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Antimicrobial and antioxidant potential of *Lavandula pedunculata* extracts and their inhibitory action against *Candida albicans*

HANANE BAKRIM♥, MERIEM MABCHOUR, OUIAM EL GALIOU, ABDELHAY ARAKRAK, MOHAMMED BAKKALI, AMIN LAGLAOUI♥♥

Biotechnology and Biomolecular Engineering Research Team, Department of Biology, Faculty of Science and Technology, Abdelmalek Essaâdi University. B.P 416 St, Tangier, Morocco. Tel.: +21-253-9393954, ♥email: bakrimhanane@gmail.com, ♥♥email: laglaouiamin@yahoo.fr

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Abstract. *Bakrim H, Mabchour M, El Galiou O, Arakrak A, Bakkali M, Laglaoui A. 2024. Antimicrobial and antioxidant potential of* Lavandula pedunculata *extracts and their inhibitory action against* Candida albicans*. Biodiversitas 25: 4020-4032. Lavandula pedunculata* (Mill.) Cav. is an aromatic and medicinal plant that has attracted widespread interest due to its numerous potential applications in the food and pharmaceutical industries. Our study aimed to investigate the biological activities of organic extracts obtained from *L. pedunculata* using Soxhlet extraction by *n*-hexane, dichloromethane, methanol, and distilled water. Extracts were evaluated for their antibacterial, antifungal, and antioxidant potential. Although these extracts did not display any antibacterial effect, we observed a significant effect against *Candida albicans*. The aqueous *L. pedunculata* extract exerted a bacteriostatic effect (11±1 mm, MIC=>40 mg/mL) and inhibited the formation of *C. albicans* biofilms (78.8%). Moreover, the aqueous fraction had the greatest potential to curb exopolysaccharide production and protein release, with OD values lower than control. Antifungal screening revealed that methanolic and dichloromethane extracts of *L. pedunculata* exerted the highest activity against *Botrytis cinerea* and *Fusarium oxysporum* as both extracts exert a fungistatic action (100% inhibition at 10 mg/mL). Antioxidant potential was particularly strong in the methanolic extract, as revealed by DPPH and FRAP assays (IC_{50}= 0.012 mg/mL). Phytochemical screening showed a strong correlation between phenolic content and antioxidant effect. LC-MS analysis of the aqueous fraction detected the presence of glucuronide and glucoside conjugates of quercetin and kaempferol, apigenin, and vanillic acid, which are indicative of rich polyphenolic content.

Keywords: Antifungal activity, antioxidants, biofilms, *Lavandula pedunculata*, plant extracts

INTRODUCTION

The development of antibiotics has been a revolutionary breakthrough in microbiology; however, new drug-resistant strains with an elevated pathogenic potential pose a significant threat to human health (WHO 2014). Furthermore, cell damage due to reactive oxygen species (ROS) is associated with various chronic illnesses, including cardiovascular disease (Farías et al. 2017). Current drug treatments reportedly have harmful side effects (Kelesidis and Falagas 2015; Angel et al. 2020). Therefore, the scientific community is increasingly searching for novel, effective therapeutic agents with minor toxicity. Plant-based extracts are attracting increased interest due to their biological activity (Tuyiringire et al. 2020; Khan and Al-Balushi 2021). A growing body of research has highlighted their key properties, including antibacterial and antioxidant activities (Acemi et al. 2020; Farag et al. 2020; Frankova et al. 2021). Such findings are of considerable significance, especially given emerging challenges in the health sector, ranging from worldwide pandemics to health complications partially triggered by oxidative stress.

In addition, herbal extracts have proven to be of major significance for the food and agricultural industries. Within the framework of the new move towards *green* labeling, several research teams have explored the potential of incorporating herbal extracts in food products. Sivarajan et al. (2017) demonstrated that a combination of clove and cinnamon extracts can extend chicken shelf-life up to 24 days and confer desirable sensory attributes. Cadet et al. (2013) noted that the levels of microbial pathogens in ready-to-eat turkey were reduced by incorporating extracts of *Alpinia galanga* (Linn.) the food's color and pH integrity were maintained. Meanwhile, in the agricultural production sector, such herbal extracts offer a promising solution to the challenge posed by pathogenic fungi, which cause various dysfunctions in plants, leading to significant annual losses (Jamiołkowska 2020). The extensive use of synthetic fungicides is met with increasing apprehension due to the health and environmental risks they incur (Triantafyllidis et al. 2020; Marciano et al. 2024). Therefore, a major move has been made to reduce dependency on synthetic fungicides while searching for alternative control strategies (Subba and Mathur 2022). Numerous research papers have provided evidence that organic extracts derived from plants such as *Plumbago indica*, *Dahlia variabilis*, *Zingiber cassumunar*, and *Durvillaea antarctica* possess the ability to inhibit the development of pathogenic fungi in plants (Marutescu et al. 2017; Saryono et al. 2017; Dethoupet al. 2018).

