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Evaluation of antimicrobial activity of Algerian Lemon(*Citrus limon*L.) peels and seeds extracts

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ABSTRACT

This work explores underutilized peels and seeds lemon (Citrus limon L) 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 seeds extract (14.51 ±1.22 mg of GAE/g) and (0.12±0.009 mg of QE/g) respectively. Phytochemical study showed the presence of quercetin in both extracts as gallic acid was identified only in the peels extract. The antimicrobial activity of peels and seeds 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 31mm diameter of inhibition zone. Exceptionally Staphylococcus epidermedis and Pseudomonas aeroginosa that showed zone of inhibition against seeds extract with diameter of 9mm, 11mm respectively. So, the peel extract had an inhibitory effect more than seeds 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 seeds crude extracts in order to understand which of them are preferable for antimicrobial activity.

MATERIALS AND METHODS

Plant material

*Citrus limon*L peels and seeds 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 and seeds 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 seeds extract(SE)

The seeds were washed and dried at ambient temperature in the darkness until used. The seeds were finely ground. 3 g of this ground material was extracted by stirring with 30 mL of pure methanol for 30 min. The extract was then kept for 24 h at 4 °C, filtered through a Whatman N° 4 filter paper to obtain a 25 ml final volume, evaporated under vacuum to dryness and stored at 4 °C until analyzed [8]**.** The extraction yield was 1.94%.

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 seeds extract. 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 systemfor movingthe 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 (Antibiotical group – Medea, Algeria). Six species of bacteria were tested: *Escherichia coli* (ATCC 10536), *Pseudomonas aeroginosa* (ATCC 27853), *Sarcinalutea* (Pasteur Institute, Algeria), *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6633), *Staphylococcus epidermedis* (ATCC 12228) and one fungal strain: *Candidasalbicans* (ATCC 10231).

Microbial strains tested were cultured in nutrient agar. After 18hours 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 seeds extracts was determined through the agar disk diffusion [13], briefly, Muller Hinton (MH) agar poured in sterilized petri dishes was culture with a standardized inoculums (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 Dimethylsulfoxyde (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 was14.51 ±1.22 mg of GAE/g for seeds extract. Therefore, peels extracts had a higher polyphenol contents when compared with seeds extract. Peels TP content of 87.77±1.42 mg of GAE/g and 158.79±0.72mg of GAE/g were found by [7] and [17]respectively. Seeds TP content of 98.23±0.84 mg of GAE/g was found by [17].

Total flavonoid content (TF) levels in peels and seeds extracts were 19.78±0.10 mg of QE/g and 0.12±0.009 mg of QE/g respectively. Peels extract had also the highest TF content than seeds extract.[17]found the flavonoid levels to be 19.95 ± 0.45mg of QE/g for seeds extract, while [18]found a TF levels to be 11,9 ± 0.66mg of QE/g for peels extract.

The correlations between TP and TF assays were 0.998 and 0.999 for peels and seeds 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 seeds extract.

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).

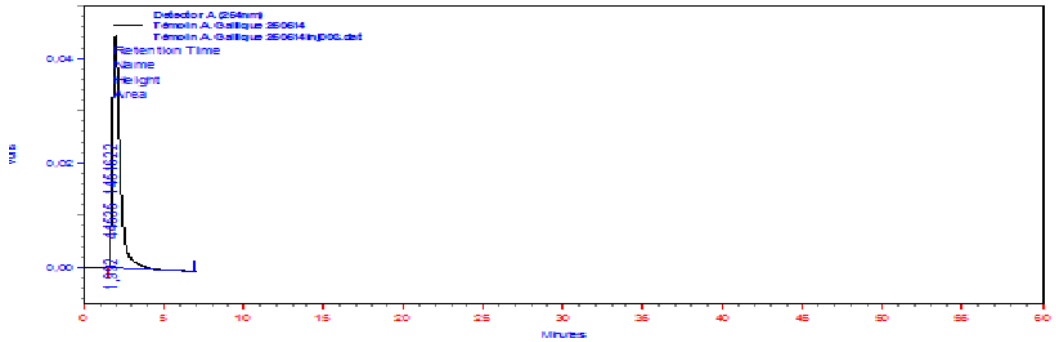
Table 1: Retention time of various phenolic controls obtained by HPLC separation

