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VALORIZATION OF CITRUS SINENSIS WASTES FOR THE PRODUCTION OF ORANGE ESSENTIAL OIL: IDENTIFICATION OF CHEMICAL COMPOSITION AND ANTIBACTERIAL ACTIVITY

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ABSTRACT

The objective of this study was to extract, characterize, and examine the antibacterial properties of orange essential oil obtained from citrus sinensis peels, using hydro-distillation method. Various components of the orange oil were characterized by FTIR, UV-Vis and GC-MS spectroscopic techniques. The extracted essential oil was conformed to AFNOR standards, and had a maximum yield of 3.4% (w/w). The obtained results showed that limonene was the major bioactive components of the orange essential oil (97.14%). The essential orange oil and its dilutions in DMSO showed an excellent antibacterial activity against Staphylococcus aureus (gram-positive bacteria) with a diameter of inhibition zone superior to 40 mm. However, it was ineffective antibacterial activity against Escherichia coli and Pseudomonas aerogenes (gram negative bacteria).

Keywords: Citrus sinensis; Orange essential oil; Antibacterial activity; Staphylococcus aureus; Escherichia coli; Pseudomonas aerogenes.

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1. INTRODUCTION

High demand for consumption of citrus fruits (*Rutaceae* family) such as oranges (*citrus sinensis*) led to production of 98 million tones worldwide in 2020 [1]. Among the citrus fruits (orange, lemon, mandarin, and grapefruit), various species of oranges account for slightly over half of the total production (54.84%) [2,3] .

Oranges contain micronutrients, macronutrients, vitamins, and minerals are either freshly consumed or used for juice production, which leads to an increase in production of wastes [4,5]. In fact, conventional ways of waste elimination are the cause of pollution in terrestrial and aquatic environments with potential negative impacts on aquatic ecosystem. Thus, the need to find cost-effective and environmental friendly waste management techniques and new processes to minimize the waste accumulation in the environment is of outmost importance [4]. One of the solutions is the valorization of citrus fruits wastes by the recovery of the bioactive molecules contained in peels. These bioactive compounds including zeaxanthin, cryptoxanthin, violaxanthin, luteoxanthin, auroxanthin, and other carotenoids [2]. For example, orange peels have many medicinal properties; they are widely used against various diseases, such as colic, upset stomach. Besides, the orange peels are used to produce diuretic, carminative, stomachic tonics as well as skin care products. Moreover, the peel wastes are utilized to treat and prevent vitamin deficiencies, colds, flu, scurvy and helping to fight viral and bacterial infections such as coronaviruses, influenza, dengue viruses and oral infections caused by *Staphylococcus mutans* respectively [6,7]. Several antibacterial effects of orange peel have been demonstrated in the literature [8]. Dubey et al., (2011) showed potent antibacterial activity of orange peel extract by disk diffusion method against several bacteria including *Pseudomonas aeruginosa, Staphylococcus aureus, Staphylococcus epidermidis, Shigella flexineri, Bacillus subtilis and Escherichia coli* [9]. Chabuck et al., (2014) reported the effectiveness of orange peel extract against *Klebsiella pneumoniae* bacteria [10].

Among other microorganisms, *Staphylococcus aureus* is one of the main bacterial pathogen strains that cause nosocomial infections [11,12] .Plant extracts and essential oils have always been used for different purposes [13]. Essential oils have been searched for their antibacterial, antifungal, antiviral, insecticidal, and antioxidant properties [11,14] . The citrus essential oils,

obtained from the peels are widely used as agents for drinks, ice creams, cakes, air fresheners, household products and perfumes [15]. They are also largely employed in aromatherapy [16], [17] . Antimicrobial activity of orange wastes are due to secondary metabolites such as flavonoids, terpenes and carotenoids [2] that are contained in peels extract [18].

