Variability of the antibacterial activity of *Citrus Limon, Citrus Limetta and Citrus Aurantifolia* depending on solvents, extraction methods and conventional growth of multi-resistant strains

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Abstract
Our study contributes to the valorization of the three Moroccan species (Citrus Limon, Citrus Limetta and Citrus Aurantifolia) by characterizing them by an in vitro evaluation of the antibacterial activity. We used two extraction techniques: maceration and Soxhlet. Four different polarity extraction solvents were used (hexane, dichloromethane, ethyl acetate and methanol) to obtain different crude extracts. However, the evaluation of this antibacterial activity of extracts and antibiotics against multi-resistant bacteria is carried out by the disk diffusion method and the macro-dilution.

We found that methanolic and ethyl acetate Soxhlet extracts of C.Limon, C.Limetta and C. Aurantifolia fruit showed strong antibacterial activity against Staphylococcus aureus and Enterococcus faecalis with minimal inhibition concentrations (MIC) of between 15,62 - 125 µg/ml and minimum bactericidal concentration (MBC) values of 62,5 to 500 µg/ml. On the other hand, the dichloromethane extracts of the three species proved to be inactive with respect to all the microbial strains tested. Gram + strains proved to be more resistant to antibiotics than gram-strains. This study showed that the solvent, the extraction method and the species significantly affect the yields and antibacterial activity of C.Limon, C.Limetta and C.Aurantifolia fruits.

Keywords: *Citrus Limon*, *Citrus Limetta*, *Citrus Aurantifolia*, Extraction method, Solvent and Antimicrobial potency.

Introduction

Most work is currently focused on finding natural products with antimicrobial properties, as they offer hope for new drugs with promising antimicrobial activity with lesser side effects for humans to overcome the problem of resistance of microorganisms to antibiotics. In the present study, the antimicrobial potency of the extracts was investigated by two gram-positive bacteria (*Staphylococcus aureus ATCC 29213* and Enterococcus faecalis C *1596*) and two gram-negative (*Escherichia coli ATCC 25922B* and *Salmonella enterica ATCC 13314*). These bacterial strains were provided by the Rabat Institute of Hygiene.

The evaluation test of this activity of the different extracts was carried out by Solid-state disk diffusion, in a first step to select the extracts having a significant activity. This same technique was applied to the behavior of the four strains with respect to the different antibiotics. The dilution in a liquid medium, in order to determine the minimal inhibitory concentrations (MIC) as well as the minimum bactericidal concentrations (MBC).

Material and Methods

Plant material: Harvesting of mature whole fruits, *C.Limetta, C.Limon*, and *C.Aurantifolia* is carried out in 2017/2018 randomly in the region of Kenitra (West of Morocco). The fruits were washed, the pulp and the skin were separated manually and seeded. These vegetable materials have been used in the fresh state. Species identification is made with National Institute of Agronomic Research Kenitra.

Preparation of extracts

Cold extraction - Maceration: The fresh pulps / epicarp (25 g) were macerated in 250 ml of the increasing polarity solvents (hexane, dichloromethane, ethyl acetate and methanol) for 24 hours at room temperature and in the dark. The extracts were filtered using Whatmann no. 1 filter paper and then concentrated to dryness in a rotavapor according to the boiling point of each solvent. The residues are taken up by different volumes of methanol in glass vials and are stored at -4 °C.

Hot extraction - Soxhlet: 25 g of fresh pulp/epicarp were extracted with 300 ml of different solvent (hexane, dichloromethane, ethyl acetate and methanol) separately using a Soxhlet extractor for 2 hours at a temperature not exceeding the point of boiling of the solvent. Then the different extracts were concentrated to dryness and taken up by different volumes of methanol. The residues obtained were stored at -4 °C.

The yield of the extracts[11] obtained was calculated as follows:

$R (\%) = 100 * M_{(ext)} / M_{(echo)}$

where $M_{(ext)}$ - is the mass of the extract after evaporation of the solvent in mg and $M_{(ech)}$ - is the dry mass of the plant sample in mg.

Qualitative Evaluation of the Antibacterial Activity of Extracts and Antibiotics by the Disc diffusion Method in a solid medium

Preparation of the inoculum: We took some colonies using a platinum loop and then introduced into test tubes containing sterile water physiology. After good homogenization of the bacterial suspension, a reading of its optical density was carried out at 625 nm. The opacity of the solution equivalent to an optical density (OD) of between 0,08 and 0,1 corresponds to 10^8 bacteria/ml. This suspension should be diluted to $1/100^{th}$ to have a final concentration of 10^6 bacteria/ml as indicated by the Committee of the Antibiogram of the French Society of Microbiology[6].

Practical implementation: 15 ml of super cooled Mueller Hinton agar were poured into the Petri dishes; after cooling and solidification on the bench 100 µl of the inoculum were spread on the surface of the MH agar medium using a rake. Then with sterile forceps, sterile Wathmann no. 3 (6 mm) paper disks were removed and then impregnated with 50 µl of the crude extracts of *C. Limon*, *C. Limetta* and *C. Aurantifolia* and disks of antibiotics in concentrations specifically (Table 1) and then plated.[7,13,26] The dishes are closed with parafilm and stored at 4 °C for 2h.[23] They were put in the oven at 37 °C for 24 hours. In the negative control box, the discs are soaked in methanol, and each trial was performed in triplicate.

Table 1
Disks of different families of antibiotics used with their charges

Antibiotics	Symbol	Code	Concentration in µg
Chloramphenicol	C	66278	30
Ciprofloxacin	CIP	68648	5
Norfloxacin	NOR	66338	10
Levofloxacin	LVX	66858	5
Fusidic acid	F	66518	10
Trimethoprim + sulfamethoxazole	SXT	68898	1,25 + 23,75
Fosfomycin	FOS	67658	200
Tetracycline	TE	67448	30
Erythromycin	E	66448	15
Kanamycin	K	66618	30
Cefalexin	CN	66208	30
Teicoplanin	TEC	68948	30
Vancomycin	VA	68928	30
Cefoxitin	FOX	66228	30
Oxacillin	OX	66848	5
Penicillin	P	67218	6 / 10 IU
Pristinamycin	PT	67278	15
Imipenem	IPM	66568	10
Cefixime	CFM	66418	10
Ertapenem	ETP	67518	10
Piperacillin + tazobactam	TZP	67238	100 + 10
Cefotaxime	CTX	66368	30
Amoxicillin + Clavulanic acid	AMC	66178	20 + 10
Ceftazidime	CAZ	66308	30
Levofloxacin	LVX	66858	5
Aztreonam	ATM	66928	30
Tobramycin	TM	67488	10
Ampicillin	AM	66128	10
Amikacine	AN	66148	30
Cephalotine	CF	66218	30
Lincomycine	L	66678	15
Piperacilline	PIP	67228	100

Expression of results: The diameter of the inhibition zone was expressed in (mm) around each disk using a graduated ruler. The interpretation of the results is as follows:
* Resistant (-): diameter ≤ 8mm, * Moderately sensitive (+): diameter between 8 and 14 mm.
* Sensitive (++): diameter between 14 and 20 mm and * Extremely sensitive (+++): diameter > 20 mm.

