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Physicochemical Indexes and Evaluation of Antioxidant, Antihemolytic and Antibacterial Activities of *Citrus sinensis* and *Citrus limon* Peel Essential Oils

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Abstract. In this study, the physicochemical indexes, phytochemical screening by thin layer chromatography of essential oils (EOs) obtained from *C. limon* L. and *C. sinensis* peels and their antioxidant, antihemolytic and antibacterial activities were investigated. The highest yield (0.71%), density (0.918 ±0.05), refractive index (1.974±0.02), saponification value (186.11±1.13 mg KOH/g) where obtained in *C. limon,* where the highest acid value (0.45±0.2mg KOH/g) was for *C sinensis*. Eight and six spots were identified in *C. sisensis* and *C. limon* respectively using thin-layer chromatography (TLC). IC50 for DPPH radical-scavenging activity were 7.04 ±1.03 and 8.39 ± 0.9 µg/mL for *C. limon* and *C. sinensis* essential oils, respectively. *C. limon* EO was also demonstrated better antihemolytic activity by peroxide scavenging, IC50 were 12.76± 0.6 for *C. limon* and 14.26 ±0.3 µg/mL for *C. sinensis* essential oils. The *C. limon* peel EOs showed, also, a higher antibacterial effect on *S. aureus* and *E. coli* with 20.5±0.2 and 11.5 ±0.01 mm, respectively. Our data showed that both *C. limon* and *C. sinensis* EO were inactive against *P. aeruginosa.*

Keywords. Citrus limon, Citrus sinensis, Essential oil, Physicochemical indexes, Biological activities.

1. Introduction

Since Antiquity, man has known the use of essential oils to preserve human health [1]. The application of essential oils has been mentioned against many diseases grace to their richness in bioactive secondary metabolites with medicinal values [2]. These chemical constituents are a complex mixture of volatile compounds belonging to terpenes and phenylpropanoids [1]. Since they are recognized as safe and many foods tolerate their presence, citrus EOs have gained acceptance in food industry [3,4]. Several types of citrus fruits, in Rutaceae family, comprises of about 140 genera and 1,300 species. Sweet orange (*Citrus sinensis* L.), lemon (*Citrus limon* L.), lime (*Citrus aurantifolia*), pomelo (Citrus *maxima* Merr.), mandarin (*Citrus reticulata* Blanco), grapefruit (*Citrus paradisi* L.), tangerine (*Citrus reticulate*), shaddock (*Citrus grandis*), sour orange (*Citrus aurantium*), Citron (*Citrus medica*), are important and famous crops around the world. Citrus essential oils were produced from different parts of plant, such as peel, leaf, and flower [5, 6]. The citrus EO is composed of tens to hundreds of various compounds, such as limonene, α/β-pinene, sabinene, β-myrcene, limonene, linalool, α-humulene, and α-terpineol, which depend on the citrus variety and growth environment [7,4]. The pharmacological

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effects of these compounds give citrus essential oils great potential for use as functional foods and cosmetic ingredients. These compounds exert also numerous activities including anti-inflammatory, anti-cancer, neuroprotective and cardiovascular effects. [6,8]. Citrus essential oils have also proven antifungal and antibacterial properties against a broad spectrum of foodborne pathogens [9]. In addition, these oils are generally non-toxic, non-mutagenic, and non-carcinogenic. They are not hazardous in pregnancy and do not alter the maternal reproductive outcome [10]. Citrus fruits, especially sweet orange and lemon are mainly processed to produce juice. Peels, seeds and pulps, corresponding to about 50% of raw processed fruits, are represented as waste from the citrus processing industry after juice extraction. These can be used as a potential source of valuable by-products and to minimize environmental impacts. Citrus peels, especially, are a potential source of valuable secondary plant metabolites and essential oils for application in the food, pharmaceutical and cosmetic industries [11,5]. The objective of our study was to identify the quality of essential oils extracted from the peels of lemon (*Citrus limon L.*), and orange (*Citrus sinensis* L.), by determination of their physicochemical indexes, phytochemical screening by thin layer chromatography and evaluate their antioxidant, antihemolytic and antimicrobial activities.

