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ANTI- BACTERIAL EFFECT OF *CITRUS* ESSENCE (*CITRUS PARADISI* AND *CITRUS RETICULATA)* FROM CHLEF REGION, ALGERIA

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ABSTRACT

Citrus essences were obtained by cold pressing of the zest of *Citrus paradisi* and *Citrus reticulata* harvested in the Chlef region (Algeria). To assess the quality and composition of these natural extracts, analyzes were performed, firstly by determining the organoleptic, physical and chemical properties, hence qualitative and quantitative analysis were done by gas chromatography coupled to spectrophotometry mass (GC / MS).

Among the 17 pathogenic bacteria tested (reference and isolated), we noted the half of the tested strains are sensitive to the essence of *C. paradisi* with a diameter ranging from 11 to 16 mm for *P. mirabilis*, *Serratia sp*. and 17-19 mm for very sensitive (*S. aureus* ATCC®25 923, *S. aureus* MRSA + ATCC® 43300, *E. coli* ATCC®25 922, *S. epidermidis*) and 21 mm or more, for extremely sensitive (*S. aureus* ATCC®29 213, P. vulgaris and *Streptococcus sp*.). The second halves are resistant to the essence of this variety.

The essence of *Citrus reticulata* showed positive antibacterial activity against 64% of tested strains (11 strains), 27% (03 strains) which are extremely sensitive (*S. aureus* ATCC®25 923 *S. epidermidis* and *P. vulgaris*), with a diameter greater than 20 mm, 55% (06 strains) are sensitive (213 ATCC®29 *S. aureus*, *E. coli* ATCC®25 922 29 212 *E. feacalis* ATCC®, *P . mirabilis*, *Streptococcus sp.*, *Serratia sp*.) with diameters of between 11 and 16 mm and 18% (02 strains) are highly sensitive (*S. aureus* and *B. subtilis* 737 ATCC®29 ATCC®6 633).

The essence of *Citrus reticulata* has minimum inhibitory concentration (MIC) ranging from 500 to 250 .mu.l of E. / ml against the strain *S. aureus* ATCC® 25 923, 50 .mu.l of E. ./ml against the strain *B. subtilis* ATCC® 6633, 25 .mu.l of E. / ml against the strain *S. aureus* 29 737 ATCC®, 3.90 .mu.l of E /. ml against the *S. epidermidis* strain.

As for the essence of *C. paradisi*, two trends emerge. When CMI less than 50 .mu.l I / ml, in the presence of *S. aureus* ATCC®25 923, 213 *S. aureus* ATCC®29. These bacteria are most susceptible to this species. When MICs was beteween 250 and 100 .mu.l of E. / Ml, we have *S. aureus* MRSA + ATCC®43 300 *S. epidermidis* and *Streptococcus sp.* These bacteria are less sensitive than the first.

KEYWORDS: *Citrus paradisi*, *Citrus reticulata*, Cold Expression, Antibacterial Effect

INTRODUCTION

Today, modern medicine uses the healing properties of essential oils and their constituents. Indeed, many volatile compounds are today common ingredients of pharmaceutical preparations. In an attempt to find new cures for diseases, the

scientific community has recently turned to the constituents of essential oils. Thus, among the gifted botanical families healing properties include: the Rutaceae (*Rutaceae*) which encompasses a wide range of aromatic plants, mainly located in tropical regions (Parray and *al*., 2012).

Citrus is a well-known family of this kind because it includes only edible species. The essence of this genus extracted by a cold expression contain natural assets principles as: flavonoids (Tripoli and *al*., 2007), limonoids (Mamers, 2007) coumarins, sterols (Ladaniya 2008) volatile oil and alkaloids (He and *al*., 2010) which allows them to operate in the pharmaceutical and biological field. : Antimicrobial activity, antioxidant, anti-inflammatory, antispasmodic ....etc. (Watson, 2011).

They are endowed with antibacterial properties to varying degrees according to their biochemical wealth terpenes, phenols, aldehydes, alcohols ... (Room 1991).