Quantitative Evaluation of Antibacterial Activity by the Macro-dilution Method in a liquid medium - Determination of minimum inhibitory concentration (MIC)

Preparation of the inoculum: An isolated colony of bacterial culture on nutrient agar was removed using a loop of platinum and homogenized in 10 ml of Mueller Hinton broth (MHB) and incubated for 3 to 5 hours at 37 °C to obtain a meadow-culture. A volume of 0,1 ml was taken for *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli* and *Salmonella enterica* respectively and was added to 10 ml of sterile MHB. This bacterial suspension is evaluated at 10^6 bacteria/ml and constitutes the pure inoculum.[37]

Preparation of the concentration range of the extracts: From the crude extracts, a series of dilutions of geometric reason 1/2 is carried out so as to have a concentration range of 15,62 to 500 µg/ml.

Practical implementation: In a series of eight tubes with caps numbered from C1 to C7, we introduced 1ml of the pure inoculum. Then we added in the tubes 1ml of plant extract according to the concentration range prepared. The C7 tube received instead of the plant extract, 1 ml of sterile MHB which served as a growth control. Due to the volume/volume dilution thus achieved, the concentration in the tubes was reduced by half. These tubes were incubated at 37 °C for 24 h. Each experiment was performed three times.[37] The determination of MIC was made by observation of the growth-induced disorder of the germs studied in each tube.

Determination of minimal bactericidal concentration (MBC)

Practical implementation: Using a loop calibrated at 2 µl, the contents of the tubes in which no trouble was observed, were removed and seeded on a Mueller-Hinton agar starting with the MIC tube. Seeding was done by parallel streaks 2 cm long at the agar surface. After 24h incubation in an oven at 37 °C, the first streak devoid of bacteria in each box will correspond to the MBC. Each experiment is performed three times.[37]

Bactericidal and bacteriostatic character: To define the bacteriostatic or bactericidal character, it is possible to calculate the MBC / MIC ratio:
* MBC/MIC < 4 → the extract is considered bactericidal (Guinoiseau, 2010)
* MBC/MIC > 4 → the extract is considered bacteriostatic (Cosentino and Tuberoso 1999, Randrianarivelo 2010).

Statistical Analysis: All experiments were performed in triplicate. The results are expressed as mean ± standard deviation. The results were analyzed by the univariate ANOVA test followed by the Tukey test for averaging comparisons. Values of $p \leq 0.05$ were considered statistically significant.

Results and Discussion

Yield in raw extracts: After extraction, we observed that the yields of the raw extracts depend on the species, the part of the fruit, the solvent and the extraction methods. The yields of the crude extracts are mentioned in figure 1. It is apparent from the observation of the extraction yields of the edible part that the ethyl acetate extract of *C.Limon* by Soxhlet recorded the yield of more important (14,72%) followed by the methanolic extract of *C.Limetta* (10,64%).

Regarding the inedible part, the best yield is recorded for methanolic extracts by Soxhlet of *C.Limetta* (12,46%) followed by *C.Aurantidolia* (12,09%) and at the end *C.Limon* (11,97%). On the other hand, the lowest yields are recorded for the hexane and dichloromethane extracts.

From the results obtained, we noticed that the yields of the crude extracts of each solvent varied according to the extraction method and the species (0,40% to 14,72%). Similarly, Ibrahim and Hegazy[9] reported that the yield of orange bark extracts by different solvents is between 8,27% and 28,32%. This variation is explained by the nature and the diffusion difference of the solvent in the samples used.[28]

It is also due to several factors including the interaction of the plant with the environment (climate, soils etc.), the period and the harvesting environment, the cultural practices and the age of the plant material.[1,15]

The appearance of Gram strains + (*Staphylococcus aureus and Enterococcus faecalis*) within antibiotics

Antibiogram: The results of the antibiogram performed on two gram + strains are shown in table 2. In our study *Staphylococcus aureus* showed resistance to chloramphenicol, norfloxacin, levofloxacin, fusidic acid, teicoplanin, vancomycin, cefalexin, kanamycin, erythromycin, ciprofloxacin, penicillin, pristinamycin and lincomycin. Regarding antibiotic susceptibility, our strains of *Staphylococcus aureus* have good sensitivity to oxacillin, cefoxitin, tetracycline, trimethoprim + sulfamethoxazole and fosfomycin.

In contrast, *Enterococcus faecalis* strain exhibited resistance to penicillin, cefoxitin, tetracycline, erythromycin, pristinamycin and fusidic acid. We observed a total resistance with no zone of inhibition to oxacillin, lincomycin and teicoplanin knowing that *Enterococcus faecalis* has shown good sensitivity to chloramphenicol, kanamycin, trimethoprim+ sulfamethoxazole, norfloxacin and levofloxacin.

The appearance of gram strains - (*Salmonella enterica and Escherichia coli*) within antibiotics

Antibiogram: The results of the susceptibility profile of gram-antibiotic strains are shown in table 3. Our results showed that *Salmonella enterica* exhibited resistance to cefixime, ciprofloxacin, fusidic acid, imipenem and ertapenem. Regarding sensitivity to different antibiotics, we noted that *Salmonella enterica* showed a very good sensitivity to piperacillin + tazobactam, cefotaxime, amoxicillin + clavulanic acid, ceftazidime, aztreonam, tobramycin, ampicillin, levofloxacin, cephalothin, cefoxitin, cefalexin, kanamycin, trimethoprim+sulfamethoxazole and fosfomycin.

