥ 12mm were considered as good inhibitory effect of extract [14, 15]. The minimum inhibitory concentration (MIC) was determined.

**Determination of minimum inhibitory concentration (MIC)**

The CMI was determined only for the most active extracts recorded during the study in solid medium (including the inhibition diameters ≥ 12mm). This method allows the determination of the MIC from a range of extract concentrations in the culture medium. According to the method of [16]. Serial dilutions of geometric ratio 2 were made with Dimethylsulfoxide (DMSO) from the initial solution (final concentration of 10 % = 0.1 g/ml) of each extracts. 2ml of each dilution was incorporated into 38 ml of medium MH (bacteria) or Sabouroud (yeast), kept super cooled. The range of final concentrations thus obtained was 0.5 – 0.25 – 0.125 – 0.0625 – 0.0312 – 0.0156 – 0.0078 – 0.0039 – 0.0019 et 0.0010 %. After solidification, mediums (MH, Sabouroud) contain the extracts or not (control) were inoculated on the surface in deposits of 1µl of microbial suspension. Petri dishes were incubated at 37°C for 24h for bacteria and 6 days for fungal growth. The MIC was defined as the lowest concentration of extract for which no growth was visible compared to the control without extract.

**Statistical analysis**

The experimental results were expressed as mean SEM (standard error of the man). Data were assessed by ANOVA. Tukey's test was then applied using XL Stat Pro 7.5 software. A p value of<0.05 was considered to be statistically significant.

**RESULTS AND DISCUSSION**

Total phenolic content (TP) of the peels was 30.10 ±2.98 mg of GAE/g, while it was 2.78 ±0.06 mg of GAE/g for juice extract. Therefore, peels extracts had a higher polyphenol contents when compared with juice extract. Peels TP content of 87.77±1.42 mg of GAE/g and 158.79±0.72 mg of GAE/g were found by [7] and [17] respectively. Juice TP content of 8.43 ± 0.02 mg of GAE/g was found by [7].

Total flavonoid content (TF) levels in peels and juice extracts were 19.78±0.10 mg of QE/g and 0.13±0.001 mg of QE/g respectively. Peels extract had also the highest TF content than juice extract. [7] found the flavonoid levels to be 1.43 ± 0.07 mg of QE/g for juice extract, while [18] found a TF levels to be 11,9 ± 0.66 mg of QE/g for peels extract.

The correlations between TP and TF assays were 0.998 and 0.990 for peels and juice extract, respectively, which were highly significant at the 0.01 level. These results indicate that the flavonoids are an important phenolic group representing the antimicrobial capacity of peels and juice extracts.

**Phytochemical study**

Four pure phenolic compounds (gallic acid (GA), salicylic acid (Sal), vanillin (Van) and quercetin (QE)) were used in the HPLC analysis as controls. Their chromatograms are shown in Figure 1 and their retention time (Tr) in Table 1. The results of the RP-HPLC-C18 analysis extracts are shown in Figure 2. Some substances were identified in our extracts by comparison of the samples with those of the chromatograms of the pure substances (their Tr).

<table>
<thead>
<tr>
<th>Standards</th>
<th>Tr (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic acid</td>
<td>1.992</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>2.933</td>
</tr>
<tr>
<td>Vanillin</td>
<td>4.333</td>
</tr>
<tr>
<td>Quercetin</td>
<td>2.892</td>
</tr>
</tbody>
</table>

The results showed that both extracts (peels and juice extracts) contain quercetin and gallic acid. Quercetin is a flavonoid which is known by its antimicrobial activity that resides in the inhibition of expressing the DNA gyrase.
and synthesis of the enzymes and membrane proteins [19]. But the vanillin was only present in the juice extract. This component and gallic acid are also known by their antimicrobial effect [20, 21, 22].
Figure 1: Chromatograms of the standards used in the HPLC (A: GA; B: Van; C: QE; D: Sal)

Figure 2: Chromatograms of the HPLC of the both extracts (E: Peels extract; F: juice extract)

Antimicrobial activity
The antimicrobial activity of peels and juice extracts of *Citrus limon* v. Eureka were assayed against six positive and negative bacteria and a fungal strain by disk diffusion method and the results of inhibition zones have shown in Table 2.