Standards	Tr (min)
Gallic acid	1.992
Salicylic acid	2,933
Vanillin	4,333
Quercetin	2,892

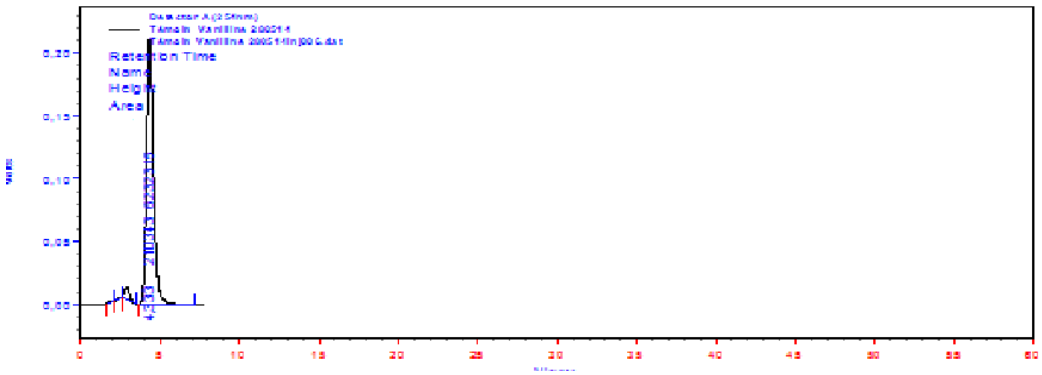
The results showed that both extracts (peels and seeds extract) contain quercetin, a flavonoid which is known by its antimicrobial activity that resides in the inhibition of expressing the DNA gyrase and synthesis of the enzymes and

membrane proteins [19], but the gallique acid was only present in the peels extract, this component is also known by its antimicrobial effect[20, 21].

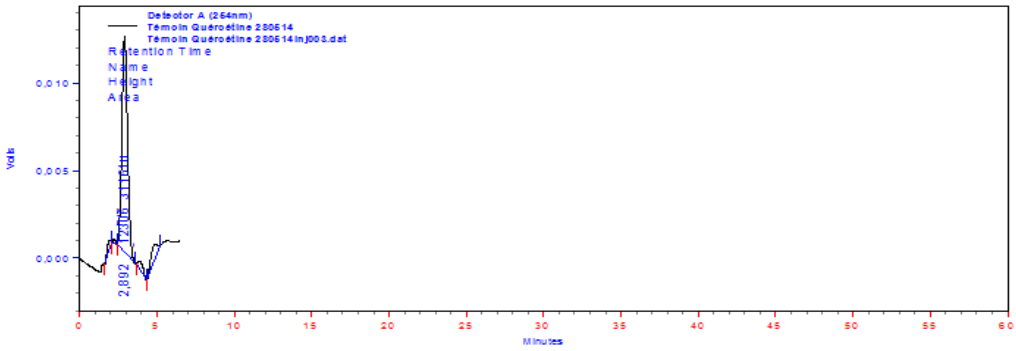
(A)



(B)



(C)



(D)