2. Materials and Methods

2.1. Plant Material Collection

We worked with the peels of two species of citrus fruit, Lemon (*C. limon*) and sweet orange (*C. sinensis*). The fruits used for the study were purchased from the local market in the region of Ouargla, Algeria. We insisted on the good quality of the product, namely the freshness, maturity and healthiness of any mechanical or unhealthy crack. The fruits were well washed then peeled. The peels were dried at room temperature and then crushed to recover their essential oils.

2.2. Extraction of Essential Oils

A quantity of crushed peels was subjected to hydrodistillation carried out using a Clevenger-type apparatus. The solvent was distilled water and the extraction took about 3 hours. The essential oil was collected, dehydrated with anhydrous Na_2SO_4 then stored at low temperature in sealed brown glass bottles to prevent their degradation [12]. Extraction process was carried in triplicate. The extraction yield was defined as the ratio between the weight of the essential oil obtained and the weight of the plant material treated. The yield is calculated using the equation:

$$Y\ (\%) = WEO/\ WPM\ X100$$

Where: Y: yield / WEO: Weight of essential oil / WPM: Weight of plant material.

2.3. Physicochemical Indexes of Essential Oils

Physicochemical indexes specific to each essential oil are used to determine its quality and criteria. The methods used are determined according to a precise protocol and comply with standards published by the French Association for Standardization [13].

2.4. Determination of Physical Index

The refractive index is the ratio between the sine of the angle of incidence of the light beam in the air and the sine of the refraction angle of the refracted ray into the essential oil maintained at a constant temperature of 20°C. The Abbe refractometer was used, which is previously calibrated with distilled water which refractive index is known (Ir (water) = 1.335) at 20°C. The prisms are then dried and a few drops of EO was placed between the two faces of the prisms. Using the eyepiece and the adjustment knob, the interface between the dark and illuminated area was brought to the center of the reticle. The correction at 20°C was calculated by the formula:

$$I_{20} = It + 0.00045\ (t - 20°C)$$

Where: I_{20}= Index at 20°C /It = Index at ambient temperature /T= ambient temperature.

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2.5. pH Determination

The pH was determined using pH paper and an approximate value was obtained.

2.6. Specific Gravity (Density)

The relative density of EO was defined as the ratio of the mass of a certain volume of oil at 20°C to the mass of equal volume of distilled water at 20°C. The measurement was carried out using an electronic densimeter. The relative density at 20°C was calculated using the following formula:

$$d_{20} = dt + (t\text{-}20) \times 0.00068$$

Where: dt: density at room temperature / t: ambient temperature / d20: density at 20°C.

2.7. Chemical Index

2.7.1. Acid Value

The acid index was determined by mixing of 0.125g of EO with 2.5mL of ethanol, then 2 drops of phenolphthalein were added. The whole was titrated with ethanolic solution of KOH 0.1N until the color changes to pink. The acid index (AI) was determined by the formula:

$$AI = \frac{5{,}61 \times \boldsymbol{vKOH}\ (0{,}1\ \boldsymbol{N})}{\boldsymbol{W.H.E}}$$

Where: V.KOH: Volume of KOH / W.H.E: Weight of essential oil.

2.7.2. Saponification Index

The saponification index was carried out by mixing of 0.25g of EO and 5 mL of ethanolic solution of KOH 0.5 N. The mixture was heated for 30 minutes. After cooling, 10 mL of distilled water and 5 mL of ethanolic solution of KOH 0.5 N were added then the mixture was returned to the water bath for 30 min. After cooling, three drops of phenolphthalein were added and the soapy solution was titrated with HCl (0.5N). The control test was prepared without essential oil. The saponification index (SI) was determined by the formula:

$$SI = ((V0\text{-}V1) \times M \times N \times F)/m$$

Where : V0: volume (mL) of the HCl solution used for the control / V1: volume (mL) of the HCl solution used for the test sample / M: molar mass of KOH /N: normality of KOH solution/ F: correction factor for the normality of KOH solution /W: Weight of the test sample.

2.7.3. Phytochemical Screening of Essential Oils by Thin Layer Chromatography

Thin layer chromatography (TLC) is a fast, simple analytical technique. The stationary phase used is 60F 254 silica gel plates on aluminum foil. Two mobile phases were tested: the first consists of butanol/ethyl acetate (8/1.9: V/V) and the second is a mixture of dichloromethane/Hexane (9/1: V/V). Approximately one microliter (µL) of each essential oil is deposited on the plate at a reference point located above the surface of the mobile phase. The plates are revealed chemically using (Godin reagent), composed of sulfuric vanillin (1%) in a concentrated sulfuric acid and ethanol 95% (2/98 :v/v). After spraying, the plate was heated in an oven for a few minutes at 110°C. Each spot is characterized by its coloration after revelation and its retention factor (Rf), this is calculated using the equation:

$$Rf = d/D$$

Where d: Distance traveled by the component/D: Distance traveled by the eluent front [14, 15, 16].