Results of this research indicated that peels extract of this plant had inhibitory effect more than juice extract (Figure 3). Exceptionally *Staphylococcus epidermedis* that showed a higher zone of inhibition against juice extract (32mm) compared with its inhibition zone against peels extract (31mm) (Figure 4).
The peel extract presented an important activity on all tested strains, that gram-positive bacteria were more susceptible than gram-negative bacteria. The most susceptible gram-positive bacteria was *Staphylococcus epidermidis* bacteria responsible for the cutaneous, urinary and nasal infections with 31mm diameter of inhibition zone (Figure 4).

Despite the great resistance against antibiotics [23], *Pseudomonas aeruginosa* presented a high sensitivity with peels and juice extracts with 21mm and 20 mm diameter of inhibition zone respectively (Figure 4) and in the case of *Candidas albicans*, yeast responsible for opportunistic oral and genital infections in humans, the results were spectacular (diameter of 30mm) with the both extracts.

Susceptibility difference between gram-positive and gram-negative bacteria may be due to cell wall structural differences between these classes of bacteria. The gram-negative bacteria cell wall outer membrane appears to act as a barrier to many substances including antibiotics [24]. Optimal extract efficiency is not only due to main active compounds, but the combined action (synergy) of the various compounds at the origin of this extract [25]. For this, comparison individual case of the antimicrobial activity of these two extracts based on the determination of a single active compound seems unnecessary.

According to our results, the peels extract presented a good antimicrobial agent which is confirmed by the work of [2].

<table>
<thead>
<tr>
<th>Strains</th>
<th>Escherichia coli</th>
<th>Pseudomonas aeruginosa</th>
<th>Staphylococcus aureus</th>
<th>Bacillus subtilis</th>
<th>Staphylococcus epidermidis</th>
<th>Sarcina lutea</th>
<th>Candidas albicans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peels</td>
<td>20</td>
<td>21</td>
<td>22</td>
<td>20</td>
<td>31</td>
<td>21</td>
<td>30</td>
</tr>
<tr>
<td>Juice</td>
<td>15</td>
<td>20</td>
<td>12</td>
<td>17</td>
<td>32</td>
<td>20</td>
<td>30</td>
</tr>
</tbody>
</table>

Figure 3: Comparative sensitivity of peels and juice extracts of *Citrus limon* v. Eureka
We bring back in Table 3 the MIC of the both extracts (peels and juice extracts), whose diameters of inhibition are equal to or higher than 12 mm. [26] proposed a classification of extracts of plant material on the basis of the results of MIC, as followed:

- Strong inhibition: MIC less than 500µg/ml.
- Moderate inhibition: MIC ranges from 600 to 1500 µg/ml.
- Low inhibition: MIC greater than 1600 µg/ml.

Table 3: MIC (expressed in µg / ml) of the peels and juice extracts (whose diameters of the inhibition zones are ≥ 12 mm) on the bacteria tested

<table>
<thead>
<tr>
<th>Extract</th>
<th>Escherichia coli</th>
<th>Pseudomonas aeroginosa</th>
<th>Staphylococcus aureus</th>
<th>Bacillus subtilis</th>
<th>Staphylococcus epidermidis</th>
<th>Sarcina lutea</th>
<th>Candidas albicans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peels</td>
<td>5000</td>
<td>5000</td>
<td>5000</td>
<td>2500</td>
<td>2500</td>
<td>2500</td>
<td>2500</td>
</tr>
<tr>
<td>Juice</td>
<td>5000</td>
<td>1250</td>
<td>5000</td>
<td>2500</td>
<td>2500</td>
<td>2500</td>
<td>2500</td>
</tr>
</tbody>
</table>

Thus, according to this classification and according to the results of the MIC, it shows that the peels and juice extract had a broad antimicrobial spectrum with doses ranging from 1250µg/ml to 5000 µg/ml.

CONCLUSION

The phytochemical results showed that both extracts (peels and juice extracts) contain flavonoids, these active substances which can inhibit the growth of different types of bacteria citing *Staphylococcus aureus* [27] and *Escherichia coli* [19].

The results of this work suggest that peels extract of *Citrus limon* v. Eureka have a broad spectrum of antimicrobial activity as beneficial and positive as the effect of juice extract, which can be used as an alternative for antibiotics. Moreover, this peel extract should be investigated in vivo to better understand its safety, efficacy and properties.

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