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Chemical Profiling, Antioxidant, and Antimicrobial Activity against Drug-Resistant Microbes of Essential Oil from *Withania frutescens* L.

Abdelfattah EL Moussaoui [1], Mohammed Bourhia [2], Fatima Zahra Jawhari [1], Ahmad Mohammad Salamatullah [3], Riaz Ullah [4], Ahmed Bari [5], Hafiz Majid Mahmood [6], Muhammad Sohaib [7], Bohza Serhii [8], Alexander Rozhenko [8], Mourad A. M. Aboul-Soud [9], Essam Ezzeldin [5,*], Gamal A. E. Mostafa [5], Dalila Bousta [1] and Amina Bari [1]

1 Laboratory of Biotechnology, Environment, Agrifood and Health, Faculty of Science, University of Sidi Mohamed Ben Abdellah, Fez 30050, Morocco; abdelfattah.elmoussaoui@usmba.ac.ma (A.E.M.); jawhari.fatimazahra@gmail.com (F.Z.J.); boustadlila@gmail.com (D.B.); aminabar3@gmail.com (A.B.)
2 Laboratory of Chemistry-Biochemistry, Environment, Nutrition and Health, Faculty of Medicine and Pharmacy, Hassan II University, Casablanca 21100, Morocco; bourhiamohammed@gmail.com
3 Department of Food Science & Nutrition, College of Food and Agricultural Sciences, King Saud University, P.O. Box 2460, Riyadh 11451, Saudi Arabia; asalamh@ksu.edu.sa
4 Department of Pharmacognosy (MAPPRC), College of Pharmacy, King Saud University, P.O. Box 2457, Riyadh 11451, Saudi Arabia; rullah@ksu.edu.sa
5 Department of Pharmaceutical Chemistry, College of Pharmacy, King Saud University, P.O. Box 2457, Riyadh 11451, Saudi Arabia; abari@ksu.edu.sa (A.B.); gmostafa@ksu.edu.sa (G.A.E.M.)
6 Department of Pharmacology, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia; harshad@ksu.edu.sa
7 Department of Soil Sciences, College of Food and Agriculture Sciences, King Saud University, P.O. Box 2460, Riyadh 11451, Saudi Arabia; msohaib@ksu.edu.sa
8 Institute of Organic Chemistry of the National Academy of Sciences of Ukraine, Murmans'ka str. 5, 02660 Kyiv, Ukraine; bogza@i.ua (B.S.); a_rozhenko@ukr.net (A.R.)
9 Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, King Saud University, P.O. Box 10219, Riyadh 11433, Saudi Arabia; maboulsoud@ksu.edu.sa
* Correspondence: esali@ksu.edu.sa

Abstract: This work was conducted to study the chemical composition, antioxidant, antibacterial, and antifungal activities of essential oil and hydrolat from *Withania frutescens*. The essential oil was extracted by hydrodistillation. The chemical characterization was performed using gas chromatography-mass spectrometry (GC/MS). The antioxidant activity was studied using four different assays (DPPH, TAC, FRAP, and β-carotene bleaching). The antibacterial activity test was carried out on multidrug-resistant bacteria including Gram-negative and Gram-positive strains. Antifungal activity was tested on *Candida albicans* and *Saccharomyces cerevisiae*. The yield of essential oil (EO) obtained by hydrodistillation of *W. frutescens* was 0.31% majorly composed of camphor, α-thujone, carvacrol, and thymol. Regarding the antioxidant activities, the concentration of the sample required to inhibit 50% of radicals (IC_{50}) of EO and hydrolat were 14.031 ± 0.012 and 232.081 ± 3.047 µg/mL (DPPH), 4.618 ± 0.045 and 8.997 ± 0.147 µg/mL (FRAP), 0.091 ± 0.007 and 0.131 ± 0.004 mg AAE/mg (TAC), 74.141 ± 1.040% and 40.850 ± 0.083% (β-carotene), respectively. Concerning the antibacterial activity of essential oil and hydrolat, the minimum inhibitory concentration (MIC) values found were 0.006 ± 0.001 and 6.125 ± 0.541 µg/mL (*Escherichia coli* 57), 0.003 ± 0.001 and 6.125 ± 0.068 µg/mL (*Klebsiella pneumoniae*), 0.001 ± 0.0 and 6.125 ± 0.046 µg/mL (*Pseudomonas aeruginosa*) and 0.012 ± 0.003 and 6.125 ± 0.571 µg/mL (*Staphylococcus aureus*), respectively. MIC values of essential oil and hydrolat vs. both *C. albicans* and *S. cerevisiae* were lower than 1/20,480 µg/mL. Based on the findings obtained, essential oils of *Withania frutescens* can be used as promising natural agents to fight free radical damage and nosocomial antibiotic-resistant microbes.

Keywords: *Withania frutescens*; essential oil; hydrolat; nosocomial infection; bacteria; candidosis; yeasts

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1. Introduction

Nosocomial infections caused by certain microbial strains remain a major cause of mortality and morbidity worldwide [1]. Many countries across the world have a great incidence of nosocomial diseases induced by multi-resistant microorganisms [2]. Several synthesized drugs have not been effective against nosocomial infections due to antibiotic-resistant bacteria [3]. It is thus fitting that medicines give high priority to alternative natural drugs to fight nosocomial antibiotic-resistant microbes [4]. Several studies have reported that plants could be promising sources of effective drugs against various microorganisms [5,6].