Limonene, which is the main component found in the essential oil of citrus peels, is a by-product of the orange juice industry. It is used as a less toxic substitute for xylene in histopathology and microscopy [19]. Limonene taken as supplement or by consuming fresh oranges is quickly absorbed in the gastrointestinal tract in humans [20–22]. It also has a high distribution rate in various tissues within the human body and it is metabolized [23] .Thus, to obtain limonene and other components, essential oil should be produced from the orange peels. Essential oil is easily evaporating compounds that are insoluble in water. Essential oil can be generated from the plants tissue by using either distillation or extraction processes. Orange oil is usually extracted from the peels by either simple conventional methods such as distillation, solvent extraction, maceration, cold pressing, or by using modern techniques including effleurage, super critical CO_2 extraction and turbo distillation extraction [24,25]. These methods of extraction have their own advantages and disadvantages. The modern methods are expensive and difficult. Maceration, a process that transforms tissue into a suspension of intact cells, is not desirable as it changes the composition of oil. Solvent extraction leaves traces of solvent in the extract, which causes allergies and affects immune system. The residue of solvent could be problematic for either organoleptic and/or health reasons. Steam distillation is another separation process used for temperature sensitive materials including oils, resins, and hydrocarbons since these are insoluble in water and may decompose at their boiling point. The main advantages of using steam distillation process are that this process assists to obtain unaltered properties of the oils as well as quick and cost-effective compared with other extraction methods [26,27]. Thus, the objective of this study was to valorize *citrus sinensis* peel wastes by producing orange essential oil using hydro distillation as a simple and economical extraction technique. Therefore, responding to the environmental issue of the accumulation of citrus fruits wastes. In addition, the extracted oil was evaluate for antibacterial activity by agar disc diffusion method *Staphylococcus aureus (S.aureus), Escherichia coli*

(E.coli) and *Pseudomonas aerogenes (P.aerogenes)*, which often cause food-transmitted disease and food spoilage.

2. MATERIAL AND METHODS

2.1. Extraction of essential oil from orange peels using hydro-distillation method

Various species of oranges such as sweet Thompson and Washington navel were purchased in whole from local markets of Bouira in Algeria in February and March of 2021. Whole oranges were washed by tap water, then peeled and their edible portions were separated. Then, the orange peels were cut into small pieces and dried using distilled water oven at 25 °C. To obtain orange oil from the peels a steam distillation using hydro-distillation technique was used for 3 h [28] . 50 g of the peels were placed in a round bottom flask (500mL) and filled with water to about three quarter full. A clear oily film was formed on top of the aqueous distillate inside the round flask. The oily film was separated and dried using anhydrous sodium sulphate (Figure 1). The extracted essential oil was placed in airtight sealed glass vials covered with aluminum foil. Then these vials were placed in a freezer at 4 °C until further analyses. The chemical composition and structure of the obtained essential oil were identified and quantified using UV-Vis, Fourier Transform Infrared (FT-IR) and GC-MS spectroscopic techniques. Furthermore, the evaluation of antibacterial activity of the essential oil by agar disc diffusion method was carried out against three important bacterial strains (*Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aerogenes*). For the analyses, all analytical grade chemicals including Dimethyl sulfoxide (DMSO) $(CH_3)_2SO$, anhydrous sodium sulphate ($Na_2SO_4 \cdot 7H_2O$), and distilled water were purchased from Sigma-Aldrich.

Fig.1. Extraction of orange essential oil by hydro-distillation

2.2. Physical and chemical characterization of orange essential oil

2.2.1. Calculation of oil yield

The oil yield indicates the potential of essential oil production of the used products. It is calculated to determine the amount of oil that can be derived from different parts of the used plant or fruit. In this study, oil yield of *citrus sinensis* wastes was estimated. The yield of the essential oil extracted from the orange peels was calculated using Eq. 1 [29].

$$\%yield = (Weight\ of\ oil\ extracted/Weight\ of\ sample\ used)X100 \qquad Eq.1$$

2.2.2. Measurement of Relative Density at 20°C

Quality assessment of essential oil is determined by measuring different physical and chemical parameter such as relative density. Effectively, relative density of oil can permit to distinguish between original and fraudulent oils. In addition, it can determine the potential use of the obtained oil. Essential oil has higher density than water According to the AFNOR Standard (NF T 75 – 111), the relative density of the essential oil at 20°C is determined as the mass ratio of a specific volume of oil and the equal mass of volume of a known substance such as distilled water [30]. The relative density is measured using a precise method called volume pycnometer (i.e., 5 ml at the temperature of 20°C).