2.7.4. DPPH Radical Scavenging Assay

The method described by Sanchez-Moreno (1998) [17] was used to evaluate antioxidant activity of essential oils. A volume of 50 µL of each essential oils at different concentrations (diluted in methanol) was added to 1950 µL of freshly prepared methanolic solution at 0.024 g/L of DPPH (1,1-diphenyl-2-pycrylhydrazile). Ascorbic acid was used as a standard antioxidant while a negative control was prepared by the addition of 50 µL of methanol to 1950 µL of DPPH solution. The

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absorbance was measured at 517 nm after incubation for 30 min in the dark at room temperature, Radical-scavenging activity was calculated as a percentage of DPPH discoloration using the following equation:

$$I\ \% = A\ control - A\ sample/A\ control \times 100$$

Where, A control: absorbance of the DPPH solution without essential oil, A sample: absorbance of the solution containing the essential oil. The antioxidant activity of the essential oils was expressed as IC50 (half-maximal inhibitory concentration) values (µg/ mL) which were calculated from the graph representing the percentage of inhibition according to different concentrations of extracts.

2.7.5. Antihemolytic Activity

Blood from a healthy donor, collected in EDTA, was used to evaluate the antihemolytic activity of essential oils. after centrifugation for 5 min at 1000x g and elimination of supernatant, the pellet was washed three times with PBS (0.2 M, pH 7.4) and resuspended in a saline solution (4%). then 0.5 mL of essential oils at different concentrations in DMSO were added to 2 ml of suspension of erythrocytes and incubated at ambient temperature for 20 min. to cause the oxidative degradation of the membrane lipids, 0.5 ml of H_2O_2 solution was added to the reaction media. after centrifugation for 10 min at 1000xg, the absorbance of the supernatant is read at 540 nm. the positive control was ascorbic acid while DMSO was taken as a negative control. The percentage of hemolysis was calculated using the formula:

$$I\ \% = ((Ac-At)/Ac)\ X\ 100$$

Ac: absorbance of the negative control/At: absorbance of the essential oil tested [18].

2.7.6. Antimicrobial Activity

Agar diffusion method was used to determinate the antimicrobial activity of *C. sinensis* and *C limon* EOs. The microbial strains tested represented by *Staphylococcus aureus* (G+) ATCC 25923, *Pseudomonas aeruginosa*(G) ATCC 27853 et *Escherichia coli* (G-) ATCC 25922 were obtained from bacteriology laboratory of "Mohamed Boudiaf" hospital in Ouargla, Algeria. After inoculating of bacterial strains with three successives 24-h transfer into nutrient agar medium, an aliquots of 100 µL of microbial inoculum, standardized to 0.5 McFarland (106 $CFU.mL^{-1}$), were spread on Mueller-Hinton medium Petrie dishes and then left to dry at 30°C for 15 min. Sterile disks of Whatman No3 filter paper (6 mm in diameter) soaked beforehand in different concentrations of the essential oils tested in DMSO were placed. The dishes were then kept at 4°C for 2 h and then incubated for 24 h at 37°C. The diameters of the zones of inhibition were measured in millimeters. concentrations of 229.5, 114.75, 56.91, 28.68 and 14 µg/mL have been tested to determine the minimum inhibitory concentration (MIC) [19-21].

2.8. Statistical Analysis

Data were analyzed using Excel (Microsoft Inc.). The different tests were carried out in triplicate and the results were illustrated as the means standard deviation of three independent measurements.