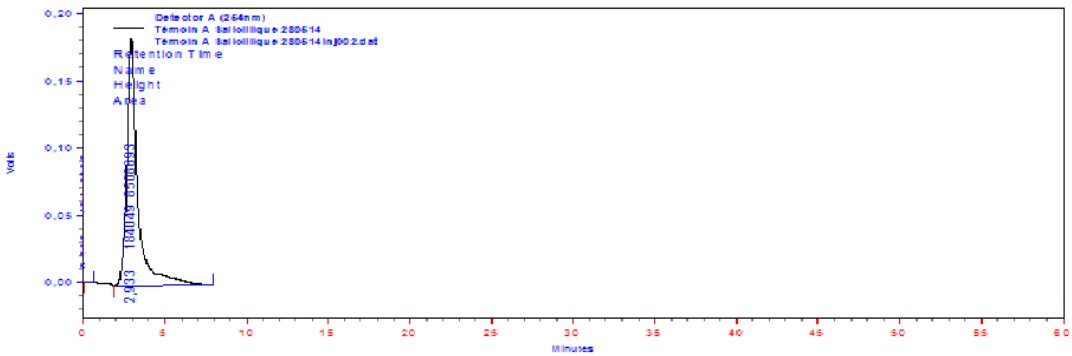
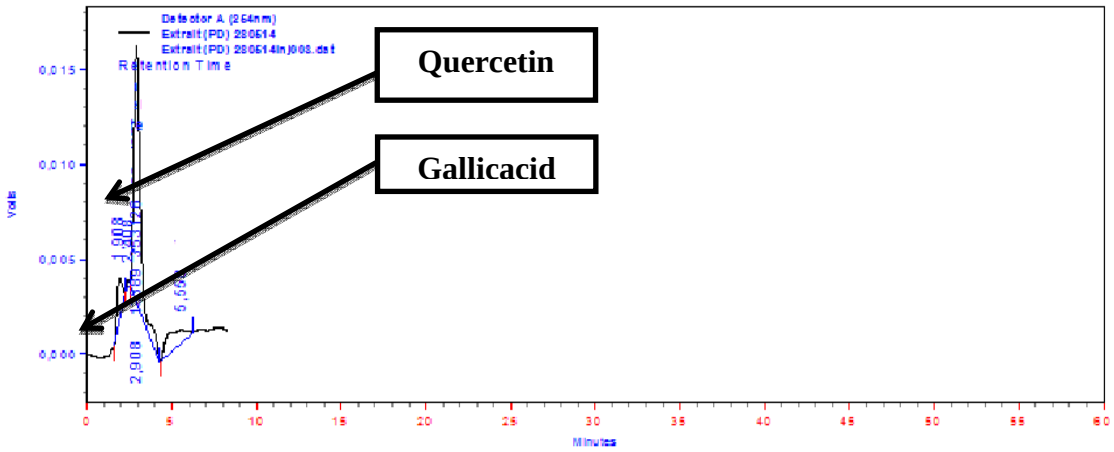


Figure 1:Chromatograms of the standards used in the HPLC (A: GA; B: Van; C: QE; D: Sal)

(E)



(F)

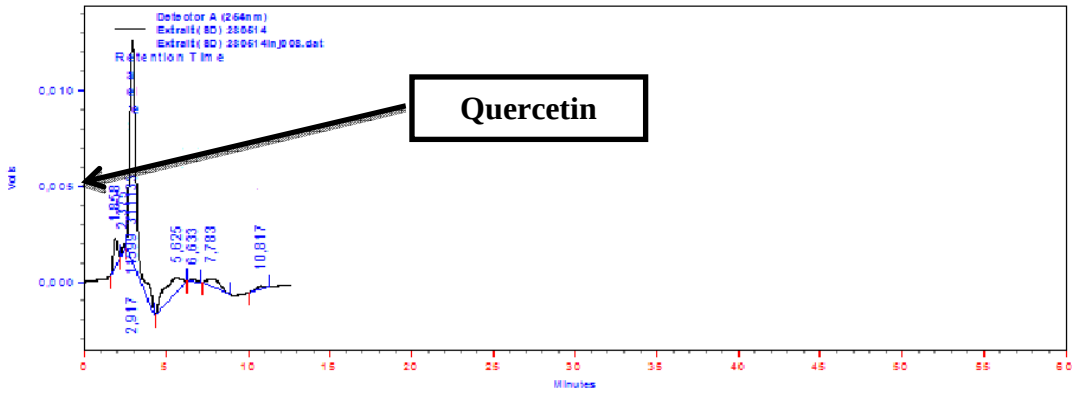


Figure 2: Chromatograms of the HPLC of the both extracts (E: Peels extract; F: Seeds extract)

Antimicrobial activity

The antimicrobial activity of peels and seeds extracts of *Citrus limon* L 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 seeds extract (Figure 3). Exceptionally *Staphylococcus epidermedis* and *Pseudomonas aeroginosa* that showed zone of inhibition against seeds extract with diameter of 9mm, 11mm respectively (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 epidermedis* bacteria responsible for the cutaneous, urinary and nasal infections with 31mm diameter of inhibition zone (Figure 4).