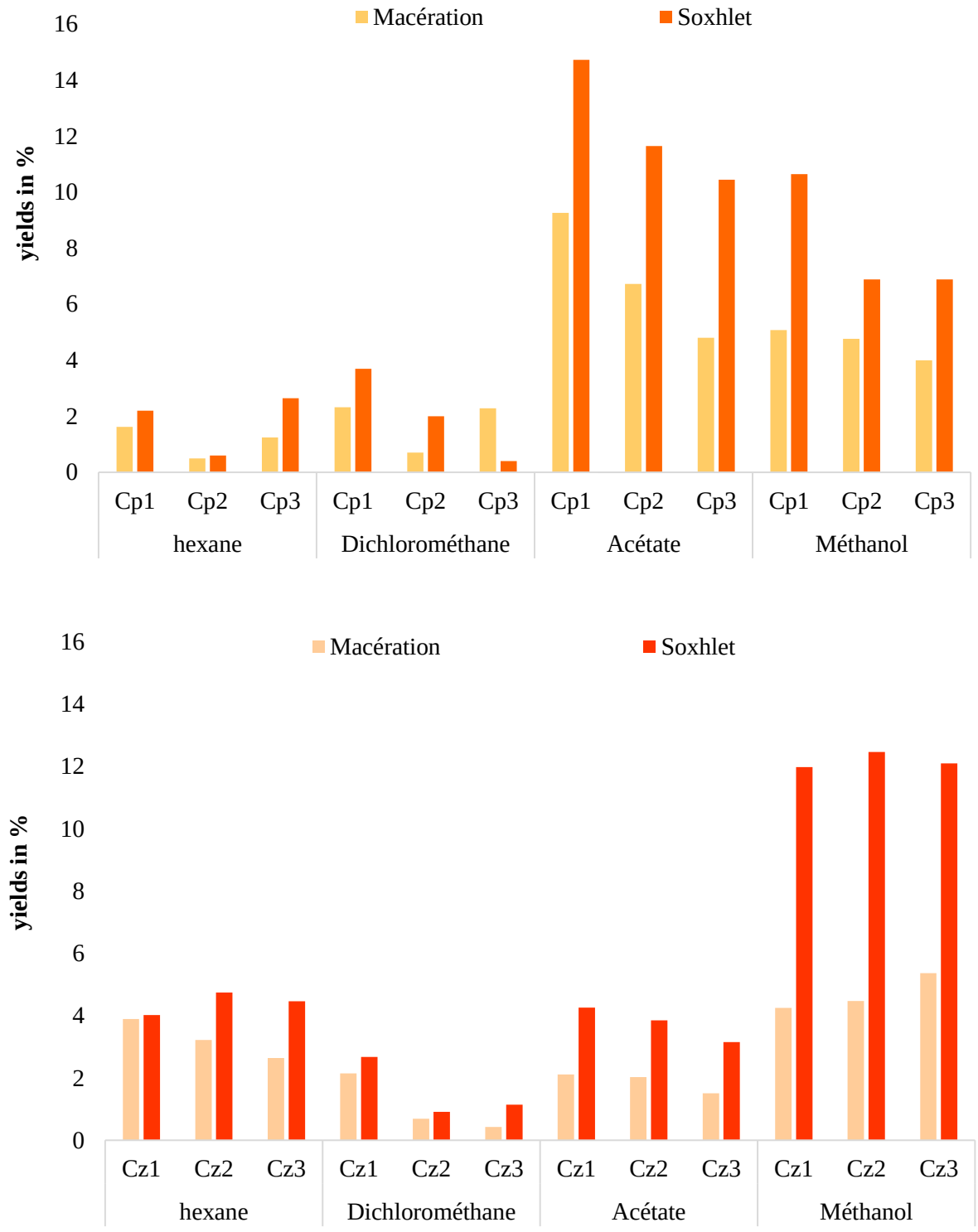


Figure 1: The effect of solvents and extraction techniques on the yield of crude extracts (%) of (A) pulp and (B) epicarp of *C.Limon, C.Limetta and C.Aurantifolia*

Table 2
Sensitivity profile of antibiotics used on Gram + bacteria (*Staphylococcus aureus* and *Enterococcus faecalis*)

Antibiotics	Diameters of the zones of inhibition (mm)		Critical diameters (mm)[6]	
	Staphylococcus aureus	*Enterococcus faecalis*	Sensitive	Resistant
Chloramphenicol	16	26	≥ 23	< 19
Ciprofloxacin	16	-	≥ 25	< 22
Norfloxacin	16	34	≥ 25	< 22
Levofloxacin	16	30	≥ 20	< 17
FusidicAcid	12	14	≥ 22	< 15
Trimethoprim+Sulfamethoxazole	20	22	≥ 16	< 10
Fosfomycin	30	-	≥ 14	< 14
Tetracycline	22	12	≥ 19	< 17
Erythromycine	14	12	≥ 22	< 17
Kanamycin	12	20	≥ 14	< 10
Cefalexin	12	-	≥ 18	< 12
Teicoplanin	6	0	≥ 17	-
Vancomycin	10	-	≥ 17	-
Cefoxitin	24	20	≥ 22	< 15
Oxacillin	24	0	≥ 20	< 20
Penicillin	4	12	≥ 29	< 18
Pristinamycin	16	6	≥ 22	< 19
Lincomycin	14	0	≥ 21	< 17

Table 3
Sensitivity profile of antibiotics used on Gram- bacteria (*Salmonella enterica* and *Escherichia coli*)

Antibiotics	Diameters of the zones of inhibition (mm)		Critical diameters (mm)[6]	
	Salmonella enterica	*Escherichia coli*	Sensitive	Resistant
Ciprofloxacin	20	30	≥ 25	< 22
Fusidic Acid	16	20	≥ 22	< 15
Trimethoprim+Sulfamethoazole	30	20	≥ 16	< 10
Fosfomycin	30	30	≥ 14	< 14
Cefalexin	22	24	≥ 18	< 12
Cefoxitin	24	25	≥ 22	< 15
Kanamycin	30	20	≥ 14	< 10
Imipenem	21	30	≥ 24	< 17
Cefixime	17	23	≥ 25	< 22
Ertapenem	22	20	≥ 28	< 26
Piperacillin + Tazobactam	24	28	≥ 22	< 18
Cefotaxime	26	30	≥ 26	< 23
Amoxicillin + Clavulanic Acid	30	25	≥ 23	< 16
Ceftazidime	28	30	≥ 21	< 19
Levofloxacin	30	30	≥ 20	< 17
Aztreonam	30	30	≥ 23	< 21
Tobramycin	20	-	≥ 18	< 16
Ampicillin	20	15	≥ 21	< 16
Cefalotin	20	18	≥ 18	< 12
Piperacillin	30	20	≥ 22	< 18