3. Results

3.1. Extraction Yield

As it can be seen in Figure 1, the yield of the hydrodistillation essential oil of *C. limon* peel was 0.71% and it was characterized by a pale yellowish color and pleasant aromatic fragrance. Whereas, orange peels seem less rich in essential oils with a yield of 0.55 %, these oils was characterized by pleasant aromatic fragrance, a pale yellowish color for lemon and darker yellow color for *C. sinensis.*

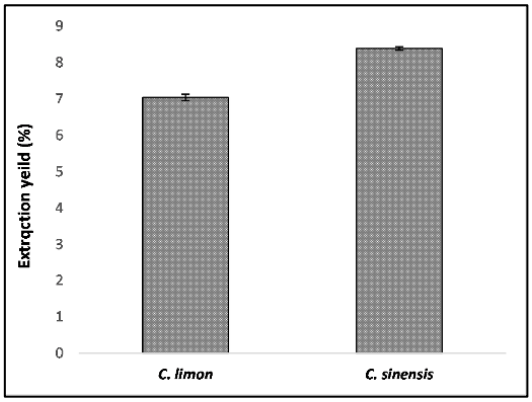

Figure 1. Extraction yield of *C.limon* and *C. sinensis* essential oils.

3.2. Physicochemical Indexes of Essential Oils

Examination of Table 1 reveals that the measured physicochemical indexes conform to international standards described by AFNOR (According to the French Association for Standardization) [13]. The essential oil from the peels of *C. sinensis* has a relatively lower density and refractive index, compared with *C. limon*. The acid index of *C. sinensis* evaluated at 0.45, is slightly higher than that of *C. limon* (0.39±0.01) While saponification index showed the opposite.

Table 1. Physicochemical indexes of *C.limon* and *C. sinensis* essential oils.

	C. sinensis EO tested	**AFNOR**	***Cirtus limon EO tested***	**AFNOR**
Density	0.845±0.01	0.842-0.850	0.918±0.05	0.905-0.921
Refractive indexe	1.4780±0.08	1.4700-14760	1.5740±0.02	1.4600-14760
Ph	7	Acide faible	6	Acide faible
Acid index (mg KOH/g)	0.45±0.0	0.5 - 2.2	0.39±0.01	0.5 – 3.00
Saponification index (mg KOH/g)	181.33±0.1	185-194	186.11±1.13	185-194

3.3. Phytochemical Screening of Essential Oils by Thin Layer Chromatography

The best chromatographic separation was obtained with dichloromethane/Hexane (9/1: V/V) mobile phase. These chromatograms were shown in Figure 2. Eight compounds were detected in *C. sinensis* with Rf at 0.86 (light blue), 0.82 (dark purple), 0.79 (green), 0.75 (light purple), 0.65 (dark purple), 0.58 (dark blue), 0.49, 0.44 (light blue), 0.26 (light yellow) and six compound in *C. limon* at Rf 0.75 (purple), 0.65 (blue), 0.60 (green), 0.44 (purple), 0.35 (light blue), 0.30 (dark blue). The marketed oils used as a reference show chromatographic profiles very close to those of our oils.

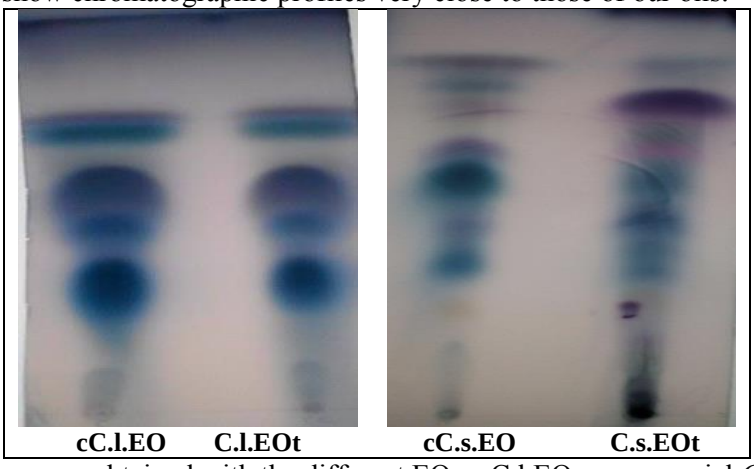

Figure 2. Chromatograms obtained with the different EOs. cC.l.EO : commercial *C.limon* EO, C.lEOt : *C. limon* EO tested, Cc.s.EO : commercial *C. sinensis* EO, C.s.EOt: *C. sinensis* EO tested.

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3.4. DPPH Radical Scavenging Assay

The results of *DPPH Radical Scavenging power of EOs* are illustrated in Figure 3. Essential oil obtained from *C. limon* showed highest radical-scavenging activity than that of *C. sinensis* such the IC_{50} values were 7.04±1.03 and 8.39±0.9µg/mL respectively. These two oils remain less important than that of ascorbic acid giving an IC_{50} equal to 6.46±0.05µg/mL.

