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In-vitro Antioxidant and Antimicrobial Activities of Iraqi *Citrus aurantium* Leaf Extracts

Hasan Mohammed Rashid[1*], **Shahad Layth Abdulkhaleq**[1].

[1]Department of Pharmaceutical Chemistry and Pharmacognosy, Faculty of Pharmacy, The University of Mashreq, Baghdad, Iraq.

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[*]**Corresponding author:**

Department of Pharmaceutical Chemistry and Pharmacognosy, Faculty of Pharmacy, The University of Mashreq, Baghdad, Iraq.

Email: hasan.m.rashid@uom.edu.iq

ABSTRACT

Background: This study compares the antimicrobial and antioxidant properties of ethanol and aqueous extracts of the dried leaves of *Citrus aurantium* (*C. aurantium*), grown in Iraq.

Methods: The microtiter and agar well diffusion methods were used with standardized strains to determine the minimum inhibitory concentrations (MICs), the minimum bactericidal concentrations (MBCs), and inhibition zones for the aqueous and ethanol extracts. Moreover, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was used to evaluate the extracts' radical-scavenging activity. All assays were performed in triplicate.

Results: Ethanol extract exhibited potent antimicrobial activity, especially against *Staphylococcus aureus* and *Streptococcus pyogenes* bacteria, as well as *Candida albicans* , with MIC values of 0.128 mg/mL for each and a zone of inhibition up to 29 mm against *Candida albicans*. Also, the ethanol extract showed higher antioxidant activity against DPPH radicals (53%) than ascorbic acid (79%).

Conclusion: The present study on *C. aurantium* leaf extracts revealed potent antimicrobial and antioxidant activity against DPPH radicals in the ethanol extract, suggesting the presence of high concentrations of potential phenol and flavonoid compounds. These results could lead to several new applications for bitter orange.

Keywords: *Citrus aurantium*, Antioxidant, Antimicrobial, In-vitro, Leaf Extracts.

Introduction

Antibacterial agents are low-molecular-weight bioactive substances that have been utilized for a wide range of therapeutic applications and have been considered the mainstay of treating infectious diseases for almost 50 years; numerous issues about the roles of microorganisms in nature are raised by the wide variety of biological activities and structural types of the organic compounds they create (1). In the last few years, rates of mortality and morbidity have accelerated due to long-term use and misuse of antibiotic agents. Several resistant bacteria, such as *Neisseria gonorrhoeae*, *Staphylococcus aureus*, and *Escherichia coli*, are responsible for these increases (2).

On the other hand, fungal infections are prevalent among immunocompromised individuals due to intensive treatments (e.g., anticancer chemotherapy, prolonged corticosteroid medication, or organ transplantation) and immunosuppressive conditions such as acquired immune deficiency syndrome (AIDS) patients. Approximately 90 % of these fatalities are attributable to species within the genera *Candida*, *Cryptococcus*, *Mucormycosis*, *Pneumocystis*, and *Aspergillus* (3). For example, fungi such as *Candida albicans* can cause epidermal infections (affecting the mucosal surfaces and skin) that occur more frequently than invasive infections, decreasing the quality of living for those affected (4). Anti-fungal medication development is more complicated than that of new anti-bacterial treatments due to the eukaryotic nature of fungi, which presents numerous potential therapeutic targets that are also present in humans, thereby delivering considerable host-toxicity risks (5,6).

Antimicrobial drug development and clinical application are continually evolving to meet new requirements, emerging knowledge, and scientific advancements. Pharmacokinetic and pharmacodynamic factors are becoming increasingly significant in defining performance targets for new and current drugs (7). Based on pharmaceutical studies, approximately 10–20% of medicinal plants used in healthcare have been shown to support treatment progress (8). The usage of alternative medications, mainly herbal-based medications, is gaining impetus all over the world (9). Using herbal therapies to manage fungal infections is a safe alternative, as plant extracts have been used in complementary medicine to treat fungal and bacterial infections for centuries (9).

