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In vitro antioxidant and antibacterial activities of *Citrus limon* L. leaves extracts from two Algerian provenances

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Abstract

An investigation was conducted to ascertain the impact of the geographical area on the biological properties of *Citrus limon* L., with the aim of evaluating its potential as a natural source of antioxidants and antibacterial agents. The study involved quantifying the total phenolic and flavonoid compounds in the leaves of *C. limon* L. collected from two Algerian regions (Chlef and Boumerdes). *In vitro* antioxidant activity of extract samples were assessed by radical scavenging DPPH and radical cation scavenging ABTS assays, followed by the evaluation of antibacterial activities against *Staphylococcus aureus* at different concentrations (200, 300 and 500 µg mL^{-1}) by aromatogram analyses. The results exhibited significant variation between plant provenances, with Chlef leaf sample extract being richer in total phenolic and flavonoid contents (72.51±3.05 mg QE g^{-1} DM, 66.13 ± 2.25 mg GAE g^{-1} DM, respectively). In addition to its antioxidant properties, Chlef leaf extract also showed high antibacterial activity against *S. aureus*. Antioxidant tests showed the higher anti-free radical capacity with 86.62 ± 1.14 % and 95.87 ± 0.21 % inhibition rate values by DPPH and ABTS tests, correspondingly. A comparable scavenging capacity of Chlef leaf extract to that of Trolox activity was detected by DPPH test, with an IC_{50} equal to 665.55 ± 4.19 µg TE mL^{-1}. Besides, this sample showed higher anti-free radical activity than the reference molecule by the ABTS. Furthermore, greater sensitivity of *S. aureus* to Chlef leaf extract was observed, with zones of inhibition superior to 10 mm. *C. limon* from Chlef (leaf) can be used as a source of natural antioxidants and antibacterial activity in different fields, especially medicinal applications and agri-foods.

Key words: *Citrus limon* L, *Staphylococcus aureus*, phenolic compounds, antioxidant activity, antibacterial capacity

Introduction

Foodpoisoning remains a significant health concern due to microbial contamination, with pathogens like *Staphylococcus aureus* causing widespread *Staphylococcal* food poisoning (SFP) (Sadaka *et al.,* 2013; Mekonnen *et al.*, 2021). The search for effective and economical natural antimicrobial has intensified as consumers increasingly reject synthetic alternatives (Riaz *et al.,* 2023).

Citrus limon L. is one of the important plants of the family Rutaceae, commonly named "Lemon" they are largely cultivated for alimentary or industrial applications (fresh fruit consumption, beverages, cosmetics, sweets, and pharmaceuticals). It is characterised by various biological properties such as anticancer activities and antibacterial potential, antifungal, anti-diabetic, anticancer, and antiviral properties are related to the presence of a wider range of phytochemicals such as terpenoids, flavonoids, limonoids, and tannins (Kawaii *et al.,* 2000; Ortuño *et al.*, 2006). Lemon by-products, such as leaves, peels, and seeds, are enriched with multiple bioactive compounds. Thus, they may be employed in the food, cosmetic, and pharmaceutical industries. Yet, these agro-waste products are dismissed annually without being transformed into valuable products such as phenolic extracts and essential oils (Qadir *et al.,* 2019).

In this context, this study focuses on the quantitative characterization of both polyphenols and total flavonoids in the hydro-methanolic leaves extract of two Algerian *C. limon* by colorimetric methods, followed by *in vitro* evaluation of the Antioxidant activities by radical scavenging by DPPH, ABTS assay. The antibacterial activity against S. aureus was assessed using aromatogram techniques. Ultimately, the antioxidant and antimicrobial properties of the two hydro-methanolic extracts were compared to identify the most promising source.

Materials and methods

Plant material: *C. limon* leaves were randomly collected in Chlef and Boumerde, Algeria, in February 2022. Samples were dried separately (T ≤40 °C) until weight stabilization, and conserved for further research.

Extract preparation: Leaves (100 g) were combined with 70% (v/v) aqueous methanol for 24 h (Bourgou, 2016). Macerates were homogenized, decanted, and filtered through Whatman filter paper #1 following three extractions. The extract was concentrated at 40 °C under vacuum. Three successive washes with petroleum ether (v/v) were carried out in order to eliminate all non-phenolic compounds. Produced aqueous extracts were lyophilized and stored at -20 °C.

