Impact of the Composition of the Essential Oils of *Citrus sinensis* (orange) and *Citrus limonum* (lemon) on the Microbiological Activity

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Abstract

The composition of the essential oils of Citrus sinensis (orange) and Citrus limonum (lemon) showed a microbiological activity. These essential oils were extracted by hydrodistillation from the peel of citrus fruit of Citrus sinensis and Citrus limonum. Their antibacterial activity was determined in vitro by two different methods (Aromatogram and Vapor phase methods) on 07 strains (04 bacteria, 02 yeasts and 01 fungus). The Screening highlighted that the two essential oils either pure or diluted had a remarkable bacteriostatic activity towards the growth of certain bacterial strain. Also, these two essential oils presented a strong power antifungal. It is clear from this study that the essential oil of Citrus limonum presented an antibacterial activity higher than the essential oil of Citrus sinensis against the same bacteria.

Keywords: *Citrus limonum; Citrus sinensis; Essential Oils; microbiological activity.*

I. Introduction

The essential oils occupy an important place in man daily life. They have very interesting properties which find their applications in various fields such as medicine, pharmacy, cosmetology, agriculture, etc. For instance the use of essential oils dates back to a very long time, since prehistorically man practiced already in its own way the extraction of odorous ingredients of plants [1].

The development of new therapeutic agents is essential to manage the bacterial resistance and the food oxidation phenomena. In this goal, the investigation of plants represents an inestimable potential for the development of new antimicrobial substances. The essential oils are beginning to gain a great interest as an important potential source of natural molecules [2]. They are the object of studies considering their possible use as an alternative for the treatment of infectious diseases [3].

The essential oils are one of the most important active ingredients of herbs with interesting and useful antibacterial and therapeutic activities which depend mainly on their chemical composition [4].

The sources of these aromatic plants are widely spread in the nature. Algeria has a series of important and various species. This was the stimulating factor for this work the objective of which was the extraction and the study of the chemical analysis and the biological activity of essential oils from two species belonging to the same botanical family, namely the Rutaceae family represented by the two Citrus limonum and Citrus sinensis species.

II. Materials and methods

A. Biological material

The *Citrus* fruit freshly harvested, cleaned, washed, and had their Barks cut into small pieces. During this study, two essential oils were tested in vitro on: 04 bacterial strains 02 Gram + and 02Gram-, 02 yeast; 01 fungus. These pathogenic germs are part of the ATCC collection and were supplied by the Institute Pasteur of Algiers. These species are often responsible for major problems of public health, due to their natural resistance to various antimicrobial agents. The bacterial strains were hospital strains isolated from the samples on the sick.

B. Chemical products

Dimethyl sulfoxide was used as solvent to dissolve essential oils. For culture media the followings were used:
- Mueller Hinton agar: for the study of the sensitivity of bacteria to the extracted essential oils.
- Sabauraud agar: for the study of the sensitivity of yeast and fungi still to the essential oils.
- Physiological water: for the preparation of bacterial suspensions.

C. Extraction process and characteristic of essential oils

The essential oils of *citrus limonum* and *Citrus sinensis* were extracted by hydrodistillation method with a Clevenger. A mass of 300 g of the fresh *Citrus* fruit peel was introduced into a 1 liter flask impregnated of distilled water. The whole was boiled off for 1 to 1.30 h. The vapors containing volatiles were condensed in a condenser and the essential oils separated from the water by density difference in the two phases.

The essential oils extracted was stored at a temperature of 6 ± 1°C in opaque glass vials hermetically closed to protect them from main degrading agents such as air, light and temperature variations.

The yield was expressed in percentage (%) and calculated as follows:

$$R\ (\%) = \frac{m_h}{m_v} \times 100 \qquad (1)$$

with R the essential oil yield in %, m_h and m_v the masses in grams of the essential oil and the plant, respectively.

The measurements of the refractive index were conducted using a Carl Zeiss Jena refractometer and consisted on placing a drop of essential oil on the refractometer prism, waiting for the temperature to stabilize and then carrying reading off the refractive index value. When the determination was different from 20°C, a correction was made according to the following expression:

$$\eta_D^{20} = \eta_T + 0.00045\ (T - 20°C) \qquad (2)$$

with η_D^{20} and η_T the refractive indices at 20°C and an ambient temperature T, respectively.