One of the medicinal plant families well-known in complementary medicine is the citrus family. Citrus plants contain many nutritious components, including vitamins and minerals, as well as several phytochemicals such as essential oils, alkaloids, flavonoids, coumarins, and carotenoids. In earlier pharmacological studies, citrus plants have been shown to have various pharmacological effects, including those related to immunity, respiratory, reproductive, gastrointestinal, cardiovascular, anticancer, anthelmintic, insect-repellent, antioxidant, and other physiological functions (10).

For example, citron (*Citrus medica*), which contains bioactive compounds such as isolimonene, citral, limonene, phenolics, flavonoids, vitamin C, exhibits numerous pharmacological activities, including antihypertensive, diuretic, antibacterial, antifungal, anthelmintic, antimicrobial, analgesic, potent antioxidant, anticancer, and antidiabetic (11). Grapefruit (*Citrus paradisi*) is another example of a citrus plant that exhibits antimicrobial properties against both Gram-positive and Gram-negative bacteria, as well as fungal infections (12). Lime (*Citrus aurantifolia*) exhibits several pharmacological activities, including antibacterial, antifungal, antioxidant, immunomodulatory, anti-obesity, antifertility, and anthelmintic properties (13). Mandarin (*Citrus reticulata*) has also been recognized as a source of bioactive chemicals (14). Sweet orange (*Citrus sinensis*) contains chemicals that have been shown to possess antioxidant, anticancer, antibacterial, anti -inflammatory, and anti-osteoporotic properties (15).

Lemon (*Citrus limon*) extracts, juice, and essential oils exhibit diverse chemical compositions and biological activities, making them recommended for use in phytopharmacology (16). The bitter orange, or *Citrus aurantium* (*C. aurantium*) L. (Rutaceae), as shown in Figure 1, exhibits several potential medicinal activities, including anticancer, antibacterial, antioxidant, antianxiety, anti-obesity, pesticidal, and antidiabetic properties (17). Potent phytochemicals such as naringin and hesperidin flavonoids are abundant in *C. aurantium* (17).

The study aims to evaluate the aqueous and ethanol extracts of dried *C. aurantium* L. leaves grown in Iraq regarding their antioxidant effects and antimicrobial activities against bacteria and fungi. To the best of our knowledge, this study will be the first to evaluate the antimicrobial activities of ethanol (70%) extract of *C. aurantium* L. leaves growing in Iraq on standardized strains and the first study to assess the antioxidant activity of *C. aurantium* L. growing in Iraq against 2,2-diphenyl 1-picrylhydrazyl (DPPH) free radicals.

Methods

Plant samples and preparation

Fresh leaves of *C. aurantium* L. (Rutaceae) were obtained from a local area near campus (Hay Al-Jihad, Baghdad, Iraq; Latitude: 33.277133", Longitude: 44.275426") in November 2024. The plant material was authenticated based on the voucher specimen (MU24111) found in the Faculty of Pharmacy, The University of Mashreq, and descriptive references (18).

To obtain the extract, the leaves were air-dried, and then each 10 g was macerated separately with a 70% ethanol solution (1:10 w/v) and distilled water. The maceration was then heated to boiling and kept overnight at room temperature (25 °C). Solvents were evaporated until dryness at 40 °C using a rotary evaporator (VV 2000 Heidolph, Germany).

Antimicrobial assay

Both *C. aurantium* extracts were assessed in triplicate for their antibacterial and antifungal activities. Ciprofloxacin was used as a positive control for bacterial isolates, and fluconazole for the fungal isolate *Candida albicans*. The microorganism strains used in this study are shown in Table 1.

Figure 1. Iraqi *C. aurantium L.* leaves and fruits.

Table 1 . The microorganism strains used in the antimicrobial assay.