Lavandula pedunculata is an aromatic shrub that grows all across the western Mediterranean area (Zuzarte et al. 2010). Many peoples and tribes use *L. pedunculata* as an antibacterial in their traditional medicine. Aqueous extracts from *L. pedunculata* have MIC values of 0.2 to 0.75 mg/mL

for *Staphylococci*, 1.2 mg/mL against *Streptococci*, and 0.5 mg/mL against *Pseudomonas aeruginosa*. Interestingly, the combination of *L. pedunculata* with *Salvia lavandulifolia*, *Salvia rosmarinus*, and *Origanum compactum* can have additive and synergistic effects (Boutahiriet al. 2022). Portuguese *L. pedunculata* has likewise been shown to restrain *Bacillus cereus*, *Staphylococcus aureus*, and *Salmonella enterica* (Vilas-Boaset al. 2023). The same study revealed that maceration and microwave-assisted extraction can be thoroughly effective in yielding biologically active phenolics, particularly salvinalic acid B and rosmarinic acid. Numerous other components with pharmacological properties can be detected in *L. pedunculata*, including coumarin, tartaric acid, isoferulic acid, salvianolic acid B, herniarin, caffeic acid, apigenin, luteolin, myricetin, and chlorogenic acid. These secondary metabolites exert biological effects on several levels: on the one hand, they have an antihyperglycemic effect through the inhibition of digestive enzymes and glucose absorption; on the other hand, they exhibit antimicrobial and antioxidant activity given their high redox potential and their capacity to interfere in the cell membrane and induce structural and functional changes, thereby impacting the electron transport chain, nutrient uptake, DNA synthesis (Boutahiri et al. 2021; Vilas-Boas et al. 2023). Several studies have focused on the biological activity of *L. pedunculata* (Costa et al. 2013; Nafis et al. 2021). Our study addresses the biological importance of this plant's extracts by conducting a series of in vitro studies intended to evaluate the plant-based antibacterial, antioxidant, and antifungal properties of *L. pedunculata* for industrial and pharmacological applications.

MATERIALS AND METHODS

Chemicals and reagents

Gas chromatography grade solvents *n*-hexane (C_6H_{14}) 97%, dichloromethane (CH_2Cl_2) 99.8%, and methanol (CH_3OH) 99%, along with glycerol (HO-CH_2CH(OH)-CH_2-OH), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Resazurin sodium salt, Folin-Ciocalteu phenol reagent, Gallic acid, and Butylated Hydroxytoluene (BHT) were all obtained from Sigma-Aldrich (Saint-Quentin Fallavier, France). Dimethyl Sulfoxide (DMSO), Tween 20, Sodium carbonate, and Rutin were purchased from Merck KGaA (Darmstadt, Germany). Potassium ferricyanide was obtained from Fluka Chemika (Buchs, Switzerland). Trichloroacetic acid and Iron chloride were purchased from Riedel-deHaën (Seelze, Germany). Microbiological culture media Mueller Hinton Broth (MHB), Brain Heart Infusion Broth (BHI broth), Potato Dextrose Agar (PDA), and bacteriological agar Type A came from Biokar Diagnostics (Allonne, France).

Procedures

Plant material and Soxhlet extraction

The plant material selected for this study consists of aerial parts of *L. pedunculata*. Plant samples were collected in the Rif region of Morocco during the flowering period in May 2018. After harvesting, plant leaves were separated and dried at 45°C with continuous ventilation, then stored in the dark until use. Before extraction, the dried leaves were crushed by an electric grinder to produce fine plant powder. 25 g of plant powder is loaded into an extraction thimble and placed inside the Soxhlet apparatus. The extraction procedure takes place by initially using 250 mL of *n*-hexane at 50°C, followed by dichloromethane, methanol, and distilled water at 100°C. Although this technique is considered costly in terms of time and solvent, it offers the advantage of ensuring a quasi-total depletion of the plant material, thus increasing the chemical diversity of the obtained extracts.

Antibacterial activity

Well diffusion assay. The bacterial cultures *Enterococcus faecalis* (ATCC 19433), *Escherichia coli* (ATCC 25922), *Listeria monocytogenes* (ATCC19144), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 14028), and *Staphylococcus aureus* (B1), and the yeast isolate *Candida albicans* were grown in sterile Brain-Heart Infusion Agar (BHIA). The obtained cultures were re-suspended in sterile physiological water and adjusted to the McFarland 0.5 standard equivalent to a concentration of approximately 10^8 CFU/mL. Next, 100 µL of the adjusted suspensions were spread uniformly across sterile Mueller-Hinton plates, and 5 mm wells were then created using a sterile pipette tip, 50 µL of each test extract prepared at 30, 60, and 100 mg/mL were added into the wells. Therefore, to ensure the diffusion of the tested samples in the medium, test plates were incubated for 2 h at 4°C before being placed in a 37°C environment for overnight incubation. Solvent controls were included as negative controls to confirm that any observed effects would solely be due to the activity of the extracts and not affected by the solvent used for extract solubilization (10% DMSO for the solubilization of dichloromethane and methanol extracts, $Tween_{20}$+distilled water [2:1] for the solubilization of the hexane extract, and sterile distilled water for the solubilization of the aqueous extract).