Rotary power was obtained using a Bioblock Scientific polarimeter (set to give 0 ° and 180 ° with the water). A priori the essential oils were diluted in a solution of ethanol 1g / 20 mL. Then a tube was filled with the test sample, ensuring that there were no air bubbles, and then placed in the polarimeter to read the angle of rotation of the sample. Rotary power is given by the following expression:

$$[\alpha]_D^{20} = \frac{\alpha}{(L \times C)} \qquad (3)$$

with L the film thickness (cell) in dm, C the concentration of the essential oils expressed as (g/100mL) and α an angular value read off from the device in degrees.

D. Study of antimicrobial activity of the essential oils

- *Aromatogram*

A series of dilution of essential oils of 1/2, 1/4, 1/8 with an organic solvent (Dimethyl sulfoxide (DMSO)) was carried out. Antimicrobial tests were made from young cultures of 18 to 24 hours. The transplanting of stem was made by seeding of microbial strain in a liquid medium. The used seeds were grown on nutrient agar; the boxes so seeded were incubated at 37°C for 18 hours for the bacteria and at 25°C for 48 hours for fungi and yeast.

Using a sterile clamp, Whatman sterile paper discs of 8 mm diameter were soaked with essential oils only by keeping in contact the end of the disc with the essential oil which was absorbed gradually until complete impregnation of the entire disc which was then placed on the agar surface. After diffusion of the essential oils in agar for 4 hours at room temperature the boxes were incubated at 37°C for 24 hours for bacteria and 25°C for 48 hours for fungi and yeast.

Soaking with DMSO has been made and the boxes were incubated for 24h at 37°C. Reading took place after incubation by measuring the diameter of the inhibition zone (diameter of the disc included) which expressed the results. The appearance and size of the diameter of the inhibition zone reflected the impact of essential oils on bacterial strains. Thus, they were classified as sensitive or resistant or sensitive.

- *Vapor phase method*

This technique allowed evaluating the antimicrobial activity of the volatile phase of essential oils .The procedure consisted in inoculating an agar with a microbial strain. Disks of diameter 4, 6 and 8mm were deposited in the center of the lid and quantities of the essential oils of 20, 40 and 60µl were taken using a micropipette.

The box was incubated lid down and an evaporation of volatile substances occurred. By volatilizing, essential oils inhibited the growth of the germs by creating a zone of inhibition.

III. Results and Discussion

A. *Essential oil yield*

The average yields of *Citrus limonum* and *Citrus sinensis* obtained through extraction by hydrodistillation were as shown in the following table:

Table 1: Yields of *Citrus* essential oils in (%)

Essential oils	Citrus limonum	Citrus sinensis
Yield (%)	0.24	0.12

According to the results reported in the literature, it is clear that citrus fruits contained a small amount of essential oils. The results obtained in this work were almost similar to those reported in the literature. Indeed, Jeannot *et al.* observed a yields ranging from 0.25 to 0.57% for *Citrus aurantium* essential oils; 0.1 to 0.6% for *Citrus sinensis* essential oils and 0.2 to 0.9% for *Citrus limonum* essential oils [5]. However, they reported that Citrus essential oils yields differ depending on the species and unexpectedly reported yields of 1-3% [6]. This difference could be explained in [7] by the choice of the harvest period, the climate, the geographical area, the genetics of the plant, the organ of the plant used, the degree of freshness, the period of drying, the method of extraction used, etc...

B. *Physical analyses*

The physical properties such as the refractive index, the rotatory power etc. are means of verification and control of the quality of the essential oil. These tests were determined according to a specific protocol and obeyed to AFNOR standards.

Table 2: Physico-chemical composition of the *Citrus* essential oils

Essential oils	η_D^{20}	$[\alpha]_D^{20}$ ($g^{-1}dm^{-1}ml$)
Citrus limonum	1.474	+65
Citrus sinensis	1.471	+90

The refractive index of *Citrus sinensis* and *Citrus limonum* essential oils were substantially identical. The values of the Index of refraction reflect the composition of the essential oils with respect to certain compounds.

The values of the refractive index of the considered samples corresponded to the AFNOR standards (1470 - 1478).