Despite the great resistance against antibiotics [22], *Pseudomonas aeroginosa* presented a high sensitivity with peels extract with 20mm diameter of inhibition zone (Figure 4) and in the case of *Candidas albicans*, yeast responsible for opportunist oral and genital infections in humans, the results were spectacular (diameter of 30mm). However, no anticandidosique activity was observed with the seeds extract.

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 [23]. 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 [24]. 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 2: Diameter of inhibition zones (mm) of various pathogenic organisms by the crud extracts of *Citrus limon* L

Extracts	Strains						
	Escherichia coli	*Pseudomonas aeroginosa*	*Staphylococcus aureus*	*Bacillus subtilis*	*Staphylococcus epidermedis*	*Sarcinalutea*	*Candida albicans*
Peels	20	20	22	20	31	21	30
Seeds	0	11	0	0	9	0	0

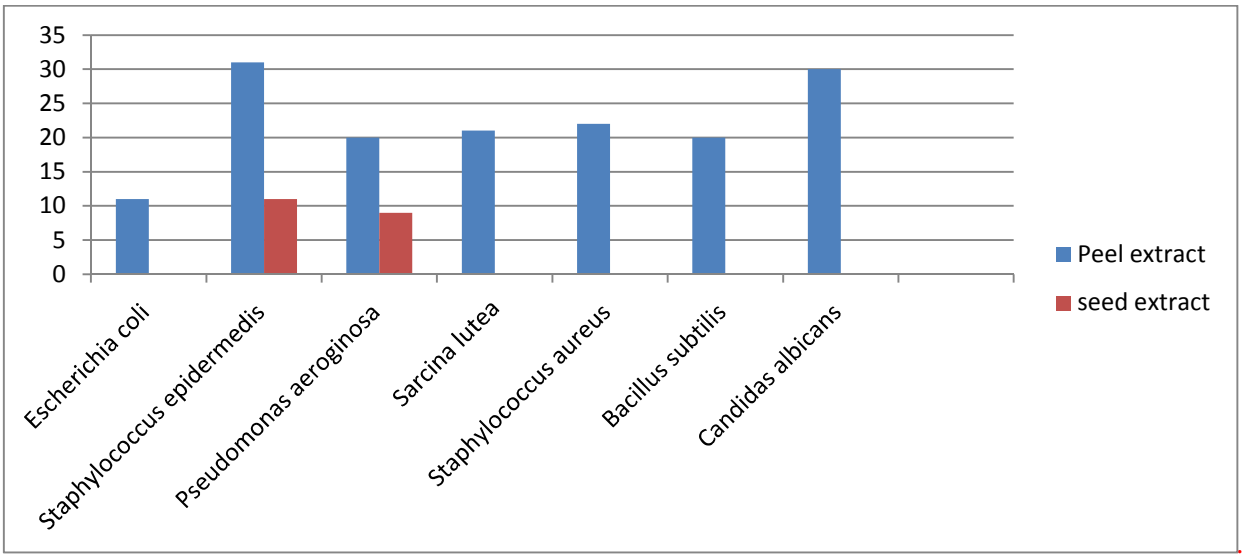


Figure 3: Comparative sensitivity of peels and seeds extracts of *Citrus limon*L

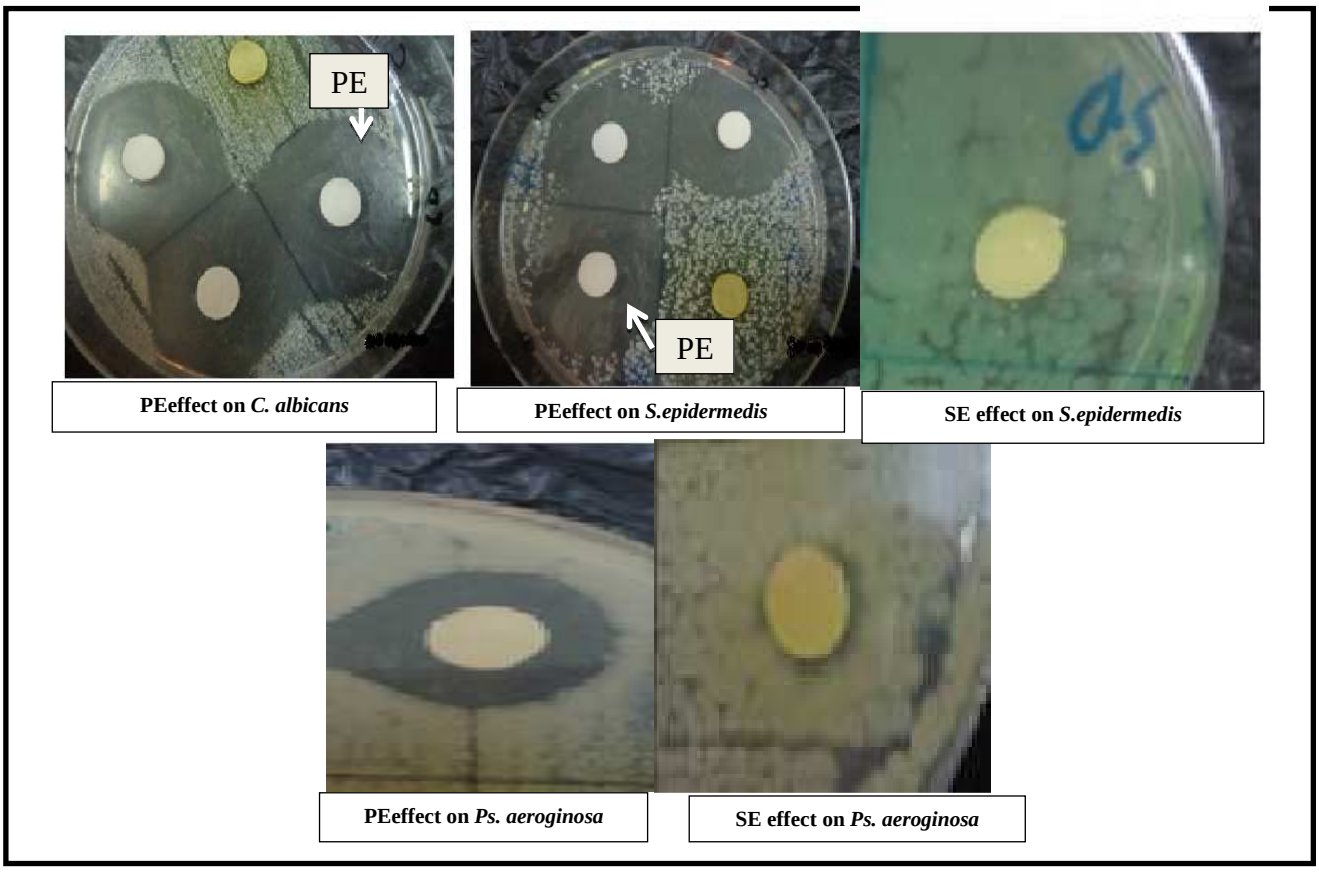


Figure 4: Examples of the effect of the peels and seeds extracts on microbial growth

We bring back in Table 3 the MICof the most active extract (peels extract) noted at the time of the study in solid medium, whose diameters of inhibition are equal to or higher than 12 mm.[25]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 1500µg/ml.
- Low inhibition: MIC greater than 1600µg/ml.

Thus, according to this classification and according to the results of the MIC, it shows that the peels extract had a broad antimicrobial spectrum with doses ranging from 2500 µg/ml to 5000µg/ml.

Table 3: MIC (expressed in µg / ml) of the Peels extract (whose diameters of the inhibition zones are ≥ 20 mm) on the bacteria tested

Extract	Strains						
	Escherichia coli	***Pseudomonas aeroginosa***	***Staphylococcus aureus***	***Bacillus subtilis***	***Staphylococcus epidermedis***	***Sarcinalutea***	***Candida albicans***
Peels	5000	5000	5000	2500	2500	2500	2500

CONCLUSION

The phytochemical results showed that both extracts (peels and seeds extracts) contain flavonoids,these active substances which can inhibit the growth of different types of bacteria citing *Staphylococcus aureus*[26]and *Escherichia coli*[19].

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

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