2.2.3. Measurement of the Refractive Index

The refractive index is a ratio to indicate the ability of the essential oil to reflect light. This index is an important measurement that indicates potential rancidity development in oil. For example, if an oil has higher reflective index, then the chances of spoilage caused by oxidation are higher [31, 32] .The Refractive Index (Standard NF T75 - 112) of the essential orange oil were measured using a Prisma-CETI convex refractometer. If this measurements is conducted at a temperature other than 20°C, then a the following Equation (Eq.2) should be applied to order to adjust for temperatures greater or lesser than 20°C [33] :

$$I20 = It + 0.00045(T - 20°C) \qquad \text{Eq. 2}$$

Where, I_{20} = Refractive index at 20°C, I_t = Refractive index at ambient temperature, and T = Ambient temperature (°C). I_t= refractive index at laboratory temperature.

2.3. Spectroscopic characterizations

Fourier Transform Infrared Spectroscopy (FTIR) spectrum of orange peels oil using a Perkin-Elmer 1000- type spectrophotometer was between 400 and 4000 cm^{-1}. UV-Vis spectrum of the extracted oil diluted in DMSO using a Unicam UV 300 spectrophotometer ranged from 250 to 400 nm.

The GC-MS analysis was performed using HP Agilent 2890 system operating at 70 eV equipped with a split/splitless injector (250°C), a split ratio of 1/80 was achieved using a HP-5MS capillary column (30 m ×0.25 mm) and film thickness: 0.25 µm.

2.3.1 Antibacterial screening

Three microorganisms (*Staphylococcus aureus, Escherichia coli, and Pseudomonas aerogenes)* used for this study were obtained from the culture collections of the pathology laboratory of Bouira hospital in Algeria). To maintain and grow the bacterial strains, a culture on nutrient agar medium was used. To screen *in-vito* antibacterial activity, disk diffusion and dilution essays were conducted [34]. Standard size sterilized Whatman's disk filter paper (6 mm diameter) was used in this study. For the dilution essay, orange essential oil was mixed with DMSO (a non-toxic dispersing agent) at the dilutions factors of $1, 1/2, 1/4, 1/8,$ and $1/16$. Following, the filter paper disks were impregnated with 10 µL of prepared solution. Bacterial strains were surface inoculated in Petri dishes containing nutrient

agar and the impregnated discs were placed on the pre-inoculated agar surface. An impregnated disc with DMSO was used as a negative control to ensure its ineffective antibacterial activity. The petri dishes were incubated at 37°C for 24 hours. After incubation the results were read and expressed as mean of inhibition zones hereafter expressed as millimeters (mm).

3. RESULT AND DISCUSSION

3.1. Physical characterization of extracted essential oil

The extraction kinetics of the essential oil by hydro-distillation was conducted at different times for 3 hours with an interval of 25 minutes that indicated an increasing curve from the start until it reached 145 min. of hydro-distillation, and then the curve has reached a plateau with a yield equal to 3.4% (Figure 2). The obtained yield percentage is good and in accordance with previous conducted studies [35].