Our present study focuses on the evaluation of the antibacterial activity of the species *Citrus paradisi* and *Citrus reticulata*

MATERIALS AND METHODS

Plant Material and Extraction Procedure

The two varieties of Citrus: *C. sinensis* and *C. aurantium* were collected in the Chlef region in late February 2013. First the *Citrus* fruits were weighted, cleaned and peeled zest to recover. The extraction of essences is made by cold expression method.

Analysis of Citrus Essence by GC / MS

GC: Hewlett Packard Agilent6890N controlled by ChemStation (NIST98).

The chromatography conditions are as follows:

- Injection of 0.5µl Split mode 1/50
- Injector temperature: 250 ° C
- Capillary Column HP5MS (30 mx 0.25 mm x 0.25µm)
- Programming temperature: 35 ° C for 10 min; 4 ° C / min up to 250 ° C for 10 min.
- Flow of carrier gas: Helium (1ml/min)
- Mass spectrum: model Agilent 5973
- Temperatures: interface (280 ° C), source (230 ° C), quadrupole (150 ° C)
- The ionization energy of 70 eV.

To assess the quality and molecular composition of *C. sinensis* and *C. aurantium* essence, qualitative and quantitative analysis by GC / MS were performed.

Pathogenic Bacteria

For the tests of antibacterial activity, the study was conducted on several pathogenic bacteria which are provided by several microbiology laboratories cited in Table 1

Table 1: The Source of Pathogenic Bacteria Studied

Les Bactéries	Source
Escherichia coli ATCC® 25 922. *Enterococcusfeacalis ATCC® 29 212.* *Pseudomonas aeruginosa ATCC® 27 853.* *S. aureus MRSA+ATCC® 43 300.*	Laboratory of Microbiology of koliâa Hospital (Algeria)
S. aureus ATCC® 25 923.	Laboratory of bacteriology Ben Boulid Hospital (Blida, Algeria)
S. aureus ATCC® 29 737. *S. epidermidis ATCC® 12 228.* *Bacillus subtilis ATCC® 6 633.*	Laboratory of Microbiology of Antibiotic complex Media, SAIDAL (Algeria)
S. aureus ATCC® 29 213. *Klebsiellapneumoniae* *Proteus mirabilis.* *Proteusvulgaris.* *Acinetobacterbaumanii.* *Serratiasp.* *Streptococcus sp.*	Laboratory of Biological Analysis (AinDefla, Algeria)
S. epidermidis.	Laboratory of Microbiology (El-Ihsan clinic, Chlef, Algeria)
Salmonella sp.	Laboratory of Molecular Microbiology, University of Hassiba Ben Bouali (Chlef, Algeria)

These bacteria are preserved and kept alive by continuous passages on various areas of solid and liquid culture, depending on the species. Moreover, to ensure the survival of bacteria and, several culture media were used for isolation media, or selective enrichment for each bacterium or, more precisely for each group of bacteria.

TESTS OF ANTIBACTERIAL ACTIVITY

Aromatogram by Method of Discs

Aromatogram is the technique chosen to determine the antibacterial activity of our Essential oil against pathogenic bacteria. This method aims to test in vitro active concentration of a particular essential oil, which will determine the susceptibility or resistance of the pathogen. It is based on the migration of essences within a box steeped in a solid nutrient medium (Burnichon and Texier, 2003). Contact is made through a paper disk on which there is an amount of extract (Degryse and *al*., 2008).

Determining the Minimum Inhibitory Concentration (MIC)

It consists in dilutions of Citrus essences and then applies the method disk to determine the minimum concentration of our essences that allows inhibition of the tested pathogenic bacteria.

The Steps are as Following

- Essences were diluted in a fixed volume of 95 ° ethanol, to give a homogeneous mixture through the use of vortex.
- This test requires the use of filter paper discs, which are pre-sterilized at 110 ° C, and are made by sterile forceps after they are impregnated in the specific dilution to essences.