The antibacterial effect was determined by measuring the diameter of inhibition zones obtained by the active extracts (Well diameter not included).

Microdilution assay. We conducted a microdilution assay to determine the minimum inhibitory and minimum bactericidal concentrations according to Mann and Markham (1998) with slight modifications. A dilution series ranging from 40 mg/mL to 4.37 mg/mL was prepared in Mueller-Hinton broth. Each sample was then transferred into 96-well microplates (180 µL) and pre-inoculated with 20 µL of diluted bacterial suspension (10^8 CFU/mL). The plates were incubated at 37°C for 24h. 0.01% of resazurin sodium salt (10 µL) was added after incubation, and the plates were left stable and untouched for 30 min. Color changes were observed to assess active concentrations, whereby MIC corresponded to the lowest concentration, showing a bacteriostatic effect as revealed by the absence of any discoloration of the resazurin dye. MBC was defined as the lowest concentration revealing a bactericidal effect, deduced by sub-culturing wells corresponding to concentrations higher or equal to MIC on agar plates. Solvents used for

extract solubilization (i.e., DMSO and $Tween_{20}$) served as negative controls.

Inhibition of initial cell adhesion by active plant extracts

Preliminary testing revealed *C. albicans* to be a representative model of sensitive strains. We, therefore, conducted further testing to investigate the influence of *L. pedunculata* aqueous extract on this strain's capacity to induce surface attachment through the protocol adopted by Bazargani and Rohloff (2016) with slight modifications. We added 100 µL aliquots of *C. albicans* suspensions (1×10^9 CFU/mL) to wells of a flat bottom titration plate. Each well was then supplemented with 100 µL of the plant extract to expose the strain to final extract concentrations of 2MIC, MIC, and ½ MIC. Controls were established with 100 µL of bacterial suspensions and an equal volume of broth media infused with solvents applied to solubilize the extracts, ensuring that the observed effects were not influenced by any external factors other than the tested extracts. Sterile Mueller Hinton Broth (MHB) (200 µL) served as blank control. The plates were incubated for 48h at 37°C under static conditions to promote cell adhesion to the well surface. After incubation, the broth was discarded along with the non-adhered cells; the wells were washed 3 times with sterile distilled water and dried at 60°C for 1h. We then added 200 µL of 0.4% Cristal Violet (CV) to each well and further incubated the plate at room temperature for 15 min. The stain was then removed, and the plates were washed three times to eliminate the non-absorbed dye. We subsequently added 150 µL of ethanol to destain the wells (to remove the CV dye absorbed by the cells attached to the wells). Finally, 100 µL of this ethanol solution is transferred to a new plate to determine the inhibition rate (IR) at OD_{590} using the equation:

$$IR\ (\%) = [(OD_{(Negative\ control)} - OD_{(Test\ sample)}) / OD_{(Negative\ control)}] \times 100$$

Where: OD: Optical density

Measurement of antimicrobial kinetics in real-time using the biosan bioreactor

We determined the growth kinetics of sensitive strains in the presence of active *L. pedunculata* extracts using the Biosan RTS-1 bioreactor, which relies on reverse-spin mixing technology combined with a near-infrared optical system, thereby allowing for a non-invasive determination of bacterial growth via optical density in real-time. Mueller Hinton broth cultures were prepared (24 h). We added 1 mL of the standardized isolates (1×10^6 CFU/mL) to 9 mL of MH broth inoculated with the active extracts at a concentration corresponding to 2MIC. The resulting suspension was placed in the bioreactor programmed at 37°C and 2000 rpm/min. The reverse tube spin period was set at 1s, and measurements were taken at 15-minute intervals over the 24-hour incubation period. Control experiments contained all components (including solvents used for extract solubilization at volumes equivalent to the quantities applied in the test group), excluding plant extracts. Kinetic parameters were determined from the raw data according to the following equations:

Bacterial growth rate (μ_{expo}): $Yf = A_i e^{k\Delta t}$ (with k= µexpo)

Generation time (G): $G = \ln(2)/\mu_{expo}$

Where: μ_{expo}: Growth rate determined during the exponential growth phase (h^{-1}), G: Generation time or the doubling time of the microbial population (h)

Determination of antifungal potential of L. pedunculata *extracts*

We screened *L. pedunculata* extracts for their ability to inhibit the mycelial growth of two fungal strains: *B. cinerea* and *Fusarium oxysporum*. Briefly, stock solutions of each extract were serially diluted in PDA to obtain final concentrations ranging from 10 mg/mL to 0.02 mg/mL. These dilutions were later poured into Petri plates and left to solidify for 15 min. After agar solidification, mycelial discs of 6 mm diameter were added to the center (Onaran and Sağlam 2016). The plates were incubated at 25°C for seven days. Fungal growth kinetics were identified in day-to-day measurements. Antifungal potential was assessed in terms of inhibition percentage (I%) using the following equation:

$$I\ (\%) = [(D_C - D_T)/ D_C] \times 100$$

Where: D_C: Growth diameter of the negative control (PDA medium supplemented with DMSO alone or $Tween_{20}$+distilled water), D_T: Growth diameter of the test samples