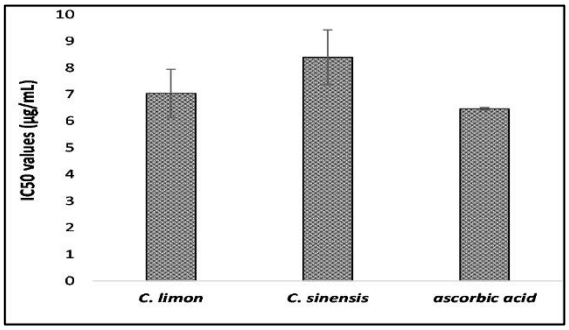

Figure 3. IC_{50} values obtained with DPPH test.

3.5. Antihemolytic Activity

Hemolysis inhibition activity caused by H_2O_2 shows that *C. limon* EO is more inhibiting than *C. sinensis* EO (Figure 4), this is evidenced by their IC50 evaluated at 12.76 ± 0.6 and 14.26±0.3µg/mL respectively. But without as much reaching the activity of ascorbic acid proved to be the most effective, its IC50 was 6.77 ±0.01 µg/mL.

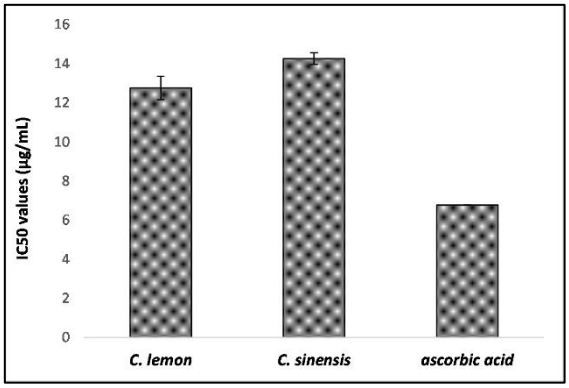

Figure 4. IC_{50} values for antihemolytic activity.

3.6. Antimicrobial Activity

The antibactrial activity of the *C. limon* and *C. sinensis* EOs was tested against *S.aureus*, *E. coli* and *P. aeruginosa*. The effectiveness of antibacterial activities was evaluated by measuring the zone of inhibition and summarized in Figure 5 (A and B)**.** The result indicates that the *C. limon* and *C. sisensis* EO showed very active against *S. aureus* forming zones of inhibition of 20.5±0.2 and 17± 0.05 mm respectively with a MIC was less than 14 µg/ml for *C. limon* and between 26.4 and 12.89 µg/ml for *C. sinensis* . The essential oils of *C. limon* and *C. sinensis* seem to have very few activity against E. coli, the measured zones of inhibition were 11.5± 0.01 and 10±0.5 mm respectively with MIC between 26.4 and 12.89 µg/ml. whereas, *P. aeruginosa.*

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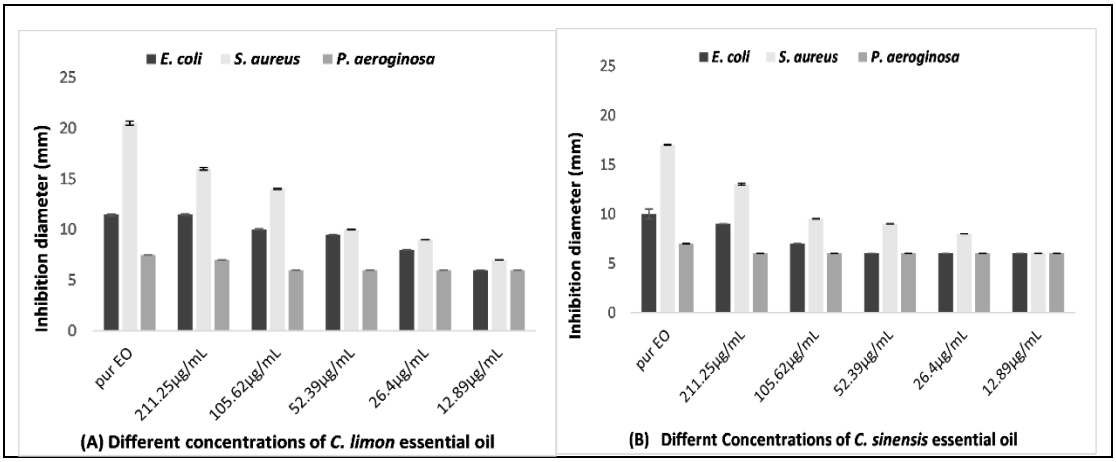

Figure 5. Antibacterial activities of *C. limon* (A) and *C. sinensis*.