The Diameters of the Discs and the Amount

Seeding Petrie dishes containing the MH medium with a thickness of 4 mm by the same inoculum for

aromatogram prepared using a swab by streaking that allow uniform spreading. Petrie boxes are divided according to the number of bacteria.

- In parallel, control assays were performed with all pathogenic bacteria using discs impregnated with 10 ml of ethanol at 95 ° C (Boulahbal, 1993). The latter is used as a control because of the difficulty encountered in the use of essence in culture media based on water solubility is low. Several substances have been used for this purpose: ethanol (Beuchat, 1976; Marino and *al*., 2001); methanol (Onawunmi, 1989).
- Incubation temperature: 24/37 ° C.
- Reading is to accurately measure the diameters of the inhibition zones using a foot slide.

RESULTS

GC/MS Analysis of *Citrus* Essences

Chemical analysis showed a determined number of components for both species: 08 species of compounds for *C. reticulata* (99.92%) (Table 2), and 31 compounds for gasoline *C. paradisi* (99.55%) (Table3).

This analysis showedthat the major component in *Citrus* essences is the "limonene" with different percentages (94.75% for the essence of *C. reticulata* and 82.98% for the essence of *C. paradisi*). On the other hand minor compounds in essence of *C. reticulata* are represented by β-pinene (2.44%), 1-5 dimethyl 1venyl (0.72%), α-pinene (0.91%), β-phellandrene (0.61%) (Table1).

In addition to limonene, the essence of *C. paradisi*has minor compounds such as: β-myrcene (2.66%), 2H-1-benzopyranone (2.07%) and in trace amounts: α-pinene (0.31%) and n-hexadecanoic acid (0.88%) (Table3).

Table 2: The Chemical Compound (%) of the Essence of *C. reticulata* Analyzed by GC / MS

Number of Pics	Retention Time (min)	Chemical Composition	% Relative	Reconnaissance Level
1	1.727	Acide formique	0.24	4
2	2.012	Ether éthyle	0.13	91
3	17.907	α pinène	0.70	97
4	20.099	β-phéllandrene	0.61	91
5	21.079	β-pinene	2.44	94
6	23.169	**D-limonene**	94.75	94
7	26.086	1-5 dimethyl 1venyl	0.72	49
8	40.103	Naphtalene	0.42	99

Table 3: The Chemical Compound (%) of the Essence of C. *paradisi* Analyzed by GC / MS

Number of Pics	Retention Time (min)	Chemical Composition	% Relative	Reconnaissance Level
1	1.870	Ethanol	0.61	90
2	17.937	α pinene	0.91	96
3	20.116	β-phellandrene	0.49	90
4	21.114	β-myrcene	2.66	86
5	23.443	**Limonene**	82.98	93
6	23.840	Octariene	0.38	96
7	24.898	Acide formique	0.20	91
8	26.103	Octadien-3-ol	0.19	72
9	29.905	Cyclohexane	0.10	72
10	30.315	Decanal	0.29	86

Table 3 : Contd.,

11	36.331	α-cubebene	0.53	96
12	36.723	1,6cyclodecadiene	0.42	95
13	37,804	Caryophullene	1.25	99
14	38.945	Cycloundecatriene	0.17	98
15	39.111	Cycloheptasiloxane	0.08	90
16	39.741	1,6cyclodecadiene	0.31	96
17	40.863	Naphthalene	0.56	94
18	43.958	Sylane	0.19	55
19	48.139	Cyclononasiloxane	0.10	55
20	48.947	Naphthalenone	0.82	95
21	51.863	Cycloheptasiloxane	0.09	52
22	52.927	Acide n-hexadecanoique	0.88	98
23	55.267	Acide benzeneacetique	0.09	50
24	55.962	Acide 9,12octadecadienoique	0.09	99
25	56.674	Osthole	0.21	99
26	58.361	7 chloro-10-ethyl	0.17	46
27	58.741	2-naphthaldehyde	0.32	50
28	63.933	Cyclononasiloxane	0.16	52
29	65.263	Acide 1-2 benzenedicarboxylique	0.27	80
30	67.003	Acide benzenesulforique	0.22	56
31	67.538	2H-1-benzopyranone	2.07	46

Susceptibility of the Studied Pathogenic Bacteria

The test was performed to compare the antibacterial effect of *Citrus* essence on pathogenic bacteria with some families of antibiotics which were used as positive controls.