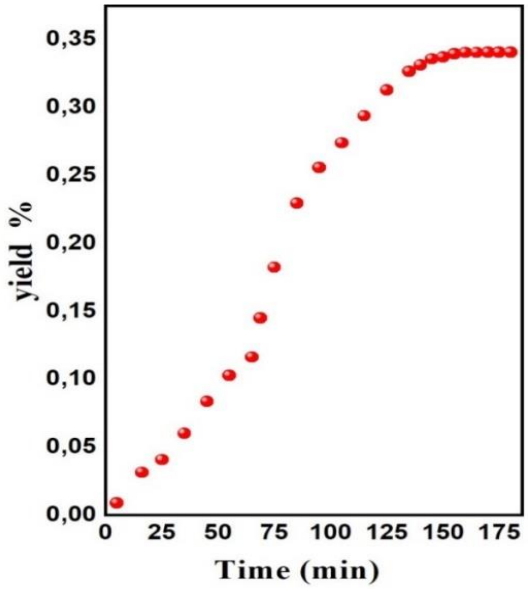

Fig.2. Evolution of essential oil yield as a function of time measured from using fresh orange peels

The organoleptic properties of essential oil are based on its smell, appearance and color. The essential oil from orange peels has a transparent liquid appearance, with a pleasantly perfumed scent of limonene. Its pH is slightly acidic (5.57), which neutralizes the microorganisms [36].

The extracted oil is characterized by a low ester index of 9.05, which allows a long shelf life. Physical characteristics of essential orange oil obtained in the current study are demonstrated in Table 1.

Table 1. Physical characteristics of essential oil from orange peel. The refractive index was calculated at 20°C

Characteristic of orange essential oil (unit)	Value
Color	Transparent
Odor	Fresh and tangy smell
Solubility	Insoluble in water
Density (g/l)	0.85
pH	5. 57
Refractive index	1.4706
Ester index	9.058

The density of an essential oil is a very important criterion that determine the quality of an essential oil for various use (i.e., cosmetics, pharmacy, and food industry). In addition, it can permit to easily distinguish the natural essential oil from fraudulent or altered ones. As shown in Table 1, the density of the extracted essential orange oil from this study at 0.85 (g/l) was within the acceptable range (0.850 to 0.870 g/l) indicated by the international standards organization such as the French Association of Standardization (AFNOR NF I. 75 – 202). Our results also indicated that the refractive index of the essential orange oil corresponded to the AFNOR standards range of (1.474 to 1.475) [31].

3.2. Fourier Transform Infrared (FTIR) and UV-Vi Spectroscopy

Although the determination of the physicochemical properties is a necessary step to characterize the essential oils, using this step alone do not provide sufficient information. Utilizing techniques such as GC/MS and infrared and UV-visible spectroscopy will better help identify essential oils characterizations. Thus, in this study, we used FTIR and UV-Vis analyses confirm the presence of limonene in the extracted orange essential oil. The results of limonene indicated peaks defines as infrared spectrums in the wavenumber at 2922 cm^{-1}, 1644 cm^{-1}, and

1423 cm^{-1} (Figure 3a). The obtained peaks in our study were within the range from 2850 cm^{-1} to 3100 cm^{1} which is a general range for limonene [37]. These peaks are assigned to C-H stretching and C=C vibration respectively. To determine limonene by UV-Vis analysis, the orange essential oil was diluted in DMSO. This analysis resulted in the UV-Vis spectrum ranged from 250 to 400 nm as shown in Figure 3b.The spectrum presented a strong band located at 259 nm and another band near 282 nm attributed to π→π* electronic transitions presented in cyclohex-1-ene ring and prop-1-en-2- yl group.