Microorganisms	Type	ATCC
Klebsiella pneumoniae	Gram-negative bacteria	ATCC®14169™
Pseudomonas aeruginosa	Gram-negative bacteria	ATCC®27853™
Streptococcus pyogenes	Gram-positive bacteria	ATCC®19615™
Staphylococcus aureus	Gram-positive bacteria	ATCC®25923™
Candida albicans yeast		ATCC®90028™

The following methods were used to determine the minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) against these microorganisms:

Microtiter method

The MIC reagent, resazurin (Alamar Blue), was prepared by dissolving 0.015 g of resazurin in 100 mL of sterile distilled water and storing it at 4°C (19). Double serial dilutions (1-64 µg/mL) of each *C. aurantium* extract, prepared from a stock solution (10 mg/mL), were performed in a microtiter plate using Mueller-Hinton broth (BD, France) as the diluent. The turbidity of bacterial suspensions was adjusted to match a 0.5 McFarland standard (OD_{600} = 0.08-0.1) using a spectrophotometer (Bio-Rad 680 microplate reader, UK). All wells were then inoculated with 20 µL of bacterial suspension, comparable to a McFarland standard of 0.5 (1.5×10^8 CFU/mL), except for the negative control wells. Microtiter plates were placed in an incubator (Gallen Kamp, England) at 37°C for 18-20 hours. After incubation, 20 µL of resazurin dye was added to all wells, and the mixture was incubated for 2 hours to observe any color changes. The MIC concentrations were determined visually in broth microdilution assays, as indicated by the lowest concentration at which the resazurin broth assay changed from blue to pink (20).

The agar well diffusion method

This method was used to detect the antimicrobial activity of *C. aurantium* extracts at MIC and MBC concentrations, as described by Wiegand et al. 2008 (21). Each bacterial isolate under study was grown in nutrient broth and incubated at 37 °C for 18-24 hours. 0.1 mL of each bacterial suspension was spread onto nutrient agar (Biolife, USA) at 37°C for 24 hours. A single colony was added to a test tube containing 5 mL of normal saline to yield a bacterial suspension of modest turbidity, comparable to the standard turbidity solution, which is nearly 1.5×10^8 CFU/mL. Using a sterile cotton swab, a portion of the bacterial suspension was carefully and evenly spread onto Mueller-Hinton agar, then incubated for 10 minutes. Five-millimeter-diameter wells were made in the previous agar layer (three wells per plate). The agar discs were removed, and 50 µL of the extract was added to each well using a micropipette (Oxford, England). Plates were incubated at 37 °C for 18 hours, and the diameter of the inhibition zones was then recorded.

Antioxidant activity

According to the procedure described by Khalid et al. 2024 (17). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, USA), prepared as a 0.004% (w/v) solution, was used to evaluate the radical-scavenging activity of the extracts. Different solutions of the standard and extracts were prepared by serial dilution (1000 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL, 31.2 µg/mL, 15.6 µg/mL). The test was performed in triplicate. The inhibition activity against the DPPH was calculated using the following equation (Equation 1):

$$\text{Equation 1: \% of antioxidant activity} = \left[\frac{(Ac - As)}{Ac}\right] \times 100$$

Ac—Control reaction absorbance (0 µg/ml)

As—Testing specimen

Statistical Analysis

The results of antimicrobial and antioxidant were expressed as mean ± SD (n = 3). SPSS statistical package version 26 (IBM Corporation, USA) was used to perform statistical analyses. A one-way Analysis of Variance (ANOVA) test was performed to assess statistical significance, with a significance threshold at (P value < 0.05).

Results

Plant samples

The results upon extracting 100 g of *C. aurantium* leaves using 1000 mL aqueous and ethanol (70%) solvents showed a yield of 26.06 g for the aqueous extract, while the ethanol extract yielded 18.62 g.

Antimicrobial assay

Microtiter method

Ethanol extracts showed antimicrobial activity against most of the tested microbial species, particularly against *Staphylococcus aureus, Streptococcus pyogenes*, and *Candida albicans*. The calculated MICs were 0.128 mg/mL for each. On the other hand, the aqueous extract shows a weaker antibacterial effect than the ethanol extract against *Staphylococcus aureus* (MIC = 0.512 ± 0.08 mg/mL) and other microbes (MIC = 1.024 mg/mL each). The MIC and MBC results of *C. aurantium* extracts are shown in Table 2.