Table 4: Antimicrobial Susceptibility of Strains Studied

Bacteria	Sensitive	Intermediate	Resistance
E. coli ATCC® 25 922	- Gentamycine	/	-Erythromycine
E. feacalis ATCC®29 212	-Gentamycine	-Tétracycline	/
P. aerugenosa ATCC®27 853	-Gentamicine	-Oxacilline	/
K. pneumoniae	-Cefotaxime	/	/
S. aureus ATCC® 43 300	- Gentamycine	- Pénicilline G	/
S. aureus ATCC® 25 923	-Gentamicine	-Oxacilline	-Vancomycine
S. aureus ATCC® 29 213	-Gentamicine	- Tétracycline	/
S. aureus ATCC® 29 737	-Gentamicine	/	/
S. epidermidis ATCC® 12 228	- Vancomycine - Oxacilline	/	/
B. subtilis ATCC® 6 633	-Gentamicine	/	/
S. epidermidis	- Vancomycine - Amikacin - Gentamycine	- Pénicilline G	- Oxacilline - SulphamethoxazoleBactrim
Serratiasp.	-Gentamycine	/	/
P. vulgaris	- Cefotaxime	/	-

			SulphamethoxazoleBactrim
P. mirabilis	-Gentamycine	/	/
Streptococcus sp.	-Pénicilline	-Oxacilline	/
A. baumanii	-Gentamycine	/	/

The activity of all antibiotics is not regular and must be specified by susceptibility (Fauchère and April, 2002).

Effect of *C. paradisi* Essence on Pathogenic Bacteria Tested

The essence of *C. paradisi* presented a wide spectrum of inhibition against a number of bacterial strains, the diameters of the inhibition zones are shown in Figure 1:

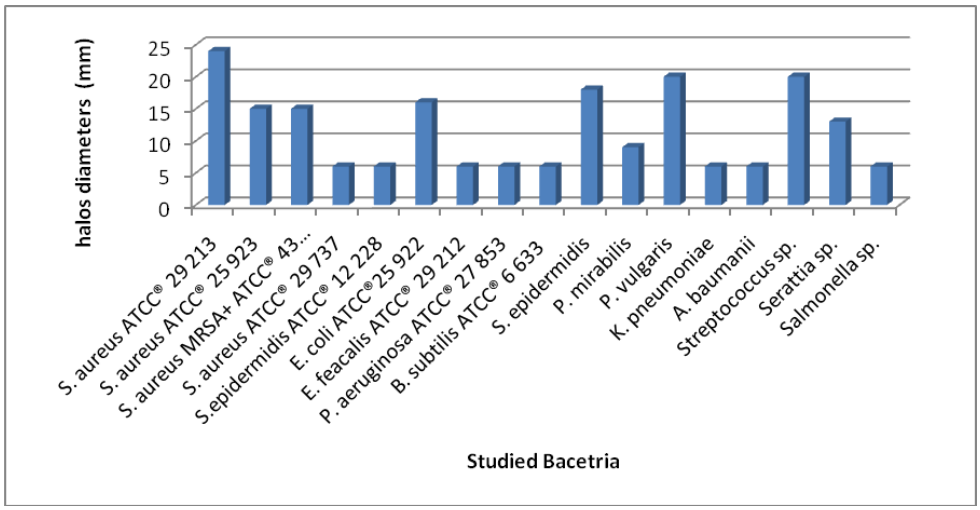

Figure 1: Graphical Representation of the Diameters of Inhibition Zones of *C. paradisi* Essence against Pathogenic Bacteria