Antimicrobial resistance (AMR) is a complicated situation where bacteria and fungi develop strategies to defeat drugs designed to eliminate them, and therefore, the germs that are not killed continue to grow strongly, even more than before [7]. Over the past few decades, AMR has been considered as one of the biggest human health threats and was classified as belonging to the tenth challenging threat by the World Health Organization for 2019 [8,9]. Many factors are involved in the emergence of AMR including the unreasonable use of antibiotics in human health, animal husbandry, hygiene, and the food industry [10,11]. Effects of the drying pipeline of antibiotics have been also contributed to the aggravation of this issue. The phenomena of AMR are seriously alarming with complex threats, but regrettably with very few definite responses [12]. The death due to AMR infections is expected to reach 10 million by 2050 with a tremendous impact on the economy if no treatment options are conducted to contain AMR and its causative agents [13].

The studied bacteria in the present work are among the drug-resistant microbes known as the SAPEEKE group, an acronym of the following strains; *Staphylococcus aureus*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp, *Enterococcus faecium*, *Klebsiella pneumonia*, and *Escherichia coli* pathogens. These species are multidrug-resistant, extensive drug-resistant, and even pan drug-resistant, as reported elsewhere [14–16]. It is well known that *Candida* spp. tested in this work is among the drug-resistant pathogens. A few years ago, mucosal candidiasis was responsible for affecting more than 90% of patients with AIDS. Due to the massive use of different drugs including oral azoles to fight mucosal candidiasis and invasive fungal infections, the resistance of *Candida* spp. is becoming largely recognized as one of the greatest growing health burdens [17].

Free radicals can be defined as molecules containing unpaired electrons of cellular oxygen metabolism in mammals. However, free radical-associated damage is largely involved in many pathological processes and cause damage to cells, membranes, and DNA. In this sense, free radical scavengers are required to fight free radicals. Free radical scavengers from natural sources including plants are regarded as potent antioxidant agents to control free radical damage [18].

In traditional medicine, *Withania frutescens* L. is frequently used by the indigenous population to fight bacterial infections, conjunctivitis, inflammation, tuberculosis, stress, bronchitis, anxiety, neurological disorders, and ulcers as well as liver and Parkinson's disease [19]. Previously published studies reported some pharmacological activities of *Withania frutescens (W. frutescens)* including anti-inflammatory, analgesic, and healing activities [19]. *W. frutescens.L* extracts have been found to be rich in withanolides, which were isolated from the plant leaves. Moreover, the chemical analysis of the *W. frutescens.L* extract showed that this plant possessed pentacarbonyl (13.22%), **2**-phenazine carbonitrile (10.64%), Terpinenol-**4** (10.04%), **4**H-**1**-benzopyran-**4**-one,**2**,**3**-dihydro-**5**,**7**-dihydroxy-**2**phenyl(S) (8.76%), and bicyclo [**3**.**1**.**1**]heptane, **6**,**6**-dimethyl-**2**-methylene (28.48%) [20,21]. Earlier works showed no toxic effects in animals treated with the *W. frutescens* extract, therefore, the studied plant was considered safe in animals treated under both acute and subacute toxicity conditions [22–24]. Preliminary phytochemical screening of *W. frutescens* extracts showed the presence of some compound classes including tannins, coumarins, saponins, and mucilage [25].

Many studies have reported that essential oils (EOs) possess pharmacological activities. However, few studies have reported on hydrolat. Unlike EO, studies on hydrolat (steam

distillation in which an aqueous phase called hydrolat) are still limited despite the interest of the food, cosmetic, and phytotherapeutic industries. Several works have reported on the evaluation of hydrolat antioxidant power. However, this natural product can be advantageous not only as a promising source of therapeutic principles, but also as a potential preservative, especially in phyto-therapy, which includes aromatherapy [26].

To the best of our knowledge, no previous study has investigated the chemical composition and pharmacological activities of essential oils and hydrolat from *W. frutescens.* It is thus fitting that the present work aimed to study the chemical composition, antioxidant, antibacterial, and antifungal activities of both essential oils and hydrolat from this plant against antibiotic-resistant microbes.

2. Results and Discussion

2.1. Phytochemical Composition of Essential Oil

The yield of essential oil (EO) obtained by hydrodistillation of *W. frutescens* was 0.31% with 26 and 23 compounds identified by using HP-5MS and DB-HeawyWAX column, respectively (Figure 1 and Table 1). The gas chromatographic analysis showed that the monoterpene chemical classes constituted the major chemical groups in *W. frutescens* essential oils including 90.35 ± 1.72% and 88.93 ± 1.67% compounds identified by HP-5MS and DB-HeawyWAX column, respectively. For sesquiterpene classes, 3.52 ± 0.82% and 3.96 ± 0.51% were detected by HP-5MS and DB-HeawyWAX column, respectively. For the other compounds, 3.18 ± 74% and 4.22 ± 0.36% were detected by both columns, respectively.