Yields determination: Methanolic extract yields were represented as a percentage of obtained extract weight relative to sample dried matter, according to the following equation (Drosoua *et al.,* 2015):

Yield (%) = Mass of extract /Weight of dry matter x 100

Total phenolic contents (TPC): Total phenolic contents were assessed spectrophotometrically according to Bouzouina *et al.* (2016) method. One mL of methanolic extract was mixed with 5 mL of Folin-Ciocalteu's reagent (2 M, diluted 10 times) and 4 mL of Sodium bicarbonate 7.5 %. One hour later, in the dark at room temperature, absorbance was determined at **λ**=765 nm. The analyses were carried in triplicate. The same protocol was applied for the standard solution of gallic acid (0-100 µg mL^{-1}) and results were expressed as gallic acid equivalent per gram of lyophilized extract (mg GAE g^{-1} LE).

Total flavonoïd content (TFC): Total flavonoïd content was estimated by colorimetric assay. The reaction mixture was prepared by mixing 0.5 mL of quercetin (0-200 µg mL^{-1}) or methanolic extracts with 0.5 mL of $AlCl_3$ at 2 %, with three repeats for each extract. Absorbances were measured at 430 nm against the blank after 10 min of incubation at room temperature (Ahn *et al.,* 2007). Results were expressed as µg of quercetin equivalent per gram of lyophilized extract (µg QE g^{-1} LE).

Antioxidant activities determination

DPPH assay: Antioxidant activities of *C. limon* L. leaves extracts were carried DPPH radical scavenging method of Jahromi *et al.* (2014), with slight revision, 5 mL of each extract concentration (2-10 mg mL^{-1}) were homogenized with 5 mL of methanolic DPPH solution (0.004 %) and repeated three times for all samples. The reaction mixture was incubated for 30 min at room temperature in the dark. The final optic density was measured at 517 nm to determine the percentage of inhibition. Trolox at different concentrations (200-1000 µg mL^{-1}) was used as à positive control. Results were expressed as µg TE mL^{-1}. Inhibition of DPPH radical (I %) was calculated using to the following formula:

$$\text{DPPH scavenging activity (\%)} = \text{A blank} - \frac{\text{A sample}}{\text{A blank}} \times 100$$

Where, A blank= Absorbance of control. A sample= Absorbance of test compound.

The half-maximal inhibitory concentration (IC_{50}) was reported as the amount of antioxidant required to decrease the initial DPPH concentration by 50 %.

ABTS assay: The ABTS test was achieved using the method developed by El-Hallouty *et al.* (2020). Seven mM of ABTS and 2.45 mM of potassium persulfate were mixed into stock solution and let it react in dark for 12-16 h. The previous solution was diluted by mixing 1 mL of $ABTS^{++}$ solution with 60 mL ethanol to obtain an absorbance of 0.700±0.05 units at 734 nm, using a spectrophotometer. The reaction mixture comprised 0.9 mL $ABTS^{++}$ and 0.1 mL of extracts (0.2- 1 mg mL^{-1}). After 15 min, absorbance was read at 734 nm. Trolox was used as standard (10-100 µmol mL^{-1}). Each sample was repeated three times. The following equation determined inhibition of ABTS radical (I %):

$$\text{ABTS scavenging activity (\%)} = (Ac - At)/Ac) \times 100$$

Where, At and Ac are respective absorbance of tested samples, and $ABTS^{+}$, was expressed as µmol Trolox equivalents (TE) per gram of lyophilized extract. The concentration necessary for 50 % reduction of ABTS was expressed as IC_{50} (TE µmol mL^{-1}).

Antibacterial activity: The antibacterial activity of lemon leaves extracts against *S. aureus* (ATCC 25923) was evaluated at different concentrations (200-300-500 and µg mL^{-1}) by the diffusion method (Abdelazeem *et al.*, 2022) with two repetitions for each concentration and extract. About 15mL of Mueller-Hinton agar was placed into Petridishes, 100 µL of fresh culture was added. After solidification at ambient temperature, 50 µL of tested extracts (200-500 µg mL^{-1}) were put directly in wells (6 mm). The plates were left for one hour in the refrigerator to allow the diffusion of extracts. The inhibition zone was measured after 24 h of incubation at 37 °C **(**Chaalel *et al.,* 2017). A positive result was considered to be achieved when an extract produced a zone of inhibition greater than or equal to 10 mm (Biyiti *et al.,* 2004). Concurrently, cefalexine (10 µg mL^{-1}) positive control test was performed.