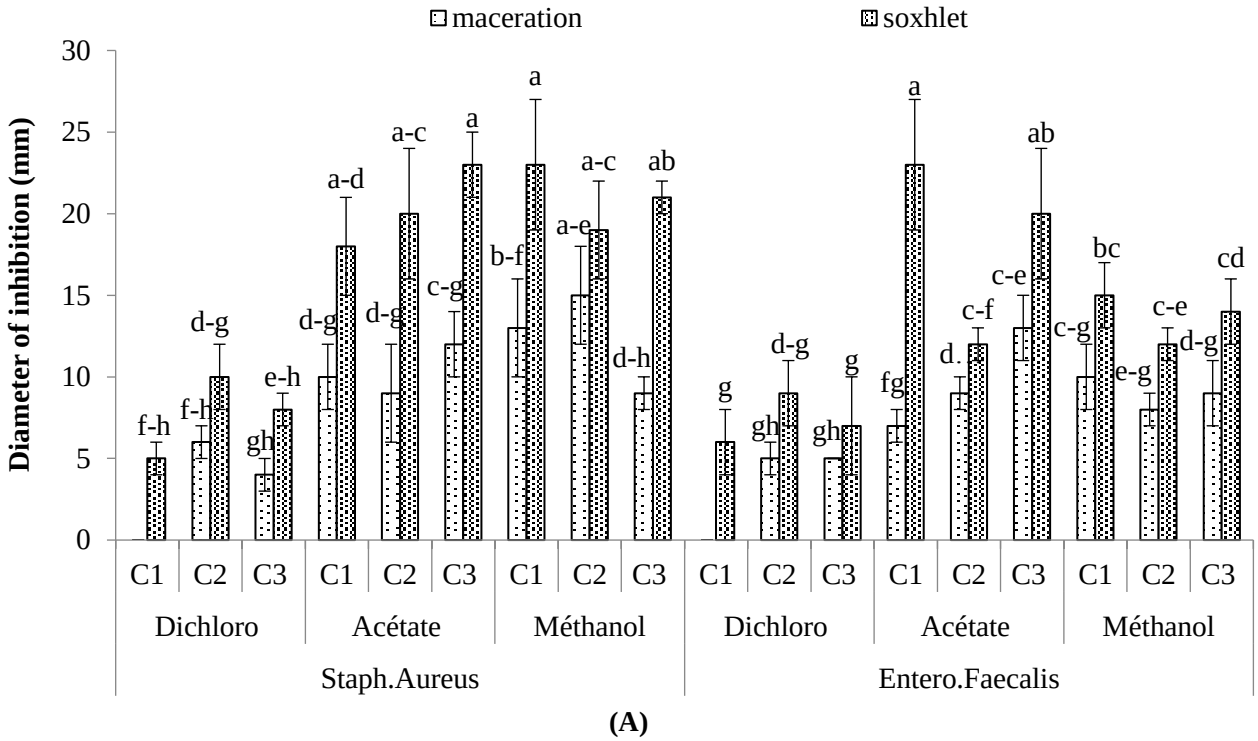
Indeed, the *Escherichia coli* strain exhibited resistance to piperacillin + tazobactam, ampicillin, cefixime, fusidic acid and ertapenem. On the other hand, *Escherichia coli* showed very good sensitivity to ciprofloxacin, imipenem, cefotaxime, amoxicillin + clavulanic acid, ceftazidime, aztreonam, tobramycin, levofloxacin, cephalothin, cefoxitin,

cefalexin, kanamycin, trimethoprim + sulfamethoxazole and fosfomycin. Finally, gram + strains (*Staphylococcus aureus and Enterococcus faecalis*) were found to be more resistant to antibiotics than the Gram- (*Salmonella enterica and Escherichia coli*) strains.

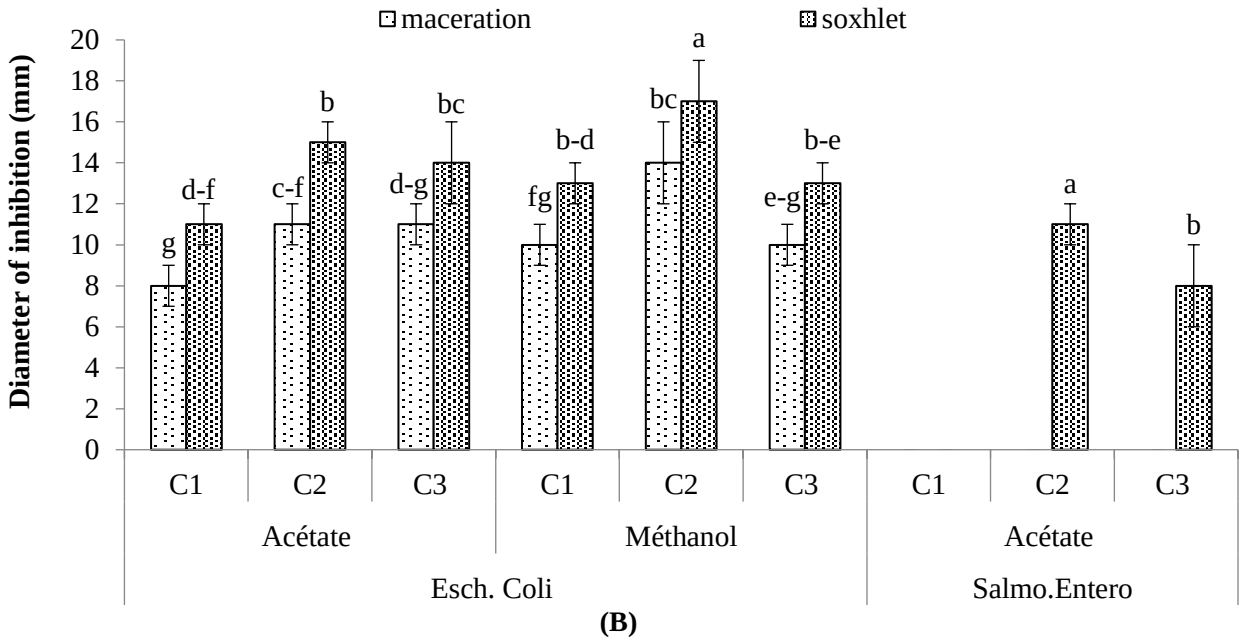
These results agree with those found by Ghadiri et al[16] and Boss et al.[3] Indeed, the qualitative comparison of antibiotics to extracts is difficult, given the nature of the composition of molecules that are not comparable.