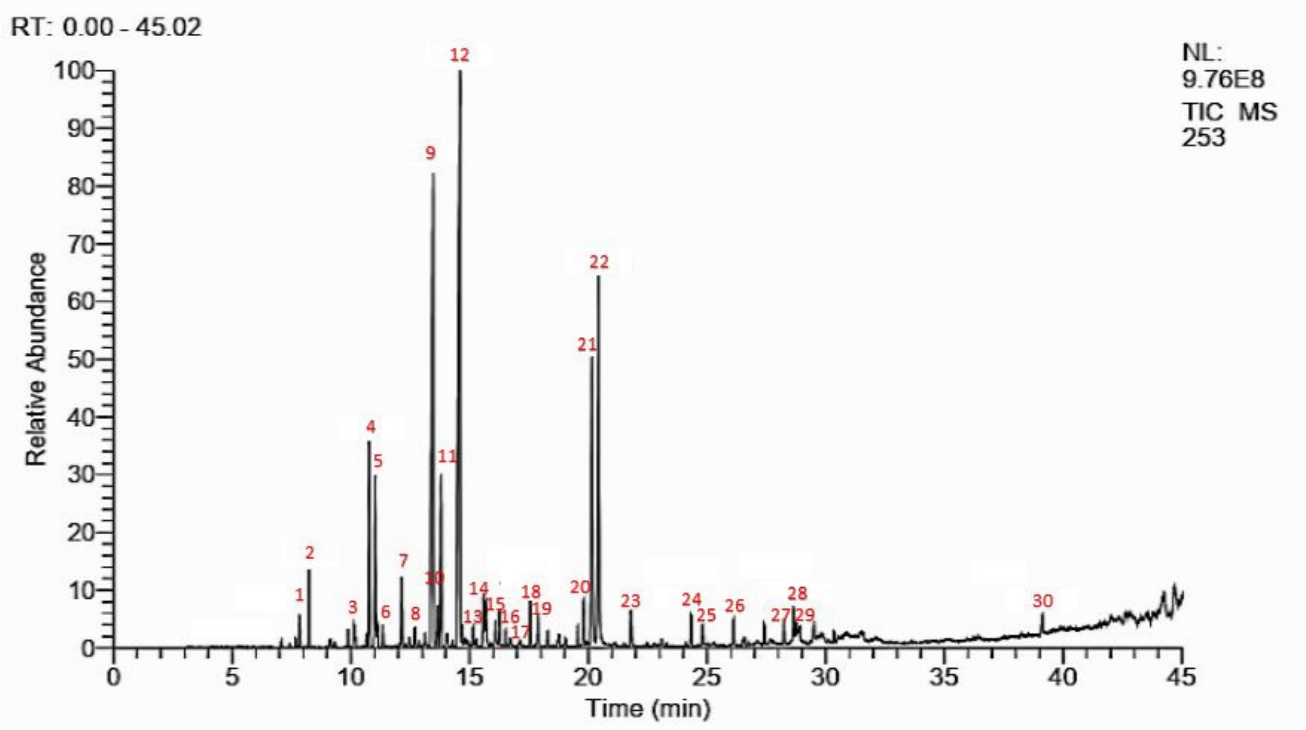

Figure 1. GCMS chromatographic profile of *W. frutescens* EO.

Table 1. Phytochemical compounds contained in the essential oil of *W. frutescens*.

		Compounds	Chemical Classes	RI		Column	
						HP-5MS	DB-H.WAX
Peak	RT (min)			Obs	Lit	(%)	(%)
1	7.84	α-Pinene	Monoterpene (MO)	937	939	1.03 ± 0.01	0.93 ± 0.01
2	8.23	Camphene	MO	946	946	1.07 ± 0.01	0.94 ± 0.02
3	10.13	Yomogi alcohol	MO	999	999	0.43 ± 0.01	0.36 ± 0.01
4	10.79	o-Cymene	MO	1020	1026	4.81 ± 0.02	4.71 ± 0.06
5	11.01	1,4-Cineole	MO	1046	1031	4.11 ± 0.01	3.94 ± 0.01
6	11.10	Limonene	MO	1023	1029	0.75 ± 0.01	Nt
7	12.16	Terpinolene	MO	1072	1088	1.42 ± 0.01	1.34 ± 0.01
8	12.69	Fenchon	MO	1087	1086	0.91 ± 0.01	Nt
9	13.48	α-Thujone	MO	1105	1102	18.64 ± 0.07	17.45 ± 0.31
10	13.66	Linalool	MO	1105	1099	1.05 ± 0.01	1.93 ± 0.01
11	13.81	β-Thujone	MO	1198	1114	4.30 ± 0.04	5.12 ± 0.23
12	14.58	Camphor	MO	1143	1146	24.26 ± 0.31	25.41 ± 0.22
13	15.16	Pinocarvone	MO	1160	1164	0.95 ± 0.01	Nt
14	15.60	Borneol	MO	1166	1169	Nt	1.16 ± 0.11
15	15.68	Bornyl acetate	Others (O)	1283	1288	1.28 ± 0.01	1.18 ± 0.06
16	16.08	4-terpineol	MO	1177	1177	0.62 ± 0.01	Nt
17	16.25	α-Ionone	O	1425	1430	Nt	0.88 ± 0.01
18	17.56	Octanol acetate	MO	1209	1213	1.65 ± 0.01	2.05 ± 0.14
19	17.86	Pulegone	MO	1237	1237	1.27 ± 0.18	0.92 ± 0.10
20	19.79	Bornyl formate	O	1595	1588	1.24 ± 0.01	1.44 ± 0.13
21	20.17	Thymol	MO	1290	1290	9.53 ± 0.8	9.24 ± 0.83
22	20.45	Carvacrol	MO	1300	1299	12.57 ± 0.97	13.43 ± 0.21
23	21.81	Nepetalactone	MO	1369	1360	0.98 ± 0.01	Nt
24	24.32	β-Caryophyllene	Sesquiterpene (ST)	1420	1419	Nt	0.82 ± 0.01
25	24.83	τ-Elemene	ST	1484	1438	0.82 ± 0.01	0.89 ± 0.01
26	26.12	Germacrene D	ST	1708	1485	0.94 ± 0.01	Nt
27	28.26	Germacrene B	ST	1823	1561	0.64 ± 0.01	Nt
28	28.66	Spathulenol	ST	1576	1578	1.12 ± 0.01	1.63 ± 0.08
29	28.76	Caryophyllene oxide	ST	1580	1583	Nt	0.62 ± 0.01
30	39.11	Hexadecanoic acid	O	1968	1968	0.66 ± 0.01	0.72 ± 0.01
			Chemical classes				
			Monoterpene (MO)			90.35 ± 1.72	88.93 ± 1.67
			Sesquiterpene (ST)			3.52 ± 0.82	3.96 ± 0.51
			Others (O)			3.18 ± 74	4.22 ± 0.36
			Total identified (%)			97.05 ± 1.07	97.11 ± 1.35

RI. = retention indices; RT. = Retention time in minutes; Obs. = retention indices determined relative to a homologous series of *n*-alkanes on HP-5MS column and on DB-HeawyWAX column, Lit. = literature RI values; Column = composition of essential oil detected on HP-5MS and DB-HeawyWAX columns; (%) = relative percentage content; NT = not detected.