All tests were performed in duplicate; results are expressed as means±SD. The development of the disc diameter was measured daily for 7 consecutive days, and the obtained data were used to assess the mycelial growth rate (MGR) (Cahagnier 1998).

$$MGR\ (mm/h) = Di_1\ T_1 + (Di_2 - Di_1)T_2 + (Di_3 - Di_2)T_3 + \ldots\ldots\ldots\ldots + Di_n - Di_{n-1})T_n$$

Where: Di: Growth diameter measured on a day-to-day basis (mm), T: Incubation time (h)

Quantification of *L. pedunculata* virulence factors

Quantification of extracellular proteins

To assess the *L. pedunculata* extracts' impact on the production of virulence factors by *C. albicans*, we quantified the amount of extracellular proteins using the Bradford assay in a sterile 96-well microplate (Bradford 1976; Adonizio et al. 2008). Briefly, *C. albicans* (with 10^6 CFU/mL) was cultivated in Luria-Bertani broth (LB broth) for 48h at 37°C in the presence (at concentrations of 1/8 and 1/4 MIC) and absence of the extracts (0 MIC). After cultivation, the cultures underwent centrifugation at 10000 rpm for 12 min. The resulting supernatants were subsequently filtered using 0.45 µm syringe filters before measuring the total protein content at OD_{595} nm. Uninoculated media served as blank controls. Each treatment was carried out in triplicate.

Exopolysaccharide extraction and estimation

Apart from determining total protein content, the supernatants were used to extract exopolysaccharides (EPS) by adding chilled ethanol (95%) (Huston et al. 2004). The mixtures were left overnight at 4°C for EPS precipitation to quantify sugar content using the Anthrone method (Yemm and Willis 1954). Media without *C. albicans* inoculation were utilized as blanks. Triplicates were established for each treatment and quantified according to the Dubois method (Dubois et al. 1951).

Evaluation of antioxidant potential and total phenolic content of *L. pedunculata* extracts

DPPH radical scavenging assay

We assessed the capacity of *L. pedunculata* extracts for free radical scavenging according to Mensor et al. (2001). A DPPH solution was prepared in methanol at 0.3 mM, and 1 mL thereof was mixed with 2.5 mL of a dilution series of plant extracts ranging from 2 mg/mL to 0.0005 mg/mL for hexane, dichloromethane, and aqueous extracts and 0.016 to 0.002 mg/mL for the methanolic extract. The mixture was vigorously vortexed and then incubated in the dark for 30 min. The antioxidant reaction was detected spectrophotometrically at 518 nm. A reference reaction was prepared with BHT. Blank samples included 1 mL of methanol and 2.5 mL of extracts in methanol at the tested concentrations. The percentage of antioxidant activity (AA %) was calculated with the following formula:

$$AA\ (\%) = [(A_{control} - A_{sample})/A_{control}] \times 100$$

Where: A: Absorbance

Analysis of ferric-reducing capacity using the FRAP method

We further investigated the antioxidant activity of *L. pedunculata* extracts by measuring ferric-reducing antioxidant power (FRAP assay) (Lfitat et al. 2021). Briefly, 2.5 mL of a dilution series of each extracted sample (2 mg/mL-0.0005g/mL) was added to equal volumes of potassium ferricyanide ($K_3Fe\ [CN]_6$) (1%) and phosphate buffer (0.2 M, pH 6.6). After a 20 min water bath at 50°C, 2.5 mL of trichloroacetic acid (10%) was added. The mixture was then centrifuged at 3000 rpm for 10 min. Aliquots from the supernatant (2.5 mL) were transferred and supplemented with 2.5 mL of distilled water before adding 100 µL of 0.1% Iron chloride ($FeCl_3$). The blank assay consisted of distilled water; absorbance measurements analyzed samples at 700 nm. Higher absorbance values indicate the presence of a high reducing capacity. Results were expressed as Means±SD of the expressed OD_{700}, and we calculated IC_{50} (mg/mL) values reflecting the concentration required to reach 0.5 absorbance.

Total phenolic content

We determined total phenolic content (TPC) according to the Folin-Ciocalteu method as described by the International Organization for Standardization (ISO) (ISO 2005; Anesini et al. 2008). Following this procedure, 5mL of Folin-Ciocalteu's reagent (1:10) and 1 mL of test samples suspended in DMSO (1 mg/mL) were added to glass tubes, and the solution was left for 8 min. Subsequently, 4 mL of sodium carbonate solution (7.5%) was added to the solution. After 1 h incubation at room temperature, absorbance was measured at 765 nm against a blank solution (distilled water). A standard curve of gallic acid at concentrations ranging from 10 to 50 µg/mL was plotted to determine TPC. Results are expressed in mg of gallic acid equivalent per 1 g of dry material (mg GAE/g dry material).