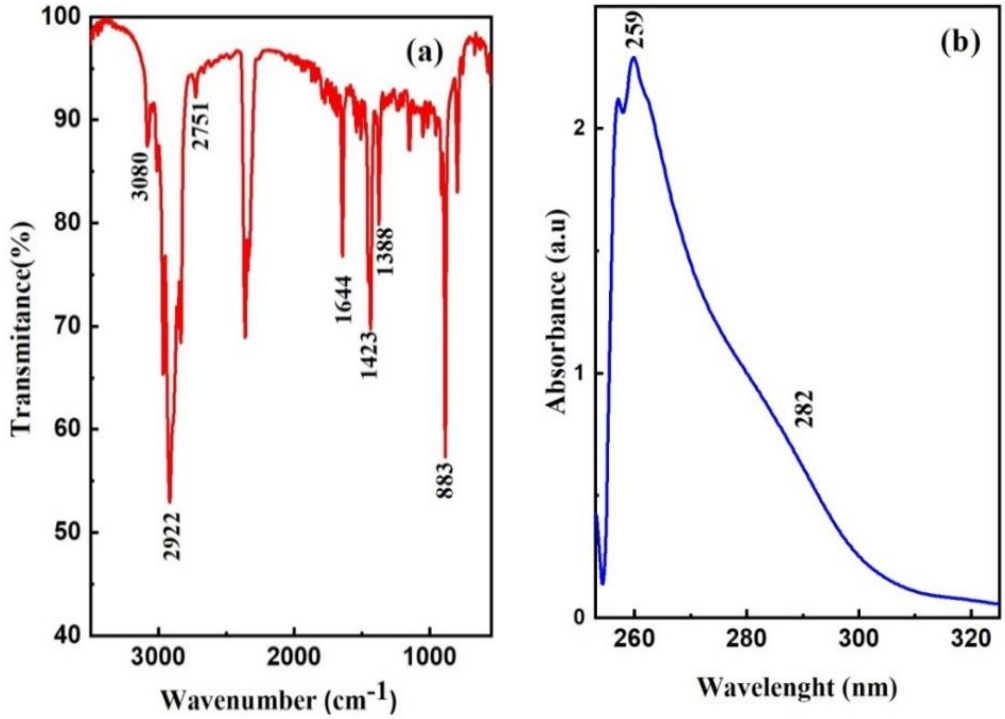

Fig.3. FTIR and UV-Vis spectra of limonene in orange essential oil

3. 3. GC-MS analysis

The orange essential oil was analyzed by GC-MS and resulted in the identification of seven components (Figure 4). The peaks were identified as limonene (97.1%), beta myrcene (1.5%), alfa pinene (0.38%), linalool (0.33%), sabinene (0.32%), decanal (0.16%), and octanal (0.14%). The obtained results were in accordance with previous research, in which limonene represented about 97% of orange peels oil [38].

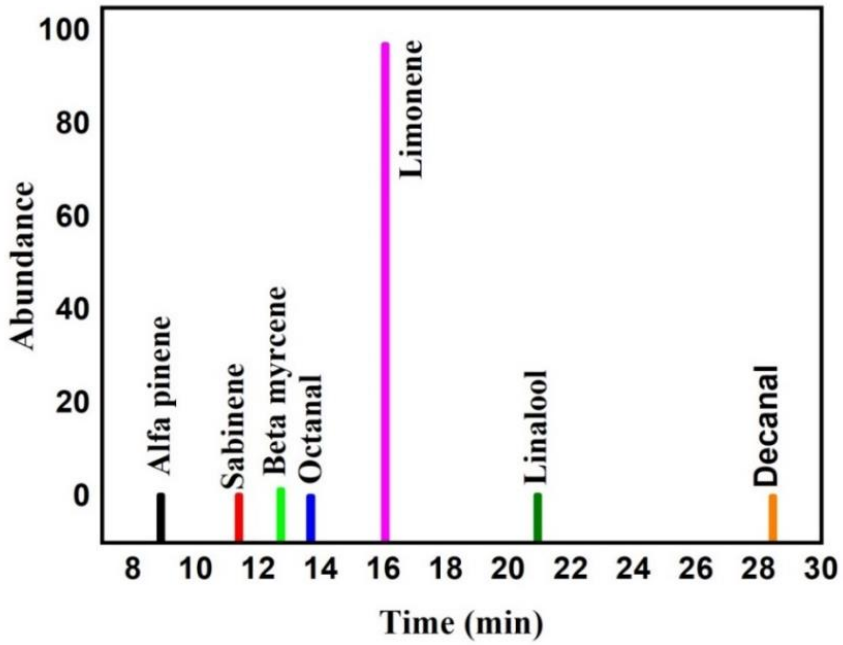

Fig.4. GC–MS Chromatogram of components of *citrus sinensis* peels oil including alfa pinene, sabinene, beta myrcene, octanal, limonene, linalool, and decanal

The screening for bioactive compounds of *citrus sinensis* wastes using UV-vis and FTIR analyses demonstrated that the major component was limonene. However, GC-MS chromatogram revealed the present of further six other components. Therefore, in phytochemical screening the three analyses must be performed together as they are complementary.