The essential oils were mainly composed of camphor (24.26 ± 0.31% and 25.41 ± 0.22%), α-thujone (18.64 ± 0.07% and 17.45 ± 0.31%), carvacrol (12.57 ± 0.97% and 13.43 ± 0.21%), and thymol (9.53 ± 0.8% and 9.24 ± 0.83%) according to the detection by HP-5MS and DB-HeawyWAX, respectively.

Many potentially bioactive compounds with pharmacological activities were identified in the characterized EO including thymol, carvacrol, linalool, γ-terpinene, and p-cymene [6,27–29]. Some natural compounds reported in the studied oils like camphor possessed interesting pharmacological activities including analgesic, antiseptic, antispasmodic, anti-inflammatory, anti-infectious [30–32].

2.2. Antioxidant Activity

The antioxidant activity was assessed using the DPPH assay. The IC_{50} (the concentration of the sample required to inhibit 50% of free radicals) values of both EO and hydrolat were 14.031 ± 0.012 µg/mL and 232.081 ± 3.047 µg/mL, respectively. The IC_{50} value of BHT used as a positive control was 11.020 ± 0.903 µg/mL. ANOVA analysis showed no significant difference between the IC_{50} value of EO and that of BHT ($p > 0.05$). As shown in Figure 2, the essential oil showed interesting anti-radical activities when compared to BHT. In this sense, hydrolat showed limited antioxidant power when compared to the oil.

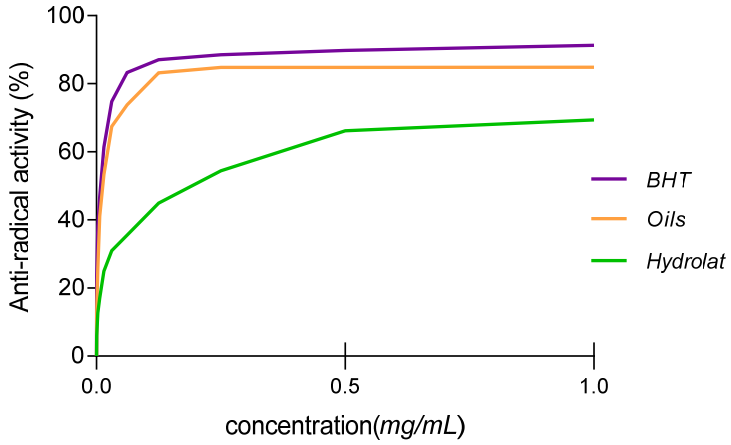

Figure 2. Anti-free radical activity of essential oil and hydrolat from *W. frutescens*.

Essential oils are complex mixtures containing several compounds with different functional groups, different polarities, and different chemical behaviors. This chemical complexity could lead to different results depending on the test used. It is thus fitting that an approach with multiple assays aiming to evaluate the antioxidant potential of hydrolat and essential oils was conducted to validate the results obtained. The results obtained revealed that the total antioxidant capacity of EO and hydrolat were 0.091 ± 0.007 and 0.131 ± 0.004 mg AAE (ascorbic acid equivalent)/mg, respectively (Figure 3).

Many studies have established a strong relationship between the chemical composition of essential oils and their antioxidant activity. The evaluation of interactions between natural antioxidant agents and other food components is an important step in the discussion of total antioxidant power in terms of health benefits. The large diversity of chemicals from a natural source, in addition to their potential interactions and action mode, make it difficult to assess the antioxidant effect by using a simple procedure [31]. It was reported that the antioxidant activity of essential oils was due to their chemical composition, particularly due to compounds with hydroxyl functions [32–34]. Consequently, essential oils that are higher in terpenes exhibit greater antioxidant power [35,36]. Metal ions are necessary for biochemical and physiological cellular functions, however, sometimes these ions go under wrong processes to cause lipid peroxidation, oxidative stress, or tissue injury in the absence of antioxidant agents [37].

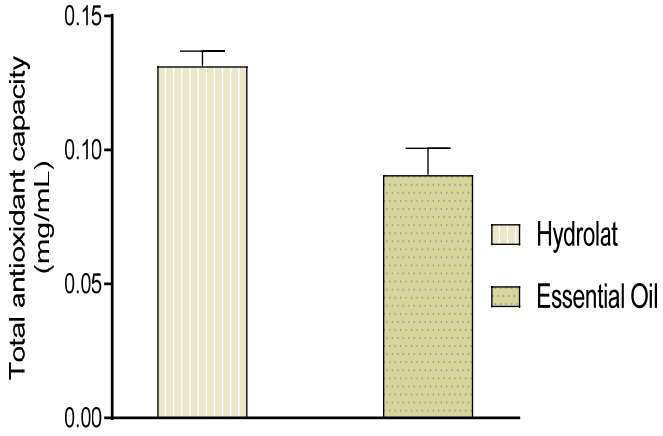

Figure 3. Total antioxidant capacity of essential oils and hydrolat from *W. frutescens*.

In this study, the antioxidant power was also evaluated using the FRAP method. As shown in Table 2, The IC_{50} values of both essential oil and hydrolat were 4.618 ± 0.045 µg/mL and 8.997 ± 0.147 µg/mL respectively. Therefore, we could confirm that EO possessed strong antioxidant power when compared to hydrolat ($p < 0.05$). Thus, we can suggest that our EO can be highly effective against free radicals.