Half of the tested Bacteria were susceptible to the essence of *C. paradisi* with a diameter ranging from 11 to 16 mm for *P. mirabilis*, *Serratia sp*. and 17-19 mm for very sensitive (*S. aureus* ATCC®25 923, *S. aureus* MRSA + ATCC® 43300, *E. coli* ATCC®25 922, *S. epidermidis*) and 21 mm or more, for extremely sensitive (*S. aureus* ATCC®29 213, *P. vulgaris* and *Streptococcus sp.*). The second halves are resistant to the essence of this variety. According to WHO vancomycin may inhibit *Streptococcus sp.* (A diameter of 20 mm). Compared with our results, the essence of this variety has a good inhibitory effect on pathogenic bacterial strains.

Effect of Essence of *Citrus reticulata* on Pathogenic Bacteria Tested

The essence of *Citrus reticulata* showed a negative antibacterial activity against 36% of the studied strains (06 strains) (Figure 2).

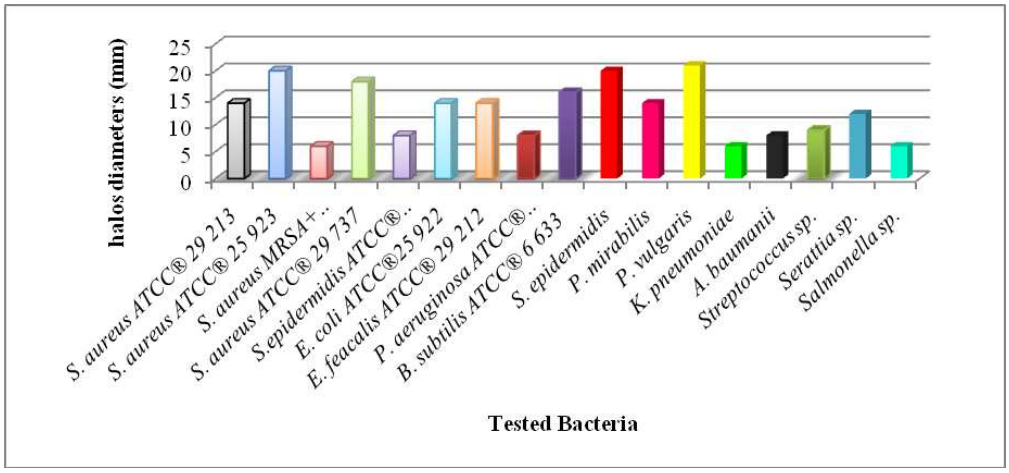

Figure 2: Graphical Representation of the Diameters of Inhibition Zones of *Citrus reticulate* Essence against Pathogenic Bacteria

The essence of *Citrus reticulata* showed positive antibacterial activity against 64% of tested strains (11 strains), 27% (03 strains) are extremely sensitive (*S. aureus* ATCC®25 923 *S. epidermidis* and *P. vulgaris*), with a diameter greater than 20 mm, 55% (06 strains) are sensitive (213 ATCC®29 *S. aureus*, *E. coli* ATCC®25 922 29 212 *E. feacalis* ATCC®, *P. mirabilis*, *Streptococcus sp.*, *Serratia sp.*) with diameters of between 11 and 16 mm and 18% (02 strains) are highly sensitive (*S. aureus* and *B. subtilis* 737 ATCC®29 ATCC®6 633).

The essence of *Citrus reticulata* showed antibacterial activity against Gram (-) and Gram (+), a special event was recorded for the species: *P. vulgaris* and *P.mirabilis*. The susceptibility of bacteria is independent effect of Gram (Dorman and Deans, 2000), or depends on the used essential oil (Deans and Ritchie, 1987).

The MIC Determination of the Essence of the Variety of *Citrus reticulata*

This figure showed the variation of antibacterial activity of *Citrus reticulata* essence in various concentrations on pathogenic bacteria.