Determination of the chemical profile of active plant extracts by LC-MS analysis

We measured the phytochemical composition of the biologically active aqueous extract from *L. pedunculata* using the HPLC-PDA-MS/MS system (SHIMADZU JAPAN) equipped with an MS 8050 mass spectrometer featuring an electrospray ionization (ESI) source. For separation, we employed a C18 (Zorbax Eclipse XDB-C18, Agilent, USA) reverse phase column with the following dimensions: 4.6 × 150 mm, 3.5 µm. During analysis, we used a water and acetonitrile (ACN) gradient (each supplemented with 0.1% formic acid). The first phase consisted of adding 5% to 30% ACN in 45 min and then progressing to 95% ACN at 80 min (1 $mL.min^{-1}$). For sample injection (5 µL), we used a thermostatically controlled autosampler (SIL-40C XS autosampler). Injected samples were prepared at a concentration of 5mg/mL (w/v). For Ion detection with a mass range of 100-1500 m/z, we applied a negative mode and a full scan mode.

Data analysis

All tests were performed in triplicate. Results were expressed as means±SD. Statistical analysis was conducted by applying one-way ANOVA analysis and Fisher's LSD (Least Significant Difference) test with STATGRAPHICS 19 Centurion software (Statgraphics Technologies, Inc., The Plains, Virginia).

RESULTS AND DISCUSSION

Extraction yield

We prepared extracts of *L. pedunculata* plant leaves by conventional Soxhlet extraction, using a solvent system that applies increasing polarity to ensure the extraction of a wide range of biologically active components. Table 1 summarizes the yields obtained for each solvent. Generally, extracts obtained by polar solvents yielded higher percentages, whereas non-polar solvents had lower percentage yields. The methanolic extract attained the highest value (9%), followed by the aqueous extraction (8.5%). On the other hand, hexane and dichloromethane achieved percentage yields of 4.2% and 4.8% respectively. These results are in agreement with those obtained by Borges et al. (2020), who found that water and methanol, compared to hexane and dichloromethane, yielded the highest extraction efficiencies in both solid-liquid and Soxhlet extraction. Yields obtained in polar and non-polar phases indicate that *L. pedunculata* extract material might be richer in compounds with a polar chemical nature. Nonetheless, the nature of the solvent applied during extraction is not necessarily indicative of the

nature of the phytochemicals obtained in the mixture, as further factors may also impact the extraction process. Elevated temperatures, for example, may induce higher extraction yields. As an example, the aqueous extract was obtained at a significantly higher temperature than other extract samples (100°C). That increase in temperature most likely contributed to the elevated level in the extraction yield (8.5%), as high temperatures tend to enhance solubility and diffusion of the plant material into the applied solvent, thereby promoting a higher mass transfer rate (Vilas-Boas et al. 2023). Therefore, although our results might suggest that solvents with high polarity can generate higher masses, other factors may play a certain role in the overall yield. This highlights the importance of considering all parameters that might reflect the quality and integrity of the resulting mixtures when selecting extraction protocols.

Antibacterial and anti-yeast effect

We evaluated the antibacterial effect of *L. pedunculata* plant extracts against various pathogenic strains. Results are shown in Table 2. Overall, the plant fractions did not show activity toward bacterial strains; however, promising results could be observed against the yeast-representative model *C. albicans*. The aqueous extract generated an inhibition zone of 11±1 mm at 60 mg/mL and MIC/MBC values of >40 mg/mL. The antimicrobial kinetics assay further highlights this anti-yeast effect, the results of which are likewise shown in Table 2. At 80 mg/mL, the aqueous extract was able to exert a fungistatic effect on *C. albicans*, which translated into a null value of the yeast's growth speed and its doubling time compared to control (μ =0.95h^{-1}/G=0.73h). *Candida albicans* is known as an opportunistic organism that tends to thrive during infections associated with immunodeficiency; it causes candidiasis, which, in turn, leads to morbidity and mortality. It is also one of the most prevalent fungal infections (Kanchanapiboon et al. 2020; Sharma and Chakrabarti 2023). It is thus essential to search for antifungal drugs capable of combating *C. albicans* infections without causing any adverse outcomes in the patient.

The bacteriostatic effect of the aqueous *L. pedunculata* extract on *C. albicans* can be considered of great importance, as a variety of new applications are possible. The nature of this extract might be the subject of further investigation for new antifungal drugs. These proposals align with several existing studies related to the *Lavandula* genus. For instance, nanoemulsions of *Lavandula spica* produced the highest level of inhibition action against *C. albicans* (Badr et al. 2021); similarly, essential oils from *Lavandula binaludensis* exerted a pronounced antifungal effect against strains isolated from patients with vulvovaginal candidiasis (Minooeianhaghighi et al. 2017).