Table 2. Antioxidant activities of essential oil and hydrolat from *W. frutescens*.

	DPPH (IC_{50} µg/mL)	FRAP (µg/mL)	TAC (mg AAE/mg EO)	β-Carotene Anti-Radical Activity (%)
Essential oil	14.031 ± 0.012	4.618 ± 0.045	0.091 ± 0.007	74.141 ± 1.040
Hydrolat	232.081 ± 3.047	8.997 ± 0.147	0.131 ± 0.004	40.850 ± 0.083
BHT	11.020 ± 0.903	0.347 ± 0.057	0.047 ± 0.001	100
Quercetin	-	0.042 ± 0.001	0.027 ± 0.001	-

The degradation of fatty acids is one of the main causes of food spoilage as reported in many works. The inhibition of lipid oxidation is frequently ensured by the intervention of natural food preservatives. In the present research, lipid oxidation was assessed by measuring the inhibitory oxidation of linoleic acid in the presence of β-carotene, which was used as a marker. The results obtained showed that the absorbance of β-carotene gradually decreased in the presence of oils, hydrolat, and BHT. Moreover, the decrease in the negative control absorbance was the most important, followed by the hydrolat, essential oil, and BHT (Figure 4).

The variation of β-carotene discoloration rate as a function of time may indicate that essential oils work against the oxidation of linoleic acid. The results obtained in this work showed that the percentage of free radical inhibition of both EO and hydrolat was 74.141 ± 1.040% and 40.850 ± 0.083%, respectively (Table 2). This activity remained significantly lower than that of BHT used as positive controls. These obtained findings were used to perform a comparison with those reported in the previous literature, which showed that species belonging to genera *Withania* possessed antioxidant power with 57% and 36% for the roots and leaves, respectively [22]. Antioxidants can exert a wide spectrum of biological functions (anti-allergic, anti-atherogenic, anti-inflammatory, antimicrobial, and antioxidant), and the identification and quantification of antioxidant content can be considered an important study to discover biological properties of natural compounds.

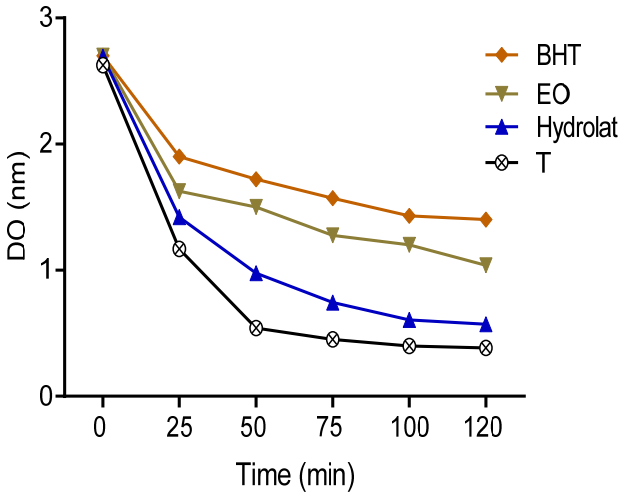

Figure 4. The absorbance of β-carotene in the presence of the samples studied, BHT, and control.

2.3. Antibacterial Activity of Essential Oil

The antibiotic resistance of strains used in the present work was well investigated before testing. All selected strains were found to be resistant to a large category of drugs, as shown in Table 3.

Table 3. List of drug resistance applied to the studied bacteria.

Bacterial Strains	Drug Resistance
Klebsiella pneumoniae	AMX, CAZ, K
Escherichia coli	CXM, AMX, CTX, K, SXT and CIP
Pseudomonas aeruginosa	SXT et AMC
Staphylococcus aureus	VA

CXM: cefuroxime; CRO: ceftriaxone; CEC: cefaclor; AMX: amoxicillin; CAZ: ceftazidime; CTX: cefotaxime; K: kanamycin; CIP: ciprofloxacin; SXT: trimethoprim/sulfamethoxazole; AMC: amoxicillin + clavulanic acid; TE: tetracycline; VA: vancomycin; E: erythromycin; P: penicillin; OX: oxacillin. K: kanamycin.

In the present work, five concentrations of essential oil were used to evaluate the antibacterial activity using the agar diffusion method and minimum inhibitory concentration (MICs) assays. The results obtained are presented in Tables 4 and 5.

The results summarized in Tables 4 and 5 show that the oil possesses a potent inhibitory effect on all selected bacteria, either Gram-negative or Gram-positive strains with different inhibition zone diameters. *E. coli* and *S. aureus* were the most sensitive bacteria to EO with inhibition zone diameters of 27 mm and 26 mm, respectively. The obtained results also revealed that the hydrolat was effective on *E. coli 57*, *K. pneumonia*, *P. aeruginosa*, and *S. aureus* except *E. coli 97*. A microdilution method was used to determine the minimal inhibitory concentration of the test sample (Table 5). The results obtained showed that the lowest inhibitory concentration of OE was recorded for *P. aeruginosa* with a concentration of 1/640 µg/mL, followed by *K. pneumoniae* with 1/320 µg/mL. Moreover, the studied OE was shown to be effective versus both types of Gram-positive and Gram-negative bacteria, unlike streptomycin and ampicillin, which were less effective [38]. Overall, the best antibacterial effect was shown by the EO vs. the broad spectrum of bacteria including both Gram-negative (*E. coli 57*, *E. coli 97*, *K. pneumonia*, *S. aureus)* and Gram-positive bacteria (*P. aeruginosa*). The antibacterial properties of essential oils observed in this work can be explained by the fact that the oil has a lipophilic character that makes it easy to penetrate the bacteria cell and ultimately lead to bacteria death. It was reported that the hydrocarbons make essential oil preferentially lodged in the biological membranes, which disturbs the membrane permeability, and ultimately leads to the immediate death of bacteria [38–40]. Closer data reported on the mechanism of action of oils with hydrocarbons can serve as a valuable reference for a better understating of oil mechanism actions toward

bacteria [41]. Chemicals in the oil can work in synergy more than individually, as previous work showed that the antimicrobial activity of essential oils was found to be higher than its single compounds tested separately [42,43].