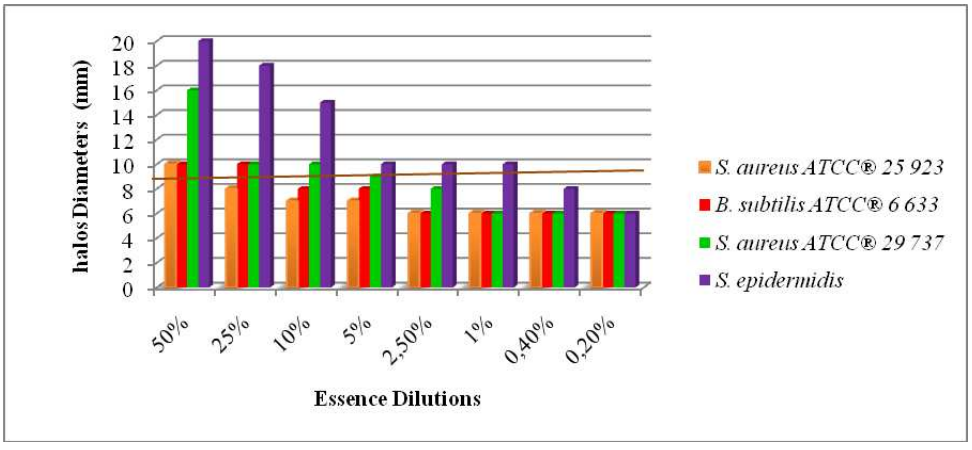

Figure 3: Graphical Representation of the Diameters of Inhibition Zones of *Citrus reticulate* Essence

According to the results shown in Figure(3), the essence of *Citrus reticulata* has an MIC of between 500 and 250 .mu.l of E. / Ml against the strain *S. aureus* ATCC® 25 923, 50 .mu.l of E. / ml against of the strain *B. subtilis* ATCC® 6633, 25 .mu.l of E. / ml against the strain *S. aureus* 29 737 ATCC®, 3.90 .mu.l I. / ml against the *S. epidermidis* strain.

Determination of the MIC of the Essence of *C. paradisi*

This figure showed the influence of the essence of *C. paradisi* on pathogenic bacteria.

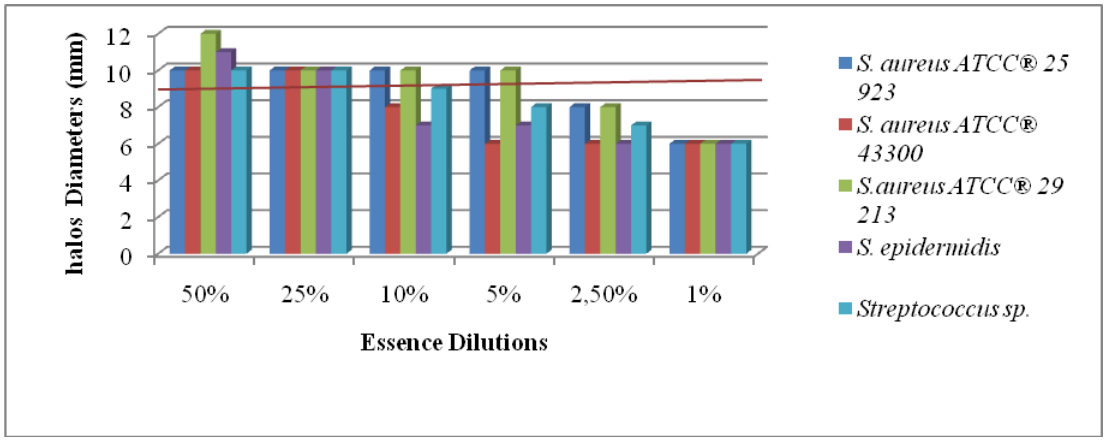

Figure 4: Graphical Representation of the Diameters of Inhibition Zones (mm) of the CMI of *C. paradisi* Essence

As for the essence of *C. paradisi*, two trends

When CMI less than 50 .mu.l of E. / Ml, in the presence of *S. aureus* ATCC®25 923, *S. aureus* ATCC®29 213. These bacteria are most susceptible to this species.