4. Discussion

Extraction yield of essential oil from dry *C. limon* and *C. sinensis* peels is within the range given by AFNOR (2000) ranging from 0.5 to 2%. This compares favorably with the findings of other researchers. The percentage yield of volatile oil from *C. sinensis* and *C. limon* has been reported by hydrodistillation as 2 and 1.5 %, respectively by Sharma and Vashist (2015) [22], as 0.81 ± 0.092% for *C. limon* [23], as 0.57 to 3.24 for *C. sinensis* by Soxhlet apparatus [24]. Golmakani and Moayyedi (2015) [25] mentioned 1.36 ± 0.06 %, 1.18± 0.08 and 1.22±0.14 % in *C. limon* peel oil in different method of oil extractions *viz.,* solvent-free microwave extraction, microwave assisted hydrodistillation and hydrodistillation respectively. It was articulated that the quality, composition, quantity and aroma of pure essential oils may vary depending on growing habitat, environmental factors, the extraction technique, the harvest, drying period, the degree of freshness and the variety [26,27]. In addition, the flavedo ratio (superficial layer rich in HEs / albedo (internal white layer with a spongy quality) in the peels influences the yield of the extraction [28].

Physicochemical indexes of an essential oil is a very important criterion for evaluating its quality as well as for identification. The measured lemon's essential oils density and refractive index shows slightly important than those obtained by Boughendjioua et Djeddi (2017) [29] and Benoudjit et al. (2020) [30] with the respectively values of 0,855±0,005 and 0.894 for density and 1,4700±0,005 and 1.475 for Refractive index. The refractive index increases with the introduction or presence of secondary products in the oil and extraction temperature [31]. It essentially varies with the content of monoterpenes and oxygenated derivatives. A high content of monoterpenes will give a high index [32]. Kanko et al. (2004) [33] showed that a low refractive index of the essential oil indicates its low refraction of light, which could favor its use in cosmetic products. An approximate pH studied essential oils is slightly acidic which supposed the richness of oil with acidic character compounds. The low acidity of oils is considered as neutralized and safe for making skin care products [34]. The present study showed, comparatively, lower acid value than that of finding of Ali (2015) [35] with 1.79 and 1.99 mg/KOH/g oil in *C. limon* and *C. sisensis* respectively. However, this same study shows very low saponification values (13.5 and 13.7 mg/KOH/g for *C. limon* and *C. sisensis* respectively). On the other, Khan (2013) [36] was mentioned a saponification index of *C. sisensis* essential oil very close to our results (183 mg/KOH/g). The acid and saponification indexes are quality criterions indicating the quantity of free fatty acids present in our essential oil and the susceptibility of the essential oil to undergo alterations, in particular oxidation. The lower acid value indicated the high quality of product, but the relatively high saponification value recorded is indicative that it has potential for use in the industry [34, 30].

Several spots were revealed by Godin reagent, it detects terpene compounds by coloring them in the visible in purple, blue, green, orange, pink and yellow [37]. These results are confirmed by the GC-MS profiles described in the literature. Indeed, Ben Miri (2018) [38] identified the presence of different group of terpenoid compounds. The monoterpene hydrocarbons are mostly represented by limonene with 54.95 % in *C. limon* EO and 82.6 % in *C. sinensis* EO. The oxygenated monoterpenes