In addition, the aqueous extract obtained from *L. pedunculata* efficiently suppressed the fixation and development of *C. albicans* biofilm during its initial formation phase (Table 2), actively inhibiting cell adhesion at a rate of 78.8% at 80 mg/mL and 74.32% at 40 mg/mL. This effect can still be observed at a lower concentration (20 mg/mL), with an anti-biofilm percentage of 68.87%, thereby highlighting the significant role this extract can play in curbing yeast and fungus-related infections. It is important to note that one of *C. albicans'* virulence factors involves its capacity to form biofilms, i.e., morphological structures that are more highly resistant than planktonic cells to antimicrobial drugs (Li et al. 2023a). Indeed, in a recent study by Marzucco et al. (2024), a fluconazole MIC dose recorded an 86.8% increase against *C. albicans* in biofilm form compared to planktonic cells.

Table 1. Extraction yield of *Lavandula pedunculata* extracts

Plant extracts	Yield (%)
Hexanic	4.2
Dichloromethane	4.8
Methanolic	9
Aqueous	8.5

Table 2. Well diffusion, microdilution, antimicrobial kinetics, and antibiofilm impact of *Lavandula pedunculata* extracts towards *Candida albicans*

Plant extracts	Well diffusion assay (mm)			Microdilution assay		Antimicrobial kinetics		Antibiofilm assay		
Concentration / parameters	30 mg/mL	60 mg/mL	100 mg/mL	MIC	MBC	μ_{expo}(h^{-1})	G(h)	80 mg/mL	40 mg/mL	20 mg/mL
Hexanic	0	0	0	-	-					
Dichloromethane	0	0	0	-	-					
Methanolic	0	0	0	-	-					
Aqueous	0	11±1	-	>40	>40	**C** 0.95* **T** 0*	**C** 0.73* **T** 0*	78.8^{a}±0	74.32^{a}±4.6	68.87^{a}±2.6

Note: C: Negative control; T: Test. Different superscript letters indicate statistically significant differences between extract concentrations ($p<0.05$). *: asterisks indicate significant differences compared to the extract-free media ($p<0.05$)

Our results demonstrate that the hydro-extraction of *L. pedunculata* leaves can significantly inhibit *C. albicans* biofilm during its development stage; however, the lack of a dose-dependent relationship was evident ($p>0.05$). Whereas we registered a 78.8% decrease in cell attachment at 80 mg/mL, that antibiofilm effect was maintained at lower concentrations with 74.32% and 68.87% at 40 mg/mL and 20 mg/mL, respectively. These results point toward an efficient alternative to commercially available antifungal drugs: *L. pedunculata* plant extracts cannot only inhibit the growth of *C. albicans*, but they can also prevent the strain's chance of acquiring drug resistance.

Estimation of virulence factors

After co-incubation with *C. albicans*, the aqueous and the hexane extracts of *L. pedunculata* were capable of inducing a remarkable decrease in the strain's virulence: the total proteins it released generated OD values lower than the control samples. At a quarter (¼) MIC, the aqueous extract induced the highest effect with a statistically significant reduction, followed by the hexane fractions ($p<0.05$). The dichloromethane extract followed the same dose-dependent trend; however, no substantial discrepancies were observed when compared with the control sample ($p>0.05$) (Figure 1.A). The lowest levels of released EPS compared to the control were detected in the presence of the aqueous extract (¼ MIC). Although the hexane extract demonstrated a high capacity to inhibit virulent protein release, it did not display a significant capacity to inhibit EPS release ($p>0.05$) (Figure 1.B).

Most commercially available antimicrobial agents are known for their ability to disrupt other virulence factors produced by pathogenic microorganisms, such as toxins, exo-proteases, adhesins, and exopolysaccharides (Silva et al. 2016; Bu et al. 2022). In this study, we have shown that the aqueous and hexane extracts derived from *L. pedunculata*, when used at a concentration of a quarter (¼) MIC (10 mg/mL), can cause a substantial decrease in the total amounts of secreted proteins (Figure 1.A). In contrast, no significant reduction was obtained using the dichloromethane extract ($p>0.05$). Additionally, we assessed the exopolysaccharide content in the supernatants of *C. albicans* cultures. Although the aqueous and dichloromethane extracts reduced sugar content in a dose-dependent manner, ANOVA analysis did not reveal major discrepancies compared to the extract-free media (Figure 1.B).

The capacity of plant extracts to reduce EPS and extracellular protein release is of great importance, given that the two factors are regarded as crucial elements in a yeast's biofilm matrix. They contribute to its formation and maintenance (Cugini et al. 2019), thereby promoting higher resistance towards antimicrobials. A decrease in these component levels suggests that the tested samples will not only influence the microbial development but will also weaken one of its most relied-upon resistance mechanisms, i.e., biofilm structure, thus increasing a yeast strain's susceptibility to treatments. Quinic and rosmarinic acids are suspected to be implicated in the observed effect against *C. albicans* biofilms (Table 4); this conclusion is in accordance with previous studies (Muthamil et al. 2018; Fialová et al. 2019).