The influence of solvents and the extraction method on the antibacterial activity of Citrus Limon Burm extracts, *Citrus Limetta Risso* and *Citrus Aurantifolia Swingle*

Aromatogram of the edible part: The results obtained showed that the zones of inhibition of the crude extracts of the three species vary considerably between the strains, the extraction methods, the solvents and the species studied with a significant variation ($p > 0.05$) (figure 2).



(A)



(B)

Figure 2: Comparison of the inhibition diameters of the various *C. Limon, C. Limetta and C. Aurantifolia* pulp extracts by two extraction methods (maceration and Soxhlet) and against (A) the gram + strains and (B) the gram-strains. The values presented are the measurements of three means ± standard deviation

The ethyl acetate and methanol extracts by Soxhlet possessed a broad spectrum of action with respect to maceration covering gram positive and negative bacteria. The dichloromethane extract exhibited resistance against gram-positive bacteria and showed no inhibitory effect on all gram-negative bacteria. In contrast, hexanoic extracts did not record any inhibition against the four bacterial strains tested.

With regard to *C.Limon*, the most active methanolic extract gives a maximum inhibition diameter of 23 mm, 15 mm and 13 mm respectively on *Staphylococcus aureus, Enterococcus faecalis* and *Escherichia coli*. The ethyl acetate extracts showed good sensitivity with inhibition diameters of 23 mm for *Enterococcus faecalis*, 18 mm for *Staphylococcus aureus* and 11 mm for *Escherichia coli*. In fact, for *C.Limetta*, the methanol extracts and ethyl acetate showed the highest activity against *Staphylococcus aureus* (21 and 20 mm respectively) followed by *Escherichia coli* (17 and 15 mm) and *Enterococcus faecalis* (13 and 11 mm). Likewise for *C.Aurantifolia*, the methanolic extract with Soxhlet is the most active gives a maximum inhibition diameter of 23 mm, 20 mm and 14 mm respectively against *Staphylococcus aureus, Enterococcus faecalis, and Escherichia coli*.

The ethyl acetate extracts by Soxhlet also showed good sensitivity with inhibition diameters of 21 mm against *Staphylococcus aureus*, 14 mm against *Enterococcus faecalis* and 13 mm against *Escherichia coli*. Based on the results, no inhibition was recorded against *Salmonella enterica* for all Soxhlet crude extracts with the exception of *C.Limetta* ethyl acetate extract which exhibited slight activity with inhibition of 11 mm.

The qualitative comparison of polar extracts by pulp Soxhlet of the three species showed that *C.Aurantifolia* exhibited good sensitivity against *Staphylococcus aureus* followed by *C.Limon* and at the end *C.Limetta*. Similarly, against *Enterococcus faecalis, C.Limon* also recorded a strong sensitivity followed by *C.Aurantifolia* and finally *C.Limetta*. As well as for gram - strains, the results revealed that *C.Limetta* had moderate susceptibility to *Escherichia coli and Salmonella enterica* followed by *C.Aurantifolia and C.Limon*.

Aromatogram of the inedible part: From the results shown in figure 3, the crude extracts of the species showed a varied antimicrobial effect between the solvents, extraction method, strains and species studied with significant differences ($p > 0.05$).

The extracts of the epicarp by Soxhlet of methanol, ethyl acetate and hexane showed the best inhibition against all bacteria tested compared to those of maceration. The dichloromethane extracts do not have active on gram - strains. But, for the gram + they recorded a moderate resistance.

Indeed for *C.Limon*, the ethyl acetate extract recorded a high sensitivity with a 28 mm inhibition zone for *Staphylococcus aureus* and a moderate sensitivity with a diameter of 15 mm and 12 mm for *Enterococcus faecalis* and *Escherichia coli* respectively. The methanol and hexane extracts showed moderate sensitivity for the different strains with zones of inhibition ranging from 17 mm to 9 mm.

Regarding the antibacterial activity of *C.Limetta*, the methanol extract varied considerably among the different bacteria tested and the highest activity was observed against *Staphylococcus aureus* (25 mm) followed by *Escherichia coli* (14 mm) and *Enterococcus faecalis* (13 mm). Hexane and ethyl acetate extracts showed moderate inhibitory activity against *Enterococcus faecalis* (20 and 17 mm respectively), *Staphylococcus aureus* (17 and 20 mm) and *Escherichia coli* (11 and 12 mm).

In addition, the antibacterial activity of *C.Aurantifolia*, the hexane extract varies between the different bacteria tested and the highest activity was observed against *Staphylococcus aureus* and *Salmonella enterica* (18 mm) followed by *Enterococcus faecalis* (17 mm) and *Escherichia coli* (16 mm). Methanol and ethyl acetate extracts showed moderate inhibitory activity against *Staphylococcus aureus* (respectively 17 and 16 mm), *Escherichia coli* (17 and 14 mm) and *Enterococcus faecalis* (11 and 16 mm). In view of the results, no inhibition was recorded against *Salmonella enterica* for all Soxhlet crude extracts with the exception of ethyl acetate and hexane extracts which showed fairly moderate antibacterial activity with a diameter of inhibition of 10 and 18 mm respectively for *C.Limetta* and 7 and 18 mm respectively for *C.Aurantifolia*.