3.4. Antibacterial Screening

Susceptibility of Gram positive (*Staphylococcus aureus*) and Gram negative (*Escherichia coli and Pseudomonas aerogenes*) bacteria was determined against the obtained orange essential oil and DMSO as control. Our results of antibacterial screening indicated that all the prepared dilutions (1, 1/2, 1/4, 1/8, and 1/16) of essential oil in DMSO showed significantly higher antibacterial activity against Gram-positive bacteria (*Staphylococcus aureus)* (Figure 5 (c1), (c2)). However, there was no activity detected against Gram-negative bacteria (*Escherichia coli and Pseudomonas aerogenes)* (Figure 5 (a1), (a2) (b1), (b2)). This could be explained by the fact that Gram-negative bacteria are usually more resistant to antimicrobial agents since their outer-membrane permeability barrier has the ability to limits access of the antimicrobial agents to their targets in the bacterial cell [39, 40]. In this study, the inhibition

zone of the orange essential oil against Staphylococcus aureus was higher than the control, which could further confirm potential antibacterial properties of the essential oil extracted from orange peels as suggested by other studies [41,42].

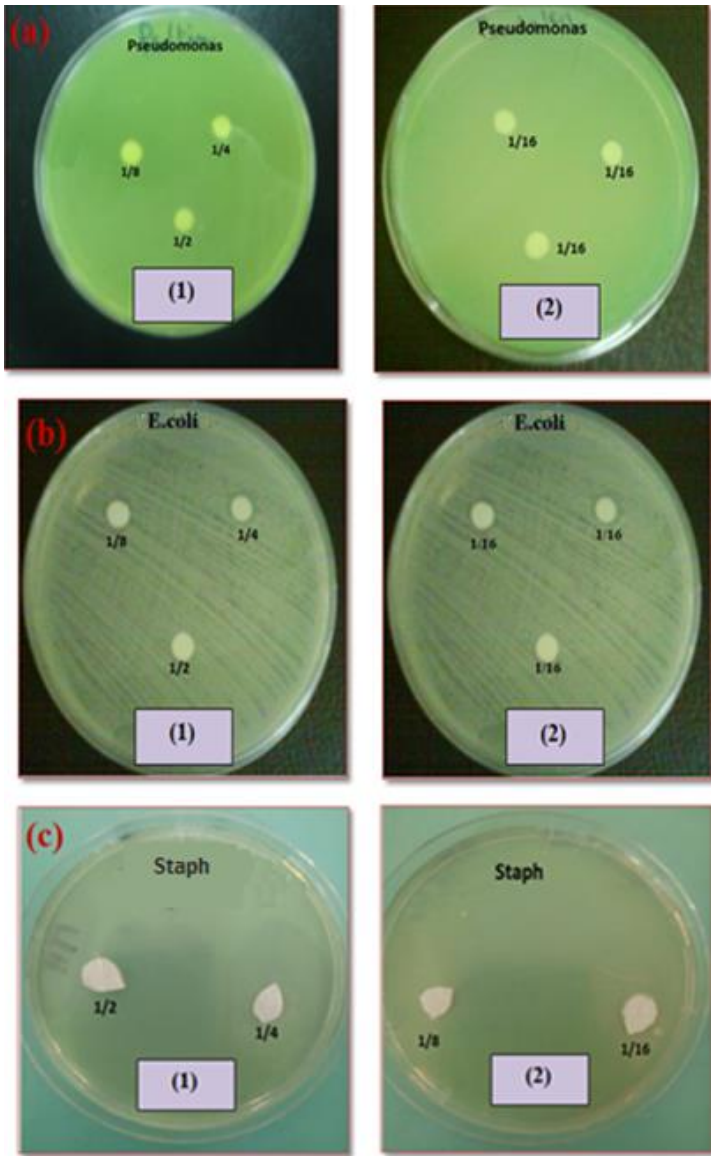

Fig.5. Antibacterial tests conducted for dilutions essential orange oil and DMSO with the ratio of 1, 1/2, 1/4, 1/8, and 1/16 for Gram negative bacteria Pseudomonas *aerogenes (a1 and a2), Escherichia coli (b1 and b2*) and Gram-positive bacteria (*Staphylococcus aureus*) *(c1 and c2)*

Table 2. Diameter of inhibition (D) zone of bacteria in the presence of essential oil

	Dilution					
Microorganisms	**1**	**1/2**	**1/4**	**1/8**	**1/16**	**DMSO**
Staphylococcus aureus	++++	++++	++++	+++	+++	----
Escherichia coli	----	----	----	----	----	----
Pseudomonas aerogenes	----	----	----	----	----	----

D: (-) no inhibition; 5-15 mm (+); 16-25 mm (+ +); 26-35 mm (+ + +) and > 40 mm (+ + + +). In general, Gram negative bacteria show more resistance to essential oils (EO) than Gram positive bacteria [43, 44] . This resistance is due to the nature of their cellular membranes structure. As a matter of fact, gram positive bacteria have thicker layer of peptidoglycan, which gives them hydrophobic surfaces [45]. Therefore, gram positive bacteria membrane allows the passage hydrophobic molecules. Other than the outer layer of peptidoglycan the main barrier against EO is the external membrane of gram negative bacteria, which allows only small hydrophilic molecules [46,47]. Hydrophobicity is a well-known