Antifungal activity

We analyzed the *L. pedunculata* extracts for their potential to inhibit mycelial growth by applying a macrodilution assay in Potato dextrose agar against *B. cinerea* and *F. oxysporum*. The overall antifungal effect was dose-dependent; in most cases, however, significant differences could only be observed between the first and second concentrations ($p<0.05$). *B. cinerea* revealed greater susceptibility to *L. pedunculata* extracts at high doses (10 mg/mL). Dichloromethane and methanolic extracts exhibited 100% inhibition against the strain. Hexane fractions were able to suppress mycelial growth by a rate of 78.57%. The methanolic extract seemed to be the most efficient of the four extracts against *B. cinerea*, as its fungistatic effect was maintained at 1.25 mg/mL (Figure 2.A). This effect was marked from the first 48 hours and continued, following a stable trend throughout the entire 7-day incubation period.

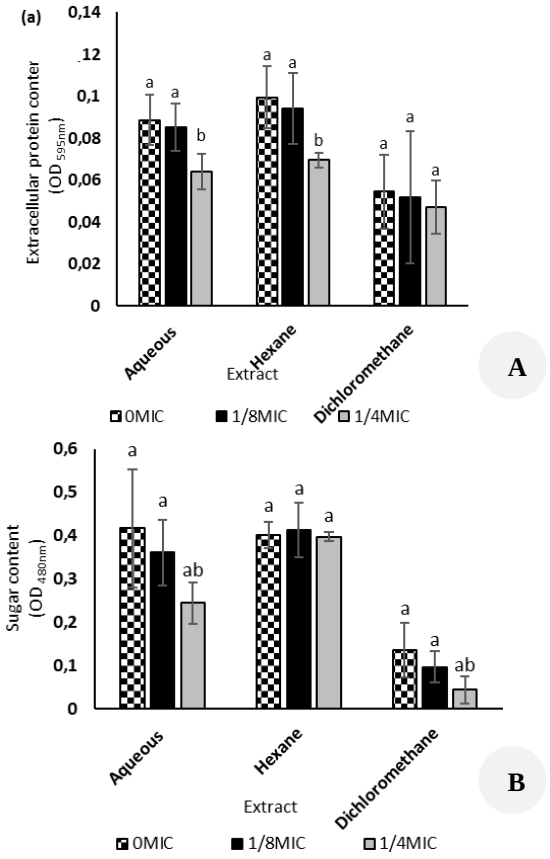

Figure 1. Effect of *Lavandula pedunculata* extracts on: A. Total extracellular proteins; B. Sugar content of *Candida albicans* cultivated in the absence and presence of the extracts at sub-inhibitory concentrations (1/8MIC= 5 mg/mL, 1/4MIC= 10 mg/mL). Different superscript letters indicate statistically significant differences compared to the extract-free media ($p<0.05$)

On the other hand, the hexane extract (10 mg/mL) suppressed fungal growth during days two, three, four, and day five before a growth spike of 13 mm occurred on day six and continued to expand to 15 mm on day seven. *L. pedunculata* extracts likewise influenced the growth of *F. oxysporum* with the methanolic extract, exerting 100% inhibition at 10 mg/mL and 27.17% at 1.25 mg/mL. The dichloromethane extract achieved full control of fungal growth at 10 mg/mL. The hexanoic extract revealed a slight tendency to restrict the growth of *F. oxysporum* (29.35%, 10.87%, 7.61%, and 6.52%, respectively, for 10, 1.25, 0.15, and 0.019 mg/mL) (Figure 2.B). Unlike *B. cinerea*, all extracts showed a minimum inhibitory effect on *F. oxysporum* at lower doses (0.15 mg/mL and 0.019 mg/mL). The aqueous extract exerted an inhibitory effect during the first days of incubation; however, this effect was not significant ($p>0.05$).

Lavandula pedunculata extracts significantly affected the mycelial growth rate of *B. cinerea*. For instance, the hexane extract decreased the fungi's growth speed from 1.045 mm/h at 0.019 mg/mL to 0.6 mm/h at 1.25 mg/mL. This effect remained pronounced after increasing the concentration to 10 mg/mL with a reduction in MGR estimated at 0.1 mm/h. The effect of the dichloromethane extract on MGR was stable from 0.019 mg/mL to 0.15 mg/mL, and a drop in speed was recorded after exposure to higher doses (fungistatic effect at 10 mg/mL). The methanolic fraction likewise recorded a fungistatic influence at 10 mg/mL. The aqueous extract did not cause any significant variations (1.12 - 1.09 - 1.08 - 1.06 mm/h, respectively, for the tested doses in increasing order). *F. oxysporum* showed a more pronounced resistance profile. The effect on its growth speed was mainly indifferent during exposure to lower doses of the extract samples; however, at a dose of 10 mg/mL, the MGR significantly dropped to 0.4 mm/h with the hexane extract and 0 mm/h (fungistatic) with the dichloromethane extract and the methanolic extract. Meanwhile, the aqueous extract recorded minimal fluctuations across the concentration range (Figure 2.C; Figure 2.D).