were represented by geranial (3.63 %), neral (2.78 %), α-terpineol (1.88 %), nerol (1.61% %), neryl acetate (1.60 %), terpene-4-ol (1.49 %), geraniol (1.45 %) and linalool (1.45 %) in *C. limon* EO and linalool (4.99 %), α-terpineol (1.08 %) in *C. sinenesis* EO. However, the sesquiterpene hydrocarbons and oxygenated sesquiterpenes were minor. Regarding antioxidant activity, our study showed that *C. limon* EO is more potent in reducing DPPH radical. This difference could be explained by the chemical composition of each EO or the interactions between the whole compounds. In the study of (Bhandari, 2021) [4], *C. lemon* EO obtained by hydrodistillation process are characterized by a greater activity, with IC50 values of 3.33 µL/mL. While, data of Ben miri et al. (2018) [38] showed lower potential to reduce the DPPH radical, with IC50 value of 1570.10±19.57 and 752.26±9.09 µg/mL for *C. limon* and *C. sinensis* respectively. Limonene which represents the major compound of *C. sinensis* and *C. limon* EOs, is known for their antioxidant power due to the donation of hydrogen [39,4]. Additionally, free radical scavenging activity of EO may be attributed to the phenolic compounds eventually extracted in Eos, known by their antioxidant activity against reactive oxygen species [38]. The essential oils tested appear effective in hemolysis inhibition, stimulated by hydrogen peroxide. The activity may be due to the presence of different phytochemicals which acted in synergy. To compare our results with literature, no data is available on the anti-haemolytic activity of *C. limon* and *C. sinensis* EOs against human red blood cells. It was demonstrated that Erythrocytes are considered as prime targets for free radical attack (Nabavi, 2013) [40].

The Erythrocytes carry oxygen in the organism and circulate repeatedly through the lungs and capillaries, where are continuously exposed to intracellular ROS derivatives (H_2O_2 and superoxide) of autoxidation of oxyhemoglobin. These ROS target polyunsaturated fatty acids and membrane proteins, causing the degradation of proteins and enzymes, alteration of fluidity and consequently the change in membrane morphology and the bursting of red blood cells. This hemolysis have already been used in measuring free radical damages. [41]. Even though ascorbic acid was better at reducing hydrogen peroxide-induced erythrocyte abnormalities than the essential oils, the antioxidant activity of *C. limon* and *C. sinensis* EOs could be widely exploited in pharmacology. The highest antibacterial activity of *Citrus limon* and *Citrus sinensis* against *Staphylococcus aureus* was also detected by Akarca and Sevik (2021) [42], with inhibition diameters of 22.55 and 26.23 mm respectively ($P < 0.05$). An antimicrobial screening evaluated by Qadir, (2018) [43] showed an extreme activity of *C. limon* against *E. coli* (30.33 ±0.58mm), and lower activity against *S. aureus* (15.42±0.58 mm). Whereas, the essential oils of *C. limon* and *C. sisensis* showed very effective against *S. aureus* and *E. coli* in the study of Javed et al. (2020) [44] who found a potent antimicrobial effect. Values of inhibition zones were 35 and 21 mm, respectively, for *C. sinsensis* and 40 and 22 mm, respectively, for *Citrus limon*. In other, the *C. limon* and *C. sinensis* essential oils showed low activity against *S. aureus* with 16 and 8mm respectively in data of Khalid et al. (2021) [45]. Our results are closely agree with those of Edogbanya (2019) [46] signaling the potential resistance of *P. aeruginosa* to *C. limon* EO, but with *C. sinensis* EO, they found a low activity. Resistance of gram- bacteria is the result of the presence of hydrophilic lipopolysaccharides in their outer membrane which prevents the penetration of hydrophobic compounds such as those found in essential oils [47]. The presence of limonene and γ-terpinene in *C. limon* and *C. sinensis* EOs could be responsible for their toxic effects by inhibiting bacterial respiration, disrupting ion transport [39], altering membrane fatty acid composition and damaging cell morphology [47]. Changes in meteorological factors (Sunlight, temperatures, relative humidity, and altitudes) and soil properties (pH, EC, texture, and nutrient contents) produce variations in physiological characters of essential oils as well as yield, constituents and biological activities. This, which interprets the differences found in the results of the different studies [45].

Conclusion

Our study revealed that the essential oils of *C. sinensis* and *C. limon* have a precious value making them good candidates for use in the pharmacological, food and cosmetic field as antibacterial, antihemolytic and antimicrobial agents. In addition, the recycling of the peels of *C. limon* and *C. sinensis* by the extraction of their essential oils has an important economic and environmental value. In perspective, it would be wise to carry out a more in-depth study on the identification of the active principles contained in *C. sinensis* and *C. limon* EOs, to evaluate their activity against a wide range of

microorganisms, to evaluate their antioxidant activity by various tests and to study other biological activities.

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