Table 2. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of *C. aurantium* extracts against various microorganisms in mg/mL.

Isolate	Aqueous		Ethanol		Positive Control	
	MBC	MIC	MBC	MIC	MBC	MIC
Staphylococcus aureus	1.024±0.02	0.512±0.08	0.256±0.02	0.128±0.02	0.32±0.04	0.16±0.00
Streptococcus pyogenes	NA	1.024±0.06	0.256±0.01	0.128±0.02	0.128±0.00	0.64±0.03
Klebsiella pneumoniae	NA	1.024±0.02	1.024±0.03	0.512±0.03	0.64±0.02	0.32±0.01
Pseudomonas aeruginosa	NA	1.024±0.02	0.512±0.07	0.256±0.02	0.64±0.03	0.32±0.02
Candida albicans	NA	1.024±0.01	0.256±0.01	0.128±0.01	0.32±0.00*	0.16±0.00*

MIC = Minimum Inhibitory Concentration; MBC = Minimum Bactericidal Concentration; NA = No Activity. Data are represented as the Mean±SD (Standard Deviation), n=3 replicates.

* The positive control for the bacterial isolate is ciprofloxacin, while the positive control for the fungal isolate is fluconazole.

The agar well diffusion method

The agar well diffusion method was used to determine the inhibitory zone at MIC and MBC concentrations obtained from the microtiter method for each extract. Both aqueous and ethanol extracts inhibited all microbial species; the ethanolic extract of *C. aurantium* at its MIC was the most effective, especially against gram-negative bacteria *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, with inhibition zones of 26 mm each, compared to 27 and 28 mm for the positive control Table 3. The lowest inhibitory zone (19 mm) was obtained from the Aqueous extract effect on *S. pyogenes*. The aqueous extract showed a smaller inhibition zone than the ethanol extract, as indicated by MIC and MBC values. The plates used for this method are shown in Figure 2 for the aqueous extract and in Figure 3 for the ethanol extract.

Antioxidant activity

Both extracts exhibited radical scavenging activity against DPPH radicals. Compared with ascorbic acid (IC_{50} = 495 ± 8 µg/mL), the ethanol extract showed potent antioxidant activity (IC_{50} = 644 ± 16 µg/mL). On the other hand, aqueous extracts showed antioxidant activity with an IC_{50} of 754 ± 20 µg/mL. The antioxidant activity percentage of different extracts of *C. aurantium* against DPPH is given in Figure 4.

Table 3 . Zone of Inhibition (mm) of *C. aurantium* extracts on different isolates.

Isolate	Aqueous		Ethanol		Positive Control
	Zone of inhibition (mm)				
	MBC	MIC	MBC	MIC	MIC
Staphylococcus aureus	24±1.60	21±0.40	30±2.30	25±0.50	29±0.80
Streptococcus pyogenes	22±2.00	19±0.80	29±1.20	26±1.60	30±0.80
Klebsiella pneumoniae	27±0.90	25±1.20	29±1.20	26±3.00	27±1.20
Pseudomonas aeruginosa	27±1.20	25±1.40	30±1.60	26±1.60	28±0.80
Candida albicans	29±1.20	24±1.60	32±2.40	29±1.70	35±1.20*

The Zone of Inhibition is measured in millimeters (mm). Data are represented as the Mean±SD (Standard Deviation), n=3 replicates.

* The positive control for the bacterial isolate is ciprofloxacin, while the positive control for the fungal isolate is fluconazole.