Table 4. Diameter of the inhibition zone of EO, hydrolat, and antibiotics (mm).

Compound	Gram-Negative Bacteria				Gram-Positive Bacteria
	Escherichia coli 57	*Escherichia coli 97*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*
Essential oil	24.32 ± 0.20	27.50 ± 0.11	19.71 ± 0.10	16.11 ± 0.21	26 ± 0.41
Hydrolat	11.21 ± 0.13	-	9.23 ± 0.50	8 ± 0.52	-
Streptomycin	-	-	-	-	9.61 ± 0.20
Ampicillin	-	-	-	-	-

Table 5. Minimum inhibitory concentration of hydrolat and essential oil of *W. frutescens* (MIC in µg/mL).

Compound	Gram-Negative Bacteria				Gram-Positive Bacteria
	Escherichia coli 57	*Escherichia coli 97*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*
Essential oil	0.006 ± 0.001	0.050 ± 0.004	0.003 ± 0.001	0.001 ± 0.0	0.012 ± 0.003
Hydrolat	6.125 ± 0.541	-	6.125 ± 0.068	6.125 ± 0.046	6.125 ± 0.571
Streptomycin	0.250 ± 0.027	0.500 ± 0.002	0.003 ± 0.001	-	0.062
Ampicillin	-		-	-	-

Our findings showed that the bacterial strains tested whether Gram-negative or whether Gram-positive was found to be completely resistant to Ampicillin and partially to Streptomycin. These results agree with those reported in earlier works [44], which showed that the most threatening drug-resistant microbes including *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, *Enterobacter* spp., and *E. coli* pathogens. The microbes tested in this work are classified as being multidrug-resistant, extensive drug-resistant, and even pan-drug-resistant, as reported elsewhere [15,16]. The limited treatment strategies vs. drug-resistant infections require new and more effective antibiotics, however, the number of antibiotics approved for clinical use since 2000 has been limited and most of them respond only to Gram-positive bacteria, and their response against Gram-negative strains are reduced by the time, which can accentuate the emergence of a greater threat to human health [45,46].

To have a antimicrobial effect, the antimicrobial agent needs to reach and interact with target microbe sites. However, the drug–target interaction is frequently interrupted via various mechanisms in bacteria (multidrug-resistant, and extensive drug-resistant), which leads to the ineffectiveness of antimicrobial agents and ultimately help develop bacteria against the tested agents [44]. The low sensitivity of Gram-negative microorganisms to antibacterial agents may be explained by the fact that they have an outer membrane surrounding the cell wall, which limits the diffusion of hydrophobic compounds through its lipopolysaccharide coating. Due to their lipophilic nature, essential oils can easily cross the cell walls and the cytoplasmic membrane causing disorders of polysaccharide structure, fatty acids, and phospholipids as well as their permeability [47]. Our findings showed that essential oils have almost similar activity against Gram-positive and Gram-negative bacteria. Therefore, we could confirm that *W. frutescens* EO can be a promising weapon to fight nosocomial pathogenic and multidrug-resistant strains.

2.4. Antifungal Activity

Yeast infections have a high frequency in hospitalized patients worldwide with many risk factors associated with a poor prognosis. Several epidemiological studies conducted on

yeast infections showed that *Candida* is responsible for many diseases. *Candida* species have been reported as the most common cause of invasive fungal infections among hospitalized patients, accounting for 8 to 10% of all nosocomial infections [48]. It was reported that invasive candidiasis is frequently associated with high crude death rates, and the control of these infections can be a great challenge since several antifungal options can no longer be effective toward the resistant strains [49]. *S. cerevisiae* has involved in human pathology causing vaginitis [50].

The antifungal activity of EO and hydrolat was conducted on *C. albicans* and *S. cerevisiae* yeasts. The inhibition of fungal growth was noted in the presence of essential oil and hydrolat. The results obtained showed that a strong essential oil inhibition was observed for *C. albicans* and *S. cerevisiae* with an inhibition zone diameter of 47 ± 3.120 mm and 40 ± 6.450 mm, respectively (Table 6). The drug used as the reference was less effective against the yeasts when compared to the essential oil with an inhibition zone diameter of 21.200 ± 4.200 mm against *Candida* and 27.650 ± 2.500 mm and *Saccharomyces*, respectively (Table 6). Our essential oil had a strong antifungal activity when compared to all standards used including fluconazole and copper sulfate. The minimum inhibitory concentration of essential oil was very low, as shown in Table 6. The results obtained revealed that our studied oil was more effective against both *C. albicans* and *S. cerevisiae* with a minimum inhibitory concentration of 10^{-4} mg/mL. The hydrolat also had a minimum inhibitory concentration of about 12.5 mg/mL. The hydrolat extract was moderately effective when compared to other results with an inhibition zone diameter of 9 ± 1.750 mm against both yeasts, *C. albicans* and *S. cerevisiae*, tested. The hydrolat extract also showed better activity by using the minimum inhibitory concentration assay on *C. albicans* of 0.400 ± 0.020 mg/mL and *S. cerevisiae* of 0.200 ± 0.010 mg/mL. The fungal strains investigated in the present work belong to the drug-resistant microbes since they showed high MICs, which existed in the range of 0.400 ± 0.020 and 0.400 ± 0.020 mg/mL for fluconazole and 10 ± 0.5–10 ± 0.25 for copper sulfate used as drug references (Table 6) [50].