However, comparison of the three epicarp Soxhlet raw extracts for gram + strains showed that *C.Limetta* has good antibacterial activity against *Staphylococcus aureus* followed by *C.Limon* and *C.Aurantifolia*. *C.Limetta* exhibited moderate activity against *Enterococcus faecalis* followed by *C.Aurantifolia* and finally *C.Limon*. On the other hand, the results revealed that *C.Aurantifolia* showed a slight antibacterial activity against *Escherichia coli* followed by *C.Limetta* and *C.Limon*. *C.Limetta* also showed moderate activity against *Salmonella enterica* followed by *C.Aurantifolia* and at the end *C.Limon*.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Edible Part: MICs were determined using the macro-dilution method for strains that showed some sensitivity (8-30 mm). Examination of the results obtained (table 4) showed that the extracts of methanol and ethyl acetate of the three species studied by Soxhlet showed antibacterial activity against all the bacteria with MIC values of 15,62 to 125 µg/ml and CMB values of 62,5 to 500 µg/ml. However, the minimal inhibitory concentrations obtained vary according to the strains, the solvents and the extraction method.

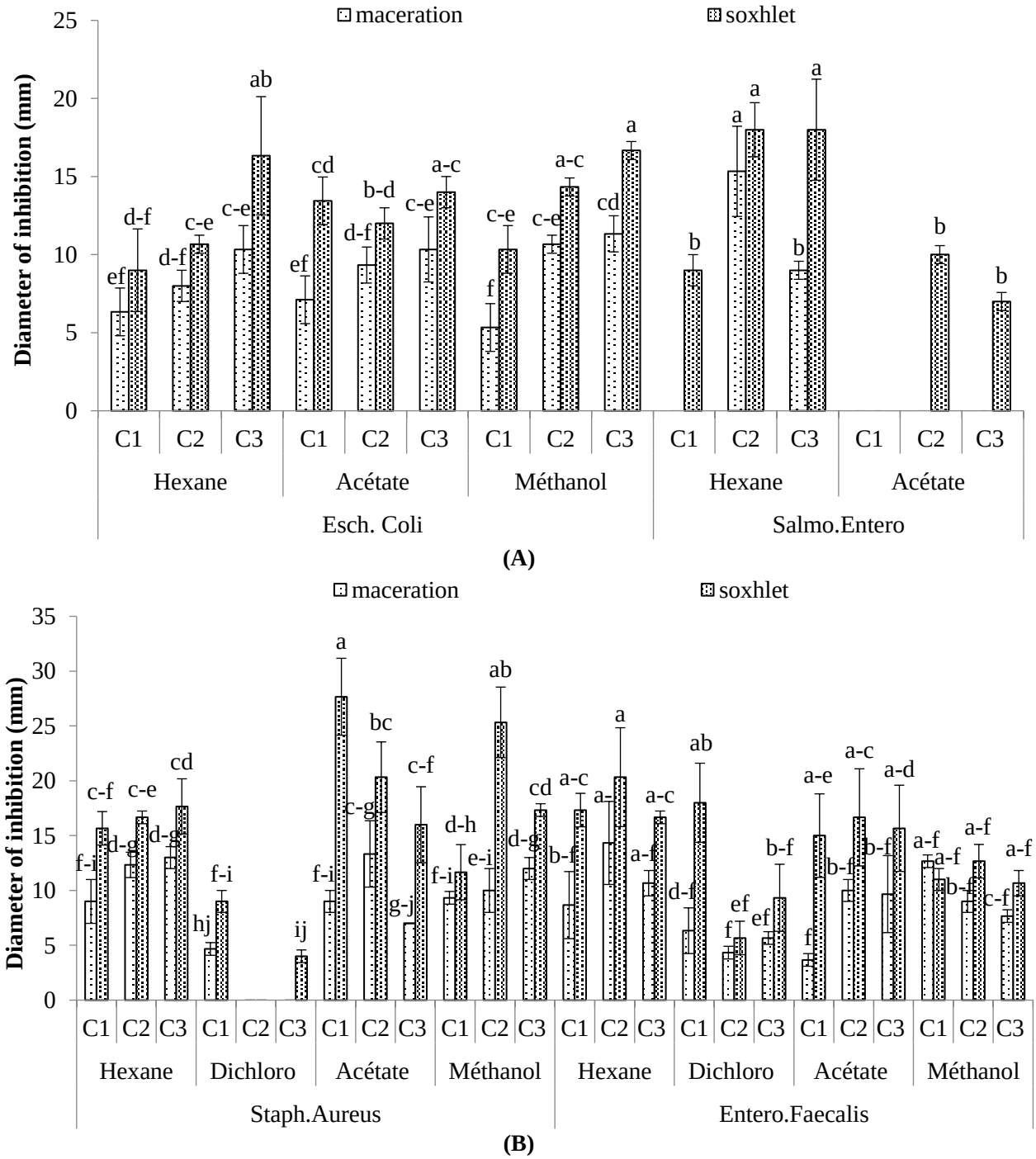


Figure 3: Comparison of inhibition diameters of the different extracts of *C.Limon, C.Limetta and C.Aurantifolia* epicarp by two extraction methods (maceration and Soxhlet) and against Gram- (A) strains and strains Gram + (B). The values presented are the measurements of three means ± standard deviation

According to the results obtained in *C.Limon*, the methanol extracts have a strong antibacterial activity against *Staphylococcus aureus, Enterococcus faecalis* and *Escherichia coli* with MIC values of 15,62, 31.25 and 62,5 µg/ml respectively.

The ethyl acetate extracts also showed strong antimicrobial activity with MIC values of 15,62 µg/ml against *Enterococcus faecalis*, 31,25 µg/ml against *Staphylococcus aureus* and 62,5 µg/ml against *Escherichia coli*.

Indeed, extracts of *C.Limetta and C.Aurantifolia* with ethyl acetate showed a strong antibacterial activity against *Staphylococcus aureus and Enterococcus faecalis* of 15,62 µg/ml followed by *Escherichia coli* and *Salmonella enterica* of 62,5 µg/ml. The methanol extracts also exhibited strong antimicrobial activity with MIC values of 15,62 µg/ml against *Staphylococcus aureus* and *Escherichia coli* and 31,25 µg/ml against *Enterococcus faecalis*. The dichloromethane extract of *C. Limetta* showed weak antibacterial activity against gram-positive strains

(*Staphylococcus aureus* and *Enterococcus faecalis*) with MIC values of 250 µg/ml.