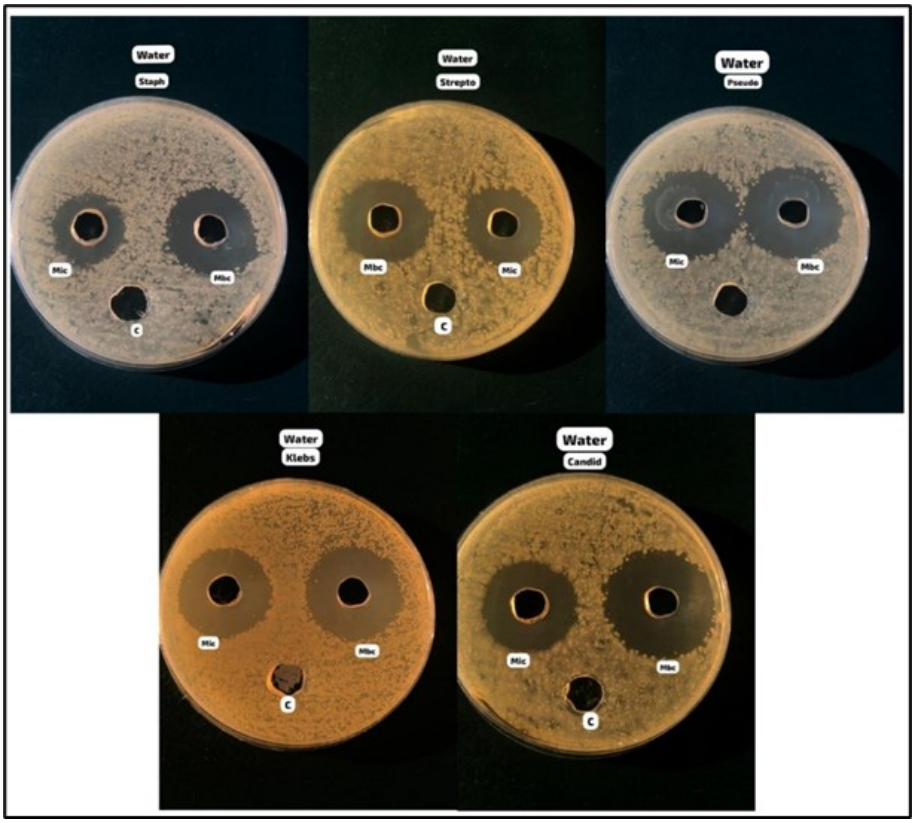

Figure 2. Agar well diffusion assay demonstrating the antimicrobial effect of Aqueous Extract. The highest inhibitory zone was obtained on *Klebsiella pneumoniae* (25 mm) and *Pseudomonas* aeruginosa (25 mm), while the lowest was obtained on *Streptococcus pyogenes* (19 mm).