Statistical analysis: All tests were performed in triplicate, with the exception of antibacterial tests, which were achieved in two replicates. The results were expressed as mean ± SD. Statistically significant data were compared using ANOVA and student's t-test., at the significance level (P<0.05) using Statbox 6 software.

Results

Yields determination: The yields of lyophilized crude samples of *C. Limon* are summarized in Table 1. Notable percentages of extractable compounds were observed in leaf extracts from Chlef and Boumerdes, measuring 81.98 and 87.98%, respectively.

Total phenolics and total flavonoids contents: The Chlef extracts showed significantly higher TFC (72.51 ± 3.05 mg QE g^{-1} DM) and TPC (66.13 ± 2.25 mg GAE g^{-1} DM) compared to Boumerdes provenance (51.71 ± 1.14 mg QE g^{-1} DM, 61.47 ± 1.21 mg GAE g^{-1} DM, correspondingly) (P<0.05).

Table 1. Extracts yields, total phenolic and flavonoid contents in leaves extracts of *C. limon from* two Algerian provenances

Provonances	Extract yields (%)	TPC (mg GAE g^{-1} DM)	TFC (mg QE g^{-1} DM
Chlef	81.98	66.13±2.25[a]	72.51±3.05[a]
Boumerdes	87.98	61.47±1.21[b]	51.71±1.14[b]

Antioxidant activities determination: *In vitro* antioxidant activity tests, namely DPPH and ABTS, were employed to assess their free radical scavenging capability. Saumya and Basha (2010) suggest the utilization of multiple methods to evaluate the antioxidant potential of a plant due to diverse oxidative processes and the intricate interplay between radical sources and antioxidants within a complex plant system.

DPPH assay: Both extracts showed dose-dependent activities against DPPH and ABTS radicals (Table 2). In comparison with Boumerdes extract, Chlef showed a high radical scavenging capacity (% I = 82.78 ± 2.55 %) at a concentration of 8 mg mL^{-1} and comparable activity to Trolox (665.55 ± 4.19 µg TE mL^{-1}) (Fig. 1).

ABTS assay: The ABTS test revealed a dose-dependent antioxidant capacity where the extracts of both provenances of *C. limon* noted full inhibition at maximum concentrations of 0.8 and 1 mg mL^{-1} (94.06 ± 0.23 % and 95.87 ± 0.21 %, respectively) (Table 2). The IC_{50} of Chlef extract (61.081 ± 1.40 µmol TE mL^{-1}) was significantly greater than Boumerdes (69.189 ± 00 µmol TE mL^{-1}) and standard (73.71 ± 0.02 µmol mL^{-1}) (P<0.05**)** **(**Fig. 2**).**

Antibacterial activity: Statistical analysis showed significant antibacterial activity of the Chlef sample against *S. aureus*, with zones of inhibition well above 10 mm (30 ± 1.41, 42 ± 1.41 mm) at concentrations of 200 and 500 µg mL^{-1}, respectively (Fig. 3).

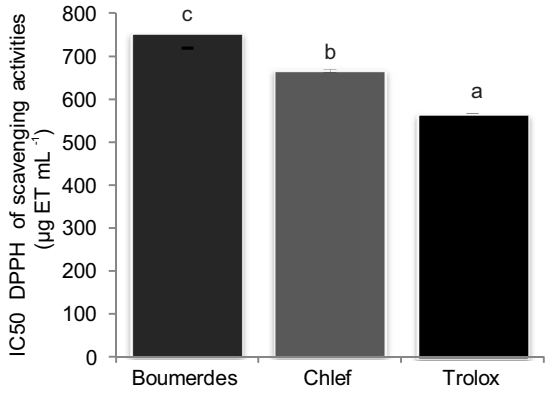

Fig. 1. Leaf extract antioxidant activities of DPPH assay, expressed as IC_{50} (µg TE/mL). Any two means having a common letter, are not significantly different at the 5% level of significance.