The values of the rotatory power of *Citrus sinensis* and *Citrus limonum* essential oils were positive, and deviated the plan of the polarized light to the right, showing that they were dextrorotatory.

The value of the rotatory power of the *Citrus limonum* essential oils was higher than that of the *Citrus sinensis* reflects the richness of the *Citrus limonum* essential oils for chiral compounds.

C. *Analyzes of the chemical composition of essential oils*

The determination of the physicochemical properties is a necessary step, but not sufficient to characterize the essential oils therefore it is necessary to complement it by chromatographic analyzes.

- *ultraviolet-visible spectrophotometry*

The analysis by the UV-VIS gave the following Spectrum:

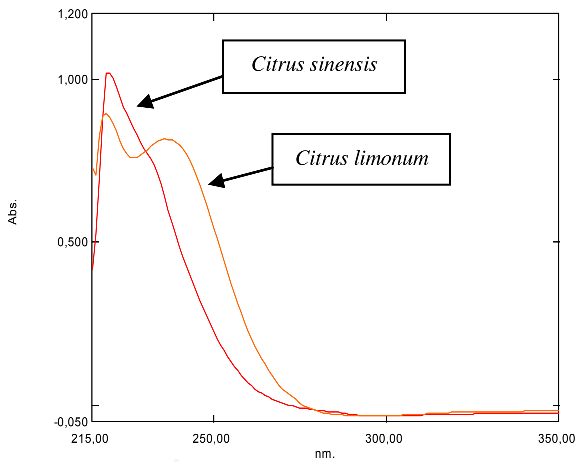

Figure 1: UV-VIS spectrum of the two essential oils of *Citrus*

The essential oils of *Citrus sinensis* and *Citrus limonum* absorb in the UV between 205 and 240 nm. On the UV spectrum, two absorption bands were visible, the first band at 236 nm and the second located at 205 nm. The one identified at 236 nm was important in the case of the essential oil of *Citrus limonum* but in the case of the essential oil of Citrus sinensis it was weakly visible. This band was allocated to electronic transitions л-л* of the carbonyl group in the two oils to justify the presence of the compounds of the same electronic transition type л-л* characteristic of existence of double bond in the chemical structure of the compounds of the essential oil.

- *Fourier Transform Infrared spectroscopy (FTIR)*

The analysis by FTIR gave the following Spectrum:

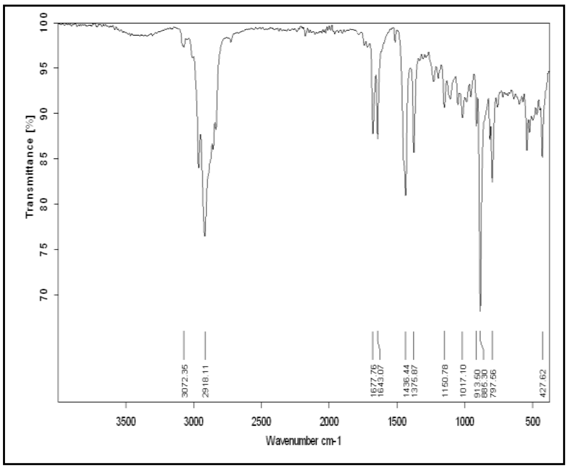

Figure 2: FTIR spectrum of the essential oil of *Citrus limonum*.

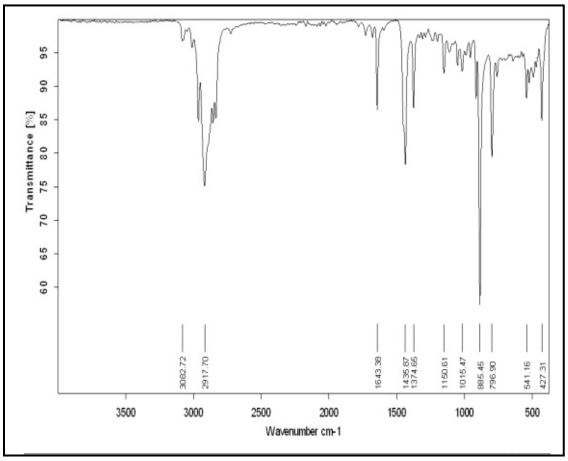

Figure 3: FTIR spectrum of the essential oil of *Citrus sinensis*.