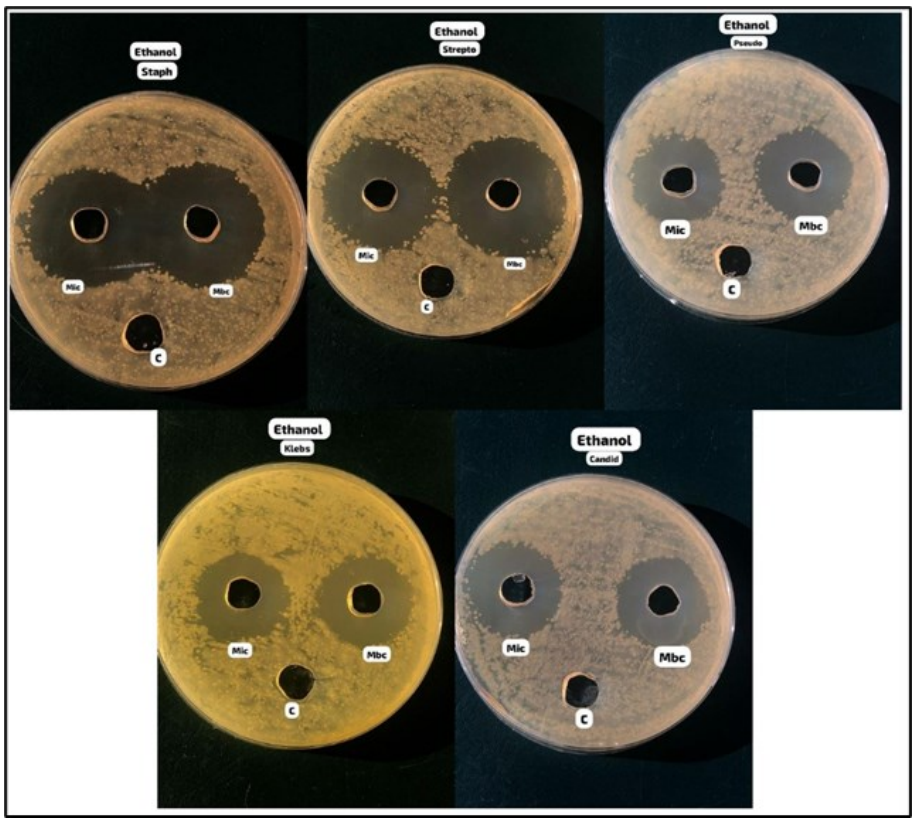

Figure 3. Agar well diffusion assay demonstrating the antimicrobial effect of Ethanol Extract. The highest inhibitory zone was obtained on *Candida albicans* (29 mm), while the lowest was obtained on *Staphylococcus aureus* (25 mm).

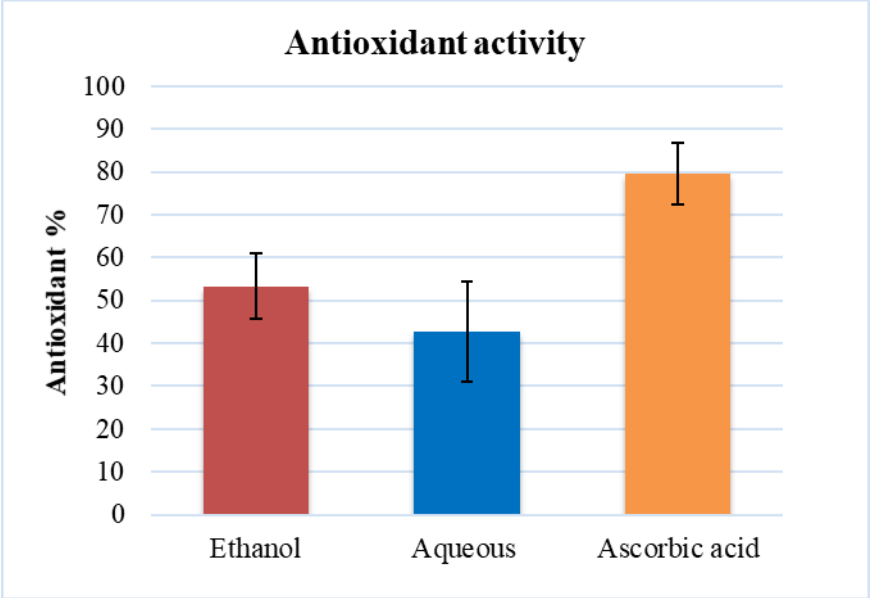

Figure 4. Comparison of *C. aurantium* extracts: DPPH scavenging activity showing antioxidant activity of Aqueous Extract (42%), Ethanol Extract (53%), and Ascorbic Acid Standard (79%) (n = 3).

Discussion

Previous studies on *C. aurantium* have reported decent antimicrobial activity, primarily due to its high levels of phenolic and flavonoid compounds (22,23). A study in Iraq on *C. aurantium* leaves extract revealed inhibition zones of only 10 and 7 mm for the aqueous and Soxhlet methanol extracts, respectively, against *S. aureus* and *P. aeruginosa* (24). The present study focuses on the ethanol extract, which showed inhibition zones comparable to those of the ciprofloxacin control against the same pathogens. A study in Iraq reported that the methanol maceration extract of *C. aurantium* leaves showed an antibacterial effect at 200 mg/mL, with an inhibition zone of 6 mm against *P. aeruginosa* clinical isolates (25). Results from the literature on the use of highly polar solvents suggested that only a small amount of phenolic and flavonoid compounds was extracted. Moreover, a resistant isolate from patients plays a significant role in lowering the inhibitory effect of these extracts.