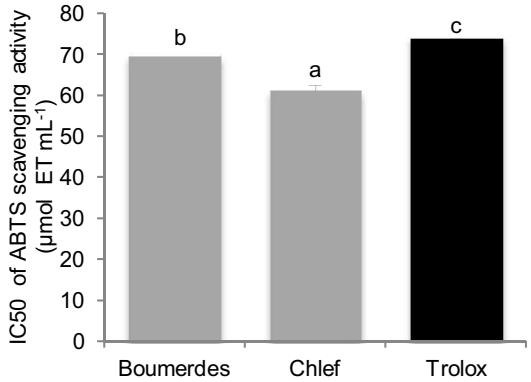

Fig. 2. Leaf extract antioxidant activities of ABTS assay, expressed as IC_{50} (µg TE/mL). Any two means having a common letter, are not significantly different at the 5% level of significance.

Table 2. DPPH and ABTS scavenging activities of the hydro-methanolic extracts of *C. limon* leaves. Superscript (a, b, c) displays the sample that scored highest for each category.

Antioxidant tests	Concentration (mg mL^{-1})	Chlef	Boumerdes
DPPH test (% I)	0	0	0
	2	23.85 ± 2.28	37.82 ± 3.00
	4	45.78 ± 2.45	54.99 ± 6.30
	6	62.36 ± 3.24	65.03 ± 2.85
	8	82.78 ± 2.55	74.18 ± 7.43
	10	89.06 ± 0.56	86.62 ± 1.14
ABTS (% I)	0.2	27.90 ± 3.40	40.37 ± 4.84
	0.4	65.72 ± 2.95	76.74 ± 0.76
	0.6	88.62 ± 0.70	91.54 ± 0.23
	0.8	94.57 ± 0.80	94.06 ± 0.23
	1	95.87 ± 0.21	94.77 ± 0.34

Data are expressed as mean±SD. (% I): Percentage inhibition of radical scavenging.

However, these results are lower than those for Cefalexin (57 ± 2.30 mm).

Discussion

The highest leaf extract yields appear to be influenced by several factors, such as environmental and cultivation conditions (Lee and Young, 2005). Indeed, the methanolic extract yields of Chlef and Boumerdes *C. limon* were found to be higher compared to hexane extracts from Pakistan (Riaz *et al.,* 2023).

This study confirms that *C. limon* is a significant source of

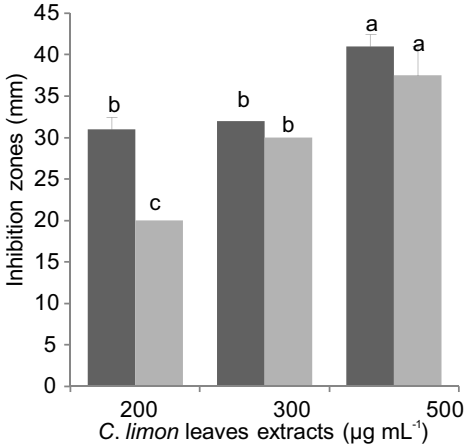

Fig. 3. Effect of leaf extract of the two provenances of *C. limon* on the growth of *S. aureus* by aromatogram method. Any two means having a common letter, are not significantly different at the 5% level of significance.

phytoconstituents, including phenolic compounds, flavonoids, tannin terpenoids, flavonoids, limonoids, and tannins. Phenolic compounds have the potential to provide benefits in terms of preventing various human diseases and preserving foods (Ehiobu *et al.,* 2021). The total phenolic compounds and flavonoid contents vary significantly depending on the species locality (Chlef and Boumerdes).

Furthermore, both Chlef and Boumerdes leaves appear to be rich in total phenolic compounds and flavonoids. The amount of total phenolic and flavonoid compounds in Chlef leaves was higher compared to values reported by Rizaldy *et al.* (2023) in peel's ethanolic extract from Indonesia species compared to Irak (Al-Anbari and Hasan, 2015) and Egyptian samples (Hamad *et al.,* 2023).