Table 6. Results of the antifungal activity of the essential oil and hydrolat.

	Antifungal Activity by Disc Method (mm)		**Antifungal Activity by the Microdilution Method (MIC in mg/mL)**	
	C. albicans	***S. cerevisiae***	***C. albicans***	***S. cerevisiae***
EO	47 ± 3.120	40 ± 6.450	0.0004 ± 0.0	0.0004 ± 0.0
Hydrolat	9 ± 1.750	9 ± 1.250	12.500 ± 0.207	12.500 ± 0.310
Fluconazole	21.200 ± 4.200	27.650 ± 2.500	0.400 ± 0.020	0.200 ± 0.010
Copper sulphate	8.5 ± 0.3	7.25 ± 0.4	10 ± 0.25	10 ± 0.5

Many studies have reported interesting data on the mechanism of action of essential oils in fungi for the understanding of the corresponding mechanisms of activity. It was concluded that oils with thymol and p-cymene penetrate cells, causing severe damage to the membrane [51]. The fungicidal activity results from direct damage to the membrane of cells rather than from metabolic impairment, leading to secondary damage of the cell membrane [52]. Such activity is in agreement with the chemical nature of monoterpenes, which most potentially act as a solvent of the cell membrane. In closer works, it was stated that fungicidal activity of oil with thymol and p-cymene vs. *Candida* spp. resulted from direct damage to the cytoplasmic membrane [53].

3. Materials and Methods

3.1. Chemicals

Ammonium molybdate, butylated hydroxytoluene (BHT), **2**,**2**-diphenylpicrylhydrazyl (DPPH), sodium phosphate, quercetin, vitamin C, iron III chloride (FeCL3), **2**,**3**,**5**-triphenyltetrazolium chloride (TTC), potassium ferricyanide (K3Fe (CN) 6), and β-carotene were purchased from Sigma Aldrich (Germany, Munich).

3.2. Selection and Identification of Plant Material

W. frutescens was collected at the end of March 2019 from the region of Fez-Morocco. The botanical identification was carried out by the botanist Amina BARI and given the voucher number BPRN69 before being deposited at the herbarium of the Faculty of Sciences, Sidi Mohamed Ben Abdellah Dhar El-Mahraz Fez University, Morocco. The aerial parts of the studied plant were dried in the shade at room temperature for 10 days before being subject to extraction.

3.3. Extraction of Essential Oils

A total of 200 g of the aerial part were finely cut and placed in a round-bottomed flask with 750 mL of distilled water. The mixture was boiled for 2 h and then the EO obtained was separated from the water before being stored at 4 °C in the darkness until further use.

3.4. Preparation of Hydrolat

Extraction of hydrolat was carried out by liquid–liquid extraction. Briefly, 200 mL recovered from the solution obtained by hydrodistillation was successively extracted again three times with 100 mL of diethyl ether at room temperature to obtain the hydrolat extract. Afterward, the organic layer was evaporated and then the remains were dried out using Na_2SO_4 to obtain the oil (0.03%).

3.5. Chemical Characterization of Essential Oil by GC/MS

The identification of different chemical compounds contained in essential oils was carried out by gas chromatography coupled to a mass spectrometer. *W. frutescens* oil was analyzed using a Thermo Fischer capillary gas chromatograph directly coupled to the mass spectrometer system (model GC ULTRA S/N 20062969; Polaris QS/N 210729) using two columns, a non-polar HP-5MS capillary fused silica column (60 m 0.32 mm, 0.25 mm film thickness) and a DB-HeawyWAX column (30 m × 0.25 mm, film thickness 0.25 µm), in addition to GC-FID (flame ionization detector). GC-MS operating conditions were maintained as follows: initial temperature of 40 °C/2 min; speed of 2 C/min; a final temperature of 260 °C/10 min; injector temperature of 250 °C; and carrier gas of helium 1 mL/min. The essential oil was diluted in hexane with a dilution ratio of 10:100. The volume of the sample injected was 1 mL with the fractional injection technique; ionization energy was 70 eV, ionization mode; ion source temperature of 200 C, sweep mass range m/z 40–650, and interface line temperature of 300 C. Retention indices (RI) were determined with reference to a homologous series of *n*-alkanes and by matching their recorded mass spectra with those stored in the spectrometer database (NIST MS Library v. 2.0) [38,54,55].

3.6. In Vitro Antioxidant Activity of Essential Oils

The antioxidant power of essential oil was evaluated in vitro using four assays: DPPH-, reducing power, total antioxidant capacity, and the β-carotene discoloration.

3.7. Diphenyl-1-Picrylhydrazyl Assay

The DPPH test was carried out according to the method described by BEKTAS [56]. Briefly, 100 µL of EO diluted in methanol was used with different concentrations. Each test portion was mixed with 750 µL of DPPH solution (0.004%). After 30 min of incubation at room temperature, the absorbance was read at 517 nm. Results were expressed as percentage inhibition according to the following formula.

$$PI(\%) = (A_0 - A/A_0) \times 100$$

where PI is the inhibition percentage; A0 is the DPPH absorbance without the sample (negative control); and A is the DPPH absorbance with the sample.

3.8. Ferric Reducing Antioxidant Power Test

This test was carried out according to the previously reported method [56]. Briefly, 500 µL was recovered from phosphate buffer solution (0.2 MPH = 6.6) and mixed with 500 µL of potassium ferricyanide [$K_3Fe(CN)_6$] (1%). Afterward, the whole was added to 100 µL of oil diluted in methanol before being incubated at 50 °C for 20 min within the water bath. The sample was mixed again with 500 µL of aqueous solution (10% TCA) including 500 µL of distilled water and 100 µL of 0.1% ($FeCl_3$) to be ready for analysis. The absorbance was determined at 700 nm against a blank. The results obtained were expressed as an effective concentration. The median effective concentration (EC-50) was performed from the graph.