The ATR analysis of essential oils of *Citrus sinensis* and *Citrus limonum* allowed the obtention of an interesting IR spectrum. In the region 2850-3100 cm^{-1}, for the two oils the presence of the vibration C-H of alkenes to 3082-3072 cm^{-1} and the vibration C-H of the groups $-CH_2$ and $-CH_3$ between 2980 and 2850 cm^{-1}, were observed.

The spectrum of the essential oil of *Citrus limonum* had a peak which was not present in the spectrum of the essential oil of *Citrus sinensis* and it was located at 1677 cm^{-1} characteristic of the carbonyl function. In addition to this difference, the size of the peaks of the vibration C-H of the groups $-CH_2$ and $-CH_3$ was important in the IR spectrum of the essential oil of *Citrus limonum* to confirm its composition compared to that of the essential oil of *Citrus sinensis*. It should be noted also the presence of the connection -C=C- to 1643 cm^{-1} in the two IR spectrum.

D. *Biological activity of essential oils*

- *Aromatogram*

Table 3. Average halos inhibition in (mm) caused by the essential oil of *Citrus sinensis* against bacterial strains of Gram-.

	Dose of essential oils				
Bacterial strains (Gram-)	1.25%	12.5%	25%	50%	100%
Pseudomonas aeruginosa	-	-	-	-	-
Escherichia coli	-	-	-	12	15

The bacterium *E. coli* is the only one that is sensitive to the essential oils of *Citrus sinensis* for doses of 5 and 100%, while *P. aeruginosa* was resistant to the essential oils of *Citrus sinensis* either pure or diluted.

Table 4. Average halos inhibition in (mm) caused by the essential oil of *Citrus sinensis* against bacterial strains of Gram+.

	Dose of essential oils				
Bacterial strains (Gram+)	1.25%	1.25%	25%	50%	100%
Staphylococcus aureus	-	-	-	15	20
Bacillus ceureus	-	11	16	20	35

The bacteria *S. aureus*, *Bacillus* showed an important zones of inhibition foe essential oils of *Citrus sinensis*.

For dilutions prepared from the essential oils of *Citrus sinensis*, the bacterium *Bacillus* was the most sensitive.

Table 5. Average halos inhibition in (mm) caused by the essential oil of Citrus sinensis against fungal strains.

	Dose of essential oils				
Fungal strains	1.25%	12.5%	25%	50%	100%
Candida albicans	12	14	18	22	30
Aspergillus flavus(ASP)	11	13	15	30	40
Sacar(S.S)	11	12	15	15	30

Fungi and Yeasts (*ASP, S.S,* and Candida) had a high sensitivity to the essential oils of *Citrus sinensis* pure, even diluted with diameters of inhibition that varied between 30 to 40 mm and between 11 to 18 mm for the pure and diluted essential oils, respectively.

Table 6. Average halos inhibition in (mm) caused by the essential oil of *Citrus limonum* against bacterial strains of Gram

	Dose of essential oils				
Bacterial strains (Gram-)	1.25%	12.5%	25%	50%	100%
Pseudomonas aeruginosa	-	-	-	13	20
Escherichia coli	-	-	11	20	50

The essential oil of *Citrus limonum*, pure and diluted, showed a significant inhibition on the growth of the bacteria tested in Gram- The diameters of inhibition around discs are important for the bacteria *P. aeruginosa.*

Table 7. Average halos inhibition in (mm) caused by the essential oil of *Citrus limonum* against bacterial strains of Gram+.

	Dose of essential oils (%)				
Bacterial strains (Gram+)	1.25	12.5	25	50	100
Staphylococcus aureus	-	-		20	25
Bacillus ceureus	-	11	15	30	50

The sensitivity of the bacterium *Bacillus* for the diluted and pure essential oil of *Citrus limonum* was important. The diameters of inhibition were important for the bacterium *S. aureus* for the doses of 50 and 100% only.

Table 8. Average halos inhibition in (mm) caused by the essential oil of *Citrus limonum* against fungal strains.

	Dose of essential oils (%)				
Fungal strains	1.25	12.5	25	50	100
Candida albicans	12	15	20	30	44
Aspergillus flavus(ASP)	12	15	15	35	42
Sacar(S.S)	-	12	25	35	44

However, the diameters of the aureoles of inhibitions of the two diluted essential oils did not exceeded 30 mm. It should be noted that the antibacterial power of the essential oil of *Citrus limonum* was more important than that of *Citrus sinensis*.