Based on these findings, the amount of phenolic compounds and flavonoids may be attributed to ecosystem factors. The characteristics of the geographical areas and sample origins can also impact the contents of these phytoconstituents (Hasanain *et al.,* 2022). Furthermore, the contents differ depending on the plant parts (Oh *et al.*, 2009) and maturation stage *(*Yoo *et al.,* 2004). Other factors, such as the extraction method, the polarity and the temperature of solvents, have been observed to increase the concentration of phytoconstituents in various plant species (Hasanain *et al.,* 2022).

The analysis of antioxidant activities results of previous studies revealed that the free radical scavenging capacity determined by the DPPH test was comparatively lower compared to *C. limon* leaf extracts of Eastern Cape (Ehiobu *et al.,* 2021). However, findings surpassed those obtained by Petretto *et al.* (2023). Additionally, the antioxidant activities of our extracts exceeded that of the essential oil of lemon peels from Iran (Moosavy *et al.,* 2017). Data obtained from the ABTS test results indicated a lower capacity for hydrogen atom donation at minimal doses in Chlef and Boumerdes leaves compared to the results of Ehiobu *et al.* (2021).

Results shown in Chlef leaf were likely attributed to the presence of hydroxyl groups in phenolic compounds, with more hydroxyl groups or conjugated double bonds. Flavonoids belong to a group of phenolic compounds with OH in ring A and or ring B. High antioxidative activity is recorded in flavonoids with a hydroxyl group at C-3'-C-4', OH at C-3, oxo function at C-4, double

bonds at C-2 and C-3. The hydroxyl group with ortho position at C-3'-C-4' provides high antioxidative activity for flavonoids. Aglycone flavonoids will contribute to higher antioxidative activity than flavonoid glycosides (Heim *et al.*, 2002) in addition to vitamins, minerals, dietary fibres, essential oils, organic acids, and carotenoids (Mamede *et al.,* 2021).

The anti-free radical properties, the methanolic extracts tested also showed better antibacterial activity against *S. aureus* than the hexanolic extracts and essential oils of *C. limon* from Pakistan (Riaz *et al.,* 2023). In addition, the mixture of methanolic extracts of *Niger limon* (Salawu *et al.,* 2021) and leaves essential oils from Egyptian (Asker *et al.,* 2020) showed lower antimicrobial activity than our results.

Indeed, the efficacy of the *C. limon* extracts tested against the bacterial strain mentioned, compared with the previous ones, may be linked not only to the leaf's richness in polyphenols and flavonoids but also to a number of other factors. The extract used likely contains specific antibacterial molecules, which may vary according to the harvesting season, the source, the mode of cultivation, the variety of plant, and the part being studied (Miraliakbari *et al.,* 2008; Hasanain *et al.,* 2022).

The antimicrobial effect could probably be due to the limonene, sabinene and γ-terpinene identified in the hexanolic extract and essential oil of the leaves (Moosavy *et al.,* 2017; Asker *et al.,* 2020; Riaz *et al.,* 2023), which exert their toxic effects by inducing irreversible damage to bacterial membranes and causing cytoplasmic losses by inhibiting respiration and ion transport processes, with a decrease of energy substrate causing bacterial lyse (Hojjati and Barzegar, 2017; Martins *et al.,* 2000; Asker *et al.,* 2020; Brah *et al.,* 2023). According to Asker *et al.* (2020), another antibacterial mechanism of action is possible through the inhibition of proteases and, consequently, the coagulation of cellular contents. Based on these results, Chlef extract is one of the natural source of antioxidants and antimicrobials promoted in manufacturing and preserving food.

This study highlights the impact of the different *C. limon* origins on the quantity of phenolic compounds, as well as their biological activities, including antioxidant and antibacterial properties. The concentration of these secondary metabolites, extraction method, and solvents used influenced the antioxidant potential and antibacterial activities of the extract from Chlef leaf. *C. limon* from Chlef showed promise as an alternative source of natural antioxidants in food manufacturing and pharmaceutical applications. To further enhance its potential, future research could focus on developing new extraction methods, exploring different collection areas and seasons, considering the controlled cultivation of *C. limon* seeds, and screening bioactive compounds of different parts by high-performance liquid chromatography (HPLC) and gas-chromatography-mass spectrometry (GC/MS) techniques with an assessment of *S. aureus* growth in a food prototypical, such as fresh minced meat, with a view to its preservation.

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