characteristic of essential oil which allows lipids of the bacterial cell membrane and mitochondria partitioning, thus, resulting in more permeability of the cell structure in gram positive bacteria [47]. Hamdy A. Shaaban indicated that the antimicrobial activity of essential oils are dependent on their chemical compositions and also their lipophilic properties [48]. Therefore, hydrophobicity is involved in the toxicity of the essential oils against bacterial pathogens [49].

Essential oils could be exploited as a way to recover and valorize wastes and by-products as this will help better waste management. Moreover, antimicrobial properties of bioactive compounds contained in the peels extract can be effective to treat several microbial infections such as soft and skin tissue infections caused mainly by *Staphylococcus aureus* and surgical wound infections caused by *Escherichia coli, Pseudomonas aeruginosa and Enterococcus spp.,* [50,51].

4. CONCLUSION

In this study, valorization of citrus peels wastes was conducted successfully. Essential oil of fresh orange (*citrus sinensis)* peels was extracted using hydro-distillation method**.** The obtained essential oil was investigated for its physical properties characterized by FTIR, UV-Vis and GC-MS spectroscopic techniques. In addition, antibacterial properties were studied with disc diffusion essay. The results showed that peels waste extract was conform to AFNOR standards with a yield of 3.4% (w/w). Moreover, spectroscopy characterization confirmed the presence of limonene, which represent 97.14% of the bioactive compounds. The extracted essential oil showed excellent antibacterial activity against *Staphylococcus aureus* but, was ineffective against *Escherichia coli* and *Pseudomonas aerogenes*. To summarize, the orange peel waste can be used as an available free source of essential oil. The peels can be recycled without side effects and used as an effective, cost effective and environmentally friendly antibacterial agent for humans and animal by using its bioactive compounds such as limonene. It should be emphasized that although orange essential oil showed antimicrobial activities against Gram positive bacteria (but not Gram negative), its antibacterial effects are significantly lower than synthetic antibiotics. Thus, orange essential oil cannot be used as an alternative to antibiotics used for medical purposes. However, it could be widely used in food and cosmetic industry due to its low toxic level.

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