It is also noted that the antibacterial activity was proportional to the concentration, the more the essential oil was concentrated, the more the zone of inhibition was extended, indicating the decrease of the bacterial growth.

The two essential oils had demonstrated very interesting antibacterial activity towards the fungal strains. Again the essential oil of *Citrus limonum* proved an antibacterial power more important than that of *Citrus sinensis*.

The observation showed that the different dilutions of the two essential oils had induced inhibition zones. The dilutions were more effective for fungal strains (*ASP, S.S* and Candida).

However the strain of *P. aeruginosa* had a great resistance for the essential oil of *Citrus sinensis*. According to [8] the sensitivity of a microorganism to the essential oils depends on the property of the essential oil and the microorganism itself and in [9] it is confirmed that the Gram+ are more sensitive to the antimicrobial action of the essential oils than the Gram-.

In fact, the Gram- had a resistance to biocidal agents with the nature of their bacterial wall. In contrast, few publications reported that there is no apparent link, nor any correlation between the antimicrobial activity of the essential oils and the nature of the bacterial cell wall [10].

The strains of the genus *Pseudomonas* proved to be more resistant to the essential oils. This resistant was not surprising and was confirmed by several previous studies such as in [11].

- *Vapor phase method*

In order to evaluate the antimicrobial activity of the vapor phase of the essential oils, the vapor phase method was used with three different doses (20, 40 and 60µl of essential oils)

In vapor phase method, bacterial strains Gram+ and Gram- were very resistant to the pure essential oil of *Citrus sinensis*.

Table 9. Average halos inhibition in (mm) caused by the essential oil of *Citrus sinensis* against fungal strains.

Fungal strains	Dose of essential oils		
	20µl	40µl	60µl
Candida albicans	25	40	46
Aspergillus flavus(ASP)	17	34	65
Sacar(S.S)	-	36	40

For the essential oil of *Citrus limonum*, the bacterial strain Gram+ *Bacillus ceureus* was the only one which had given a zone of inhibition in vapor phase method.

Table 10. Average halos inhibition in (mm) caused by the essential oil of *Citrus limonum* against bacterial strains of Gram+.

Bacterial strains (Gram+)	Dose of essential oils		
	20µl	40µl	60µl
Staphylococcus aureus	-	-	-
Bacillus ceureus	10	12	23

Table 11. Average halos inhibition in (mm) caused by the essential oil of *Citrus limonum* against fungal strains.

Fungal strains	Dose of essential oils		
	20µl	40µl	60µl
Candida albicans	37	50	66
Aspergillus flavus(ASP)	40	54	66
Sacar(S.S)	-	37	56

E. A comparative study between the Aromatogram and vapor phase method

The effectiveness of the essential oils depended on its chemical composition where the more the essential oil was rich in active substances, the more was important its activity. The biological activity of the essential oils was linked to its chemical composition, to the functional groups of the major compounds (alcohols, phenols, terpenes and ketonic).The minor compounds also played an important role in the activity of the essential oils and seemed to act in synergy with the main compounds [12].

All of the obtained microbiological results during this study showed that all products tested had a very important antibacterial activity and antifungal, in which some strains appeared to be distinguished by a very high sensitivity, including the (L)-limonene (80%) which was the major constituent of essential oil of *Citrus limonum* and (D)- limonene (90%) which was the major constituent of essential oil of *Citrus sinensis*, it might be responsible for the strong fungicide power. Note that the essential oil of *Citrus limonum* which its major compound was the (L)-limonene showed a very strong microbial inhibitory action against the fungal strains compared to the essential oil of *Citrus sinensis*. During this study, it was noticed that the volatile fraction of the essential oil studied showed a better antifungal activity compared with the method of direct contact on agar. This result was in good agreement with the literature data. The explanation of these observations could be attributed to the low solubility of the essential oils in water and therefore in the agar medium due to their hydrophobic character. Also the volatile and hydrophobic character made these oils easy to be absorbed by the fungal mycelium by the direct contact on agar.