Comparison of the polar extracts by pulp Soxhlet of the three species recorded that *C.Limon, C.Limetta and C.Aurantifolia* showed the same high inhibitory activity against *Staphylococcus aureus* followed by *Enterococcus faecalis* and at the end *Escherichia coli* and *Salmonella enterica*.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the Inedible Portion: MIC was studied only for extract-sensitive microorganisms. Our results showed that hexane, methanol and ethyl acetate extract by Soxhlet were able to inhibit the growth of the four bacteria tested with MIC ranging from 15,62 to 62,5 µg/ml. from 62,5 to 500 µg/ml. However, the minimum inhibitory concentrations obtained vary considerably according to the strains, the solvents, the extraction method and *Citrus* species (table 5).

Concerning *C.Limon and C.Limetta*, the extracts with ethyl acetate and methanol were moderately active against *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli* whose MIC is 62,5 µg/ml and the MBC values ranging from from 125 to 500 µg/ml. Hexane extracts showed good sensitivity against *Staphylococcus aureus, Enterococcus faecalis* with a MIC of 15,62 µg/ml and 31,25 µg/ml against *Escherichia coli* and with a MBC of 62,5 µg/ml. On the other hand, hexane extracts of *C.Limetta* showed a slight sensitivity against *Staphylococcus aureus* and *Escherichia coli* with a MIC value of 125 µg/ml and 250 µg/ml against *Enterococcus faecalis* and with a varied CMB of 250 to 500 µg/ml.

In fact, in *C.Aurantifolia*, the ethyl acetate extracts were very active against *Staphylococcus aureus* and *Enterococcus faecalis* whose MIC was 15,62 µg/ml followed by *Escherichia coli* of 31,25 µg/ml and *Salmonella enterica* of 125 µg/ml. The methanol extracts also showed good antimicrobial activity with MIC values of 15,62 µg/ml against *Escherichia coli* and 62.5 µg/ml against *Staphylococcus aureus* and *Enterococcus faecalis*. As well as CMB values were ranging from 62,5 to 500 µg/ml.

Table 4
Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the various extracts obtained by maceration and Soxhlet of the pulps of *Citrus Limon (C1), Citrus Limetta (C2)* and *Citrus Aurantifolia (C3)* with their interpretations against the four bacteria tested

			Staphylococcus aureus		*Enterococcus faecalis*		*Salmonella enterica*		*Escherichia coli*	
			Maceration	**Soxhlet**	**Maceration**	**Soxhlet**	**Maceration**	**Soxhlet**	**Maceration**	**Soxhlet**
dichloro methane	C2	CMI	-	250	-	250	-	-	-	-
		CMB	-	ND	-	ND	-	-	-	-
		Character	-	bacteriostatic	-	bacteriostatic	-	-	-	-
ethyl Acetate	C1	CMI	62,5	31,25	-	15,62	-	-	125	62,5
		CMB	250	125	-	62,5	-	-	500	125
		Character	bactericidal	bactericidal	-	bactericidal	-	-	bactericidal	bactericidal
	C2	CMI	125	15,62	31,25	15,62	-	62,5	125	62,5
		CMB	500	62,5	250	62,5	-	250	500	62,5
		Character	bacteriostatic	bactericidal	bacteriostatic	bactericidal	-	bactericidal	bactericidal	bactericidal
	C3	CMI	31,25	15,62	31,25	15,62	-	62,5	125	62,5
		CMB	125	62,5	125	62,5	-	250	125	62,5
		Character	bactericidal	bactericidal	bactericidal	bactericidal	-	bactericidal	bactericidal	bactericidal
methanol	C1	CMI	62,5	15,62	62,5	31,25	-	-	62,5	62,5
		CMB	250	62,5	250	125	-	-	250	125
		Character	bactericidal	bactericidal	bactericidal	bactericidal	-	-	bactericidal	bactericidal
	C2	CMI	125	15,62	62,5	31,25	-	-	31,25	15,62
		CMB	250	62,5	125	62,5	-	-	250	62,5
		Character	bactericidal	bactericidal	bactericidal	bactericidal	-	-	bacteriostatic	bactericidal
	C3	CMI	125	15,62	62,5	31,25	-	-	31,25	15,62
		CMB	500	62,5	250	62,5	-	-	62,5	62,5
		Character	bactericidal	bactericidal	bacteriostatic	bactericidal	-	-	bactericidal	bactericidal

Table 5
Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the various extracts obtained by maceration and Soxhlet of the *Citrus Limon (C1)*, *Citrus Limetta (C2)* and *Citrus Aurantifolia (C3)* epicarps with their interpretations against the four bacteria tested

			Staphylococcus aureus		***Enterococcus faecalis***		***Salmonella enterica***		***Escherichia coli***	
			Maceration	**Soxhlet**	**Maceration**	**Soxhlet**	**Maceration**	**Soxhlet**	**Maceration**	**Soxhlet**
Hexane	C1	CMI	125	15,62	125	15,62	-		-	31,25
		CMB	500	62,5	500	62,5	-		-	62,5
		Character	bactericidal	bactericidal	bactericidal	Bactericidal	-		-	bactericidal
	C2	CMI	500	125	500	250	-		250	125
		CMB	ND	250	ND	500	-		ND	500
		Character	bacteriostatic	bactericidal	bacteriostatic	bactericidal	-		bacteriostatic	bactericidal
	C3	CMI	500	125	500	250			250	125
		CMB	ND	125	ND	250			ND	500
		Character	bacteriostatic	bactericidal	bacteriostatic	bactericidal			bacteriostatic	bactericidal
ethyl Acetate	C1	CMI	125	62,5	-	62,5	-	-	-	62,5
		CMB	ND	250	-	250	-	-	-	250
		Character	bacteriostatic	bactericidal	-	bactericidal	-	-	-	bactericidal
	C2	CMI	125	62,5	125	62,5	-	62,5	125	62,5
		CMB	500	250	500	125	-	500	500	250
		Character	bactericidal	bactericidal	bactericidal	bactericidal	-	bacteriostatic	bactericidal	bactericidal
	C3	CMI	125	15,62	31,25	15,62	-	125	125	31,25
		CMB	250	62,5	125	62,5	-	500	250	62,5
		Character	bactericidal	bactericidal	bactericidal	bactericidal	-	bactericidal	bactericidal	bactericidal
Methanol	C1	CMI	125	62,5	125	62,5	-	-	-	62,5
		CMB	ND	ND	ND	125	-	-	-	250
		Character	bacteriostatic	bacteriostatic	bacteriostatic	bactericidal	-	-	-	bactericidal
	C2	CMI	125	62,5	125	62,5	-	-	125	62,5
		CMB	250	ND	ND	500	-	-	500	125
		Character	bactericidal	bacteriostatic	bacteriostatic	bacteriostatic	-	-	bactericidal	bactericidal
	C3	CMI	125	62,5	125	62,5	-	-	31,25	15,62
		CMB	250	250	250	250	-	-	125	62,5
		Character	bactericidal	bactericidal	bactericidal	bactericidal	-	-	bactericidal	bactericidal