3.9. Total Antioxidant Capacity Test

Twenty-five µL of EO was mixed with 1 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). Afterward, the solution obtained was incubated at 95 °C for 90 min. The absorbance was measured at 695 nm [57]. The total antioxidant capacity was expressed in milligrams of ascorbic acid equivalent per gram of essential oil (mg EAA/g EO). The experiment was performed in triplicate.

3.10. Beta-Carotene Discoloration Test

One milliliter of the solution of β-carotene solubilized in chloroform (2 mg/10 mL) was introduced into a vial containing 10 µL linoleic acid and 100 mg Tween 80. The chloroform was evaporated at 45 °C for 5 min using a rotary evaporator under empty. Next, 25 mL of hydrogen peroxide was added to the residue before adding water to obtain an emulsion. A total of 2.5 mL of the mixture obtained was mixed with 100 µL of essential oil diluted in methanol and was then incubated in the water bath at 50 °C for 2 h with the BHT (positive control). The percentage of antioxidant activity was calculated according to the following equation [58].

$$AA\% = (A_E / A_{BHT}) \times 100$$

where AA% is the percentage of antioxidant activity; A_E is the absorbance of the sample; and A_{BHT} is the absorbance of the sample with the positive control (BHT).

3.11. Antibacterial Activity

The antibacterial activity of essential oil was tested on five strains: Gram-negative bacteria including *E. coli* (ATB:57) B6N (CHU, Fez), *E. coli* (ATB:97) BGM (CHU, Fez), *K. pneumoniae* (LM, FMP, Fez), and *P. aeruginosa* (LM, FMP, Fez); Gram-positive bacteria including *S. aureus* (LM, FMP, Fez). All strains tested were clinically isolated from lung, urinary tract, and surgical site infections (University Hospital Complex, Fez, Morocco). These bacterial strains have been reported as multidrug-resistant, extensive drug-resistant, and even pan-drug-resistant [14–16]. The disk diffusion method was used to determine the inhibition zone. Briefly, after incubation of the inoculated plates for 30 min, the experiments were conducted as follows: sterile discs (6 mm) impregnated with 10 µL of the test material, ampicillin 1.67 mg/disc, and streptomycin 0.02 mg/disc were placed on the agar surface. Afterward, the plates were incubated again at 37 °C. At the end of the experiment, the antimicrobial power was evaluated by measuring the growth inhibition zones in mm [38].

The bacterial strains selected were cultured in tubes containing 9 mL of MHB (Mueller–Hinton broth) before being incubated at 37 °C for 18–24 h. A drop of the culture was seeded onto Petri dishes containing nutrient agar before being incubated again at 37 °C for 18–24 h. The bacterial suspension (inoculum) was prepared from the pure cultures as follows: identical colonies from wells were isolated and then discharged into 10 mL sterile physiological water with 0.9% NaCl. The optical density of bacterial suspension was adjusted to be between 0.08 and 0.1 nm, which corresponded to 10^7 to 10^8 CFU/mL

according to McFarland. The minimum inhibitory concentrations (MICs) were determined using a microdilution method [59].

3.12. Antifungal Activity

The antifungal activity of both EO and hydrolat on *Candida albicans* and *Saccharomyces cerevisiae* was performed using the direct contact method. Different concentrations of the test sample were incorporated into the agar culture medium.

The blastospores of strain cultures (5-day cultures) were recovered from Petri dishes before counting the blast strains obtained using the Malassez cell. The optical density (OD) of the fungal spore suspension was measured using a spectrophotometer with 630 nm to standardize the spore suspension at 10^7 blastospores/mL. Fluconazole and copper sulfate were used as positive controls with 5 mg/mL.

Different concentrations (v/v) of 1/100; 1/200; 1/400; 1/800; 1/1600; 1/3200 of oils were incorporated into the PDA (potato dextrose agar) culture medium. Afterward, 10 µL of the inoculum spot was deposited in each medium. Petri dishes were sealed with parafilm before being incubated at 27 °C for six days. The minimum inhibitory concentration was observed in the solid medium [60]. Petri dishes with concentrations that showed a total absence of mycelial growth were selected to determine the minimum inhibitory concentrations (MIC).

In the present work, the obtained results showed that the oil from *W. frutescens* was found to exhibit a potent antioxidant activity in all bioassays used such as DPPH, FRAP, TAC, and β-carotene. The antioxidant activity of the essential oil tested was strongly correlated with the antimicrobial and antibacterial activity. However, hydrolat showed limited effects toward fungal and bacteria tested as well as the antioxidant activity.

3.13. Statistical Analysis

The mean values ± standard deviations were calculated using GraphPad Prism 7 (Microsoft Software, GraphPad Software Inc.; San Diego, CA, USA). The results were compared using one-way ANOVA and the Tukey-test as the post-hoc test. The difference at $p < 0.05$ was considered to be significant.

4. Conclusions

In the current research study, we investigated the chemical composition, antioxidant, antibacterial, and antifungal activities of essential oil and hydrolat from *W. frutescens* on nosocomial antibiotic-resistant microbes. The results obtained showed that the oil was found to be rich in bioactive compounds with promising activities on both yeasts and bacteria. However, hydrolat was not more active toward the fungal and bacteria tested nor in antioxidant activity. Finally, we confirmed that oil in *W. frutescens* can serve as medicines as it provides potentially active agents to fight free radical damage, bacterial, and fungal infections.

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