This behavior can be explained by the lipophilic nature of the fungal tissue and also by the high water content in the agar. In the literature, it appears that the vapor phase method is less considered with only few papers. The studies carried out did highlight that the vapor phase was inhibitory to the growth of the sprouts than the liquid phase. This was confirmed in particular on the fungal strains [13].

Also the use of essential oils in the vapor phase seemed to be a protocol of promising control which could be applied in fumigation. For example, there might incorporate essential oils in the packaging of food and since these substances were volatile so it would inhibit and eliminate any bacterial and fungal contamination without the slightest contact with the food. This finding was confirmed by a recent study [14]. The antimicrobial properties of essential oils of *Citrus* were considered for the first time in 1948 [15]. The species of *Citrus* in solution were reported to be more powerful than phenols as disinfectants in [11]. According to [16] the essential oils of *Citrus* from sweet orange, lemon, mandarin and grapefruit showed an antifungal activity against *Aspergillus niger*, *A. flavus*, *Penicillium chrysogenum* and *P. verrucosum*. It was also established according to Cox that typically the fungi were more sensitive than bacteria [17].

It appeared clearly that the inhibitory action of the vapor phase method was lower than that of the liquid phase (Aromatogram) for bacterial strains. Only, the fungal strains showed a high sensitivity to the essential oils for the three doses. Similarly to the Aromatogram there was a relationship between the amount of essential oils by disk and the diameter of the inhibition.

IV. CONCLUSION

The present work focused on the impact of the composition of the essential oils of *Citrus sinensis* (orange) and *Citrus limonum* (lemon) on the microbiological activity. The extraction of the aromatic essence of these two *Citrus* fruits was carried out in the Department of process engineering. The essential oils of *Citrus limonum*, and *Citrus sinensis* were analyzed according to the standard method AFNOR in order to determine the usual physical constants: refractive index, rotating power. The obtained results were in accordance with the standards. The chromatographic analyses of the two essential oils clearly showed the better value of the essential oil of *Citrus limonum* compared to that of *Citrus sinensis*.

The estimate of the antimicrobial activity of essential oils was achieved on several microbial strains of reference (ATCC). For this two qualitative methods were used (Aromatogram and vapor phase methods) with increasing doses. The obtained results were very encouraging, the experiments showed a very important inhibition in the growth of bacteria tested. The diameters of inhibition, around the disks were important for all the bacteria tested (*S. aureus, E. coli, Bacillus, and P. aeruginosa*) with diameters of inhibition which varied between 15 and 50 mm, even for the fungal strains the sensitivity of bacteria towards the two essential oils was very important with diameters of inhibition that varied between 30 to 44 mm. By contrast, in the vapor phase method, the two essential oils presented an inhibitory action on only the fungal strains.

It is important to note that the essential oil of *Citrus limonum* marked an inhibitory effect more important to those of the essential oil of *Citrus sinensis*. This was due to the difference in the chemical composition of these two essential oils from which the major compound of the essential oil of *Citrus limonum* was the (L)-limonene while the major compound of the essential oil of *Citrus sinensis* was the (D)-limonene. The (D)-limonene might be responsible for the strong power fungicide of these essential oils on the growth of the bacteria tested. Therefore the essential oil of *Citrus limonum* appeared to be an excellent product to bactericidal activity.

List of symbols

C: Concentration of the essential oils (g/100mL)
L: Cell film thickness (dm)
m_h: Masse of essential oil (g)
m_v: Masses of dry plant g)
R: Essential oil extraction yield (%)
T: Temperature (°C)
α: Angular value (Degrees)
η_D^{20}**:** Refractive index at 20°C
η_T**:** Refractive index at Temperature T

Abbreviations

AFNOR: French standards Association.
ASP: *Aspergillus flavus.*
ATCC: American Type Culture Collection.
Bacillus: *Bacillus ceureus.*
Candida: *Candida albicans.*
Citrus limonum**:** Lemon.
Citrus sinensis**:** Sweet orange.
DMSO: Dimethyl Sulfoxyde.
E.coli: *Escherichia coli.*
Gram+: Gram positive.
Gram-: Gram negative.
IPA-Algiers: Pasteur Institute of Algeria.
P. aeruginosa*: Pseudomonas aeruginosa.*
S. aureus : *Staphylococcus aureus.*
S.S : *Sacar.*

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