When MICs ente 250 and 100 .mu.l of E. / Ml, we have *S. aureus* MRSA + ATCC®43 300 *S. epidermidis* and *Streptococcus sp.* These bacteria are less sensitive than the first.

The results of the evaluation of CMI summarized in Figures 3, 4 show that the MIC values vary by three parameters: the tested Bacteria, the nature of the essential oil and the dose.

Community strains of *Staphylococcus* genus are generally resistant to penicillin G and A, but sensitive to penicillin M. They are often sensitive to macrolides, synergistines, fluoroquinolones (Fauchère and April, 2002).

According to WHO, the pathogenic strain *S. aureus* ATCC® 43300 is sensitive to Gentamicin (12-19 mm diameter). This result is similar to those of the inhibitory activity of the essence of *C. paradisi*.

DISCUSSIONS

By studying the chemical composition of essential oils of *C. sinensis*, Moufida and Marzouk (2003) confirmed that these essential oils consist mainly of limonene. This compound varies between 68% and 98%, hence α-pinene is presented only in low levels (0.2% and 10.23%).

It is noted from this analysis that the acyclic compounds such as nerol and geraniol are absent in the species *C. aurantium* and C. *sinensis*. Gancel et al (2005) found the presence of these two compounds only in the essence of *C. limonum* .Several studies (Moufida and Marzouk, 2003; Belleti and *al*., 2004;. Rehman and *al*., 2004) showed that generally Citrus essential oil was consisting mainly of monoterpene compounds (97%). Whereas other compounds, such as alcohols, aldehydes and esters are represented with low contents of from 1.8 to 2.2%.

Nogata and *al*., 2006, contested that flavonoids found in *Citrus* oils represented the non-volatile portion.

According to WHO vancomycin may inhibit *Streptococcus sp*. (A diameter of 20 mm). Compared with our

results, the essence of this variety has a good inhibitory effect on pathogenic bacterial strains.

The essence of *Citrus reticulata* showed antibacterial activity against Gram (-) and Gram (+), a special event was recorded for the species: *P. vulgaris* and *P.mirabilis*. The susceptibility of bacteria is independent effect of Gram (Dorman and Deans, 2000), or depends on the used essential oil (Deans and Ritchie, 1987).

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Similar results were recorded with other types of essence Akin and Aktumsek, (2009), testing the essential oil of *Eucalyptus camaldulensis* against *S. aureus, P. aeruginosa* and *E. coli*, they reported that *S. aureus* was the only one to show some sensitivity to Eucalyptus. This greater Gram-positive bacteria (*S. aureus*) sensitivity against essential oil has already been observed by several authors: Cox and *al*. (2000). Freidman M. and *al*., (2002).; Burt, (2004) and Lefsih and *al*., (2010). Oussalah and *al*., (2007) which noted that *S. aureus* was four times more sensitive than *E. coli* and *Salmonella typhimurium* to the action of essential oil savory.

The antibacterial properties of EO are partly related to their composition leading to the accumulation in bacterial walls, thus disturbing the operation and the permeability of cell membranes, cell wall degradation (Helander and *al*., 1998), injury cytoplasmic membrane (Knobloch and *al*., 1989; Ultee and *al*., 2002), damage of membrane proteins (Juven and *al*., 1994; Ultee and *al*., 1999), leakage of the contents cells (Oosterhaven and *al*., 1995; Lambert and *al*., 2001), coagulation of cytoplasm and depletion of proton motive force (Ultee and *al*., 2002).

The essence of *Citrus unshiu* showed antibacterial activity against Gram (-) and Gram (+), a special event was recorded for the species: *P. vulgaris* and *P.mirabilis.* The susceptibility of bacteria is independent effect of Gram (Dorman and Deans, 2000), or depends on the used essential oil (Deans and Ritchie, 1987).