However, the comparison for gram + strains showed that *C.Limetta* has good antibacterial activity against *Staphylococcus aureus* followed by *C.Limon and C.Aurantifolia*. *C.Limetta* showed moderate activity against *Enterococcus faecalis* followed by *C.Aurantifolia* and finally *C.Limon*. In addition to the gram - strains, the comparison revealed that *C.Aurantifolia* showed a slight antibacterial activity against *Escherichia coli* followed by *C.Limetta* and *C.Limon*. *C.Limetta* also showed moderate activity against *Salmonella enterica* followed by *C.Aurantifolia* and at the end *C.Limon*.

Based on our results, most *Citrus* extracts tested with polar solvents showed superior antimicrobial activity compared to apolar extracts. These variations can be explained by the dielectric constant, which plays a key role in the solubility of phytochemicals in solvents. This confirms the effect of the solvent system on the antibacterial activity of the extracts, which may be due to the richness of our species in polar substances.[12,24] These results are consistent with those obtained in some previous studies.[18,20,21,34] Similarly, Nasrin et al[29] reported that methanol and ethyl acetate extract of *Syzygium tamilnadensis, Syzygium densiflorum* and *Eugenia candollana* showed high and significant antibacterial activity against the four bacteria tested.

However, this activity depends not only on the polarity of the solvent, but also on the extraction method involved. Indeed, in this study we have demonstrated that the antibacterial activity of extracts obtained by Soxhlet is more

effective than that obtained by maceration. In contrast, Rasha et al[33] reported that Soxhlet extracts of *Persicaria odorata* showed the highest antimicrobial activity compared to extracts obtained by ultrasonic maceration and decoction against the four bacteria. Various reports indicate that the antibacterial activity depends on the solvent used, the extraction method, the structure of the compound in the extracts and the strain under study.[30]

From the qualitative and quantitative comparison of the results of Soxhlet polar extracts for the pulp of *C. Limon, C. Limetta* and *C. Aurantifolia*, we observed that all three species recorded upper inhibitory zones ranging from 12 to 23 mm against gram-positive strains (*Staphylococcus aureus, Enterococcus faecalis*) with MIC values ranging from 15,62 to 31,25 µg/ml. The diameters of inhibition are between 8 and 18 mm against gram negative strains (*Escherichia coli and Salmonella enterica*) with MIC values of 15,62 to 62,5 µg/ml. Our results are comparable to those obtained by Al farraj et al[2] for the polar extracts of *Citrus Aurantifolia* against *Staphylococcus aureus and Enterococcus faecalis* with zones of inhibition vary between 18,5 and 28 mm and against *Salmonella typhimurium LT2* and *Escherichia coli* with diameters of 15 to 18 mm.

In addition, another study conducted by Yathiender[38] on the polar extracts of *Citrus maxima* and *Citrus aurantium* (pulps) recorded the highest diameters for *Citrus maxima* (25 to 27mm) against *Staphylococcus aureus* compared with *Escherichia coli* (18 to 21 mm).

Regarding the epicarps, we found that the three species showed high inhibition diameters between 11 and 28 mm against gram + bacteria with MIC values ranging from 15,62 to 250 µg/ml. The zones of inhibition are between 9 and 18 mm against Gram- bacteria with MIC values of 15,62 to 125 µg/ml. These results are consistent with those reported by Parna Das et al[31], Klangpetch et al[25] and Baba et al.[4] In addition, a study of ethanolic extracts of *Citrus maxima* (Burm.) was resistant to *Escherichia coli and Salmonella typhimurium*.[5]

In addition, we observed that strains of *Escherichia coli* and *Salmonella enterica* showed resistance to all extracts regardless of the concentration used and for both parts in relation to *Staphylococcus aureus* and *Enterococcus faecalis*. This observation can be explained by the inefficiency of the active molecules in these species or the complexity of the double membrane including the cell envelope expressed by lipoproteins and lipopolysaccharides, and which act as a barrier against antibacterial substances, as opposed to the unique membrane structure of gram-positive bacteria.[22,39]

However, the strong antibacterial activity of the polar extracts in all three species of *Citrus* vis-à-vis, the microorganisms tested was attributed to the revelation by phytochemical screening of the main families of chemical compounds. These include triterpenes, alkaloids, sterols, anthraquinones, coumarins, reducing sugars, proteins, carotenoids[35] likely to confer antimicrobial properties. The results of this correlation are consistent with previous studies that showed the inhibitory effect of phytochemicals of various plant foods on different microorganisms.[9,17,27,36]

Conclusion

In general, we concluded from this study that the methanol and ethyl acetate extract of the three *Citrus* species showed maximum antimicrobial activity against gram + strains (*Staphylococcus aureus and Enterococcus faecalis*). These bacterial strains are the most sensitive. On the other hand, these extracts recorded some resistance against gram - strains (*Escherichia coli and Salmonella enterica*). Hexane extracts in the epicarps also showed moderate sensitivity against the four strains studied. However, extraction techniques have revealed that Soxhlet is the best technique. The extract obtained by the latter has a good sensitivity against strains compared to maceration for the two parts studied.

However, the results revealed that the fruits of the three citrus species showed a very good sensitivity against gram + strains than gram-. Then we found that the antibacterial activity of the extracts is closely related to the nature of the solvent used and the extraction method. These data suggest that the fruits of *C.Limon, C.Limetta and C.Aurantifolia* (pulp and epicarp) have a good potential to inhibit microbial growth that alters food and can be an extremely effective alternative for human infections.

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