Bitter orange (*C. aurantium*) is well known as a source of active compounds, including phenols and flavonoids (26). Naringin, eriodictyol-7-neohesperidoside, and hesperidin were reported as major flavonoids identified in a study on Jordanian *C. aurantium* (17). Another study in Tunisia revealed that bitter orange peel and juice extracts contain phenolic acids, which constitute up to 73.80% of the

total components, followed by flavonoids, 23.13% (27). In the same study, HPLC analysis indicated that p-Coumaric and ferulic acids were the most abundant phenolic compounds (27). Compounds such as naringin and hesperidin are abundant in relatively nonpolar solvent extracts (17). Those agents have been reported to potently inhibit bacteria such as *Staphylococcus aureus*, *Proteus mirabilis,* and *Bacillus subtilis* (29–32). Moreover, Naringin's antibacterial activity was expected due to the compound's ability to reduce biofilm formation and toxin production (33). In addition, naringin's activity against *C. albicans* is believed to be mediated by apoptosis induced by mitochondrial dysfunction (34), suggesting that the abundance of potent phenolic and flavonoid compounds is responsible for its potent antimicrobial activity. Several studies from different countries have concluded that the extracts exhibit a powerful antioxidant effect. (34–36). While the current research suggests a high potential for using *C. aurantium* leaves as a source of antioxidants, with IC_{50} values of 644±16 and 754±20 µg/mL for ethanol and aqueous extracts, respectively, compared to the ascorbic acid reference IC_{50} of 495±8 µg/mL. A study in Cyprus reported that the *C. aurantium* ethanol extract had an IC_{50} of 96.07 µg/mL, while the ascorbic acid reference had an IC_{50} of 5.8 µg/mL. Moreover, a study of Algerian *C. aurantium* leaf extracts showed an IC_{50}value of555 µg/mL against the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, compared to 543 µg/mL for the gallic acid reference (36).

The current study reported antimicrobial and antioxidant activities comparable to those reported in the literature. The ethanol extract of Iraqi *C. aurantium* leaves may be a new source of antimicrobial and antioxidant compounds. Furthermore, both extracts showed promising potential for use in the food and pharmaceutical industries as natural additives with high antioxidant activity or as natural preservatives for other products. Serving in these industries will offer many more advantages due to the plant's high edibility and low cost.

Limitations

The current study had some limitations, including the absence of phytochemical characterization. In contrast, the literature suggests the presence of similar phytochemicals worldwide, but at variable concentrations. The future directions will focus on identifying and isolating the active components of Iraqi *C. aurantium* and validating their efficacy and safety *in vitro* and *in vivo*.

Conclusion

The present study on *C. aurantium* leaf revealed that the ethanol extract has potent antimicrobial activity, especially against *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Candida albicans*, with MIC values of 0.128 mg/mL for each. Moreover, the same extract exhibited high antioxidant activity (53%) against the DPPH radical compared to 79% for the reference agent. These results suggest the presence of high concentrations of phenolic and flavonoid compounds in the extracts, which could serve as a new source of pharmaceuticals, nutraceuticals, or food preservatives.

Ethical Considerations

Not applicable.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

Conceptualization; H.R, S.A. **Methodology;** H.R. **Formal Analysis;** H.R. **Investigation;** H.R, S.A. **Writing – Original Draft Preparation;** S.A. **Writing – Review & Editing;** H.R. All authors have read the final manuscript draft and approved it for submission.

Data Availability Statement

Data will be made available upon reasonable request.

Supplemental Material

Not applicable.

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