Piccaglia and *al*. (1993) studied the antibacterial power of savory (Saturejamontana) essential oil, they recorded variables activities against target bacteria. Indeed, gram negative *P. aeruginosa* showed some resistance with diameter of 7.1 mm, against *E. coli* (Gram negative) and *S. aureus* (Gram positive) showed a sensitivity which is sensitive with diameter of 13.5 mm, the highly sensitive 18.4 mm, and respectively.

The different effects that we have observed between Gram-positive and Gram-negative bacteria may be due to the hydrophobic character of the essence that may have increased the permeability of the cell membrane.

The activity of an essential oil is to be related to its chemical composition (depending on the nature of the functional groups). But with the proportions of these various components (Degryse and *al*., 2008) ; the authors considered that the chemical compounds of greater efficiencies and having the widest target are phenols (Thymol, Carvacrol), alcohols (α-Terpinene and Linalool), aldehydes, ketones, terpenes and more rarely (Pibiri, 2005).

The present study showed that the antibacterial activity of essences from *Citrus paradisi* and *Citrus reticulata* may in part be associated with its major components (Limonene, Caryophullene, β-myrcene and D-limonene, βpinene, α-pinene) respectively

The bacteriostatic activity of limonene has been demonstrated against several microorganisms (Bakkali and *al*., 2008;.Sokovic and van Griensven 2006;.Donsi and *al*., 2011;. Singh and *al*., 2010). Limonene belongs to the family of cyclic monoterpenes, which are accumulated in the microbial cell membrane and cause a loss of membrane integrity and the dissipation of the proton motive force (Sikkema and *al*., 1994).Moreover,pinene has been shown inhibitory activity against many organisms (Bakkali and *al*., 2008;.Sokovic and van Griensven., 2006; Jiang and *al*., 2011).Pinene can destroy the cellular integrity and inhibit respiration and ion transport processes. Furthermore, pinene can also increase the permeability of the membrane (Uribe and *al*., 1985).It is not only the major compounds of the species that are responsible for this antibacterial activity, but there may also be other minor compounds that can interact in a synergistic or antagonistic way to create an effective system against bacteria(Sokovic and *al*., 2007)

Conner and Beuchat (1984) and Trombetta and *al*., (2005) suggested that the antibacterial activity of essential oils of herbs and spices or their components could be the result of damage or disruption of several enzymatic cellular systems, there including the production of energy and synthesis of structural components.

CONCLUSIONS

The extraction of *Citrus* zest by cold expression was successful because it gives good quality organoleptic and physico -chimique comparable to those obtained by AFNOR. Chromatographic analysis GC / MS species revealed: an abundance of monoterpene compounds dominated by the presence of limonene (94.75% for the essence of *Citrus reticulata* and 82.98% which represents the essence of *C. paradisi*).

Among the 17 pathogenic bacteria tested (reference and isolated), we noted the half of the tested strains are sensitive to the essence of *C. paradisi* with a diameter ranging from 11 to 16 mm for *P. mirabilis, Serratia sp.* and 17-19 mm for very sensitive (*S. aureus* ATCC®25 923, *S. aureus* MRSA + ATCC® 43300, *E. coli* ATCC®25 922, *S. epidermidis*) and 21 mm or more, for extremely sensitive (*S. aureus* ATCC®29 213, *P. vulgaris* and *Streptococcus sp.*). The second halves are resistant to the essence of this variety.

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Sensitivity tests to evaluate "*in vitro*" antibacterial activity by determining the minimum inhibitory concentration (MIC) of essences extracted a set of pathogenic bacteria isolated from different collection areas. The essence of *Citrus reticulata* an MIC of between 500 and 250 .mu.l of E. / ml vis-à-vis the strain *S. aureus* ATCC® 25 923, 50 .mu.l of E. / ml in respect of the strain *B. subtilis* ATCC® 6633, 25 .mu.l of E. / ml against the strain *S. aureus* 29 737 ATCC®, 3.90 .mu.l of E. / ml against the *S. epidermidis* strain.

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