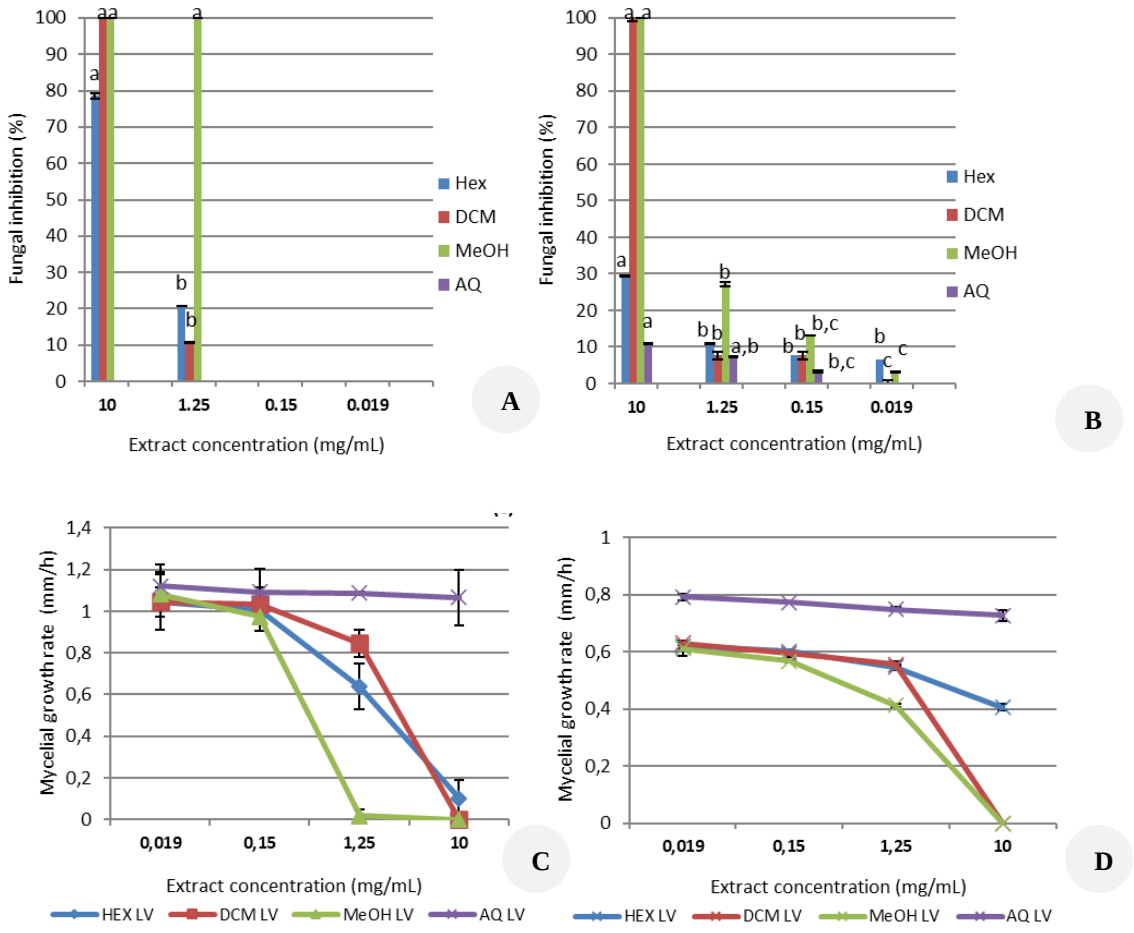

Figure 2. Fungal inhibition percentage and mycelial growth rate of fungal strains in the presence of *Lavandula pedunculata* extracts: A. Inhibition against *B. cinerea*; B. Inhibition against *Fusarium oxysporum*; C. Mycelial growth rate of *Botrytis cinerea*; D. Mycelial growth rate of *F. oxysporum*. Different superscript letters indicate statistically significant differences between extract concentrations ($p<0.05$)

Botrytis cinerea is a phytopathogenic fungus responsible for developing gray mold in almost all plant species. Its mechanism of action involves spore attachment to plant surface, germination, and penetration through stomata and plant wounds, followed by secretion of toxins, cell wall-degrading enzymes, and cell death-inducing proteins (Bi et al. 2023; Singh et al. 2024). Meanwhile, *Fusarium oxysporum* infects several plant species, including chickpeas and tomatoes; this phytopathogen is capable of penetrating plants and releasing cell wall degrading enzymes, which facilitate fungal invasion and plant degradation; it also produces fusaric acid responsible for the development of wilting in infected plants (Nag et al. 2022). Both pathogens have caused considerable crop losses in the past decade, prompting extensive research into organic compounds and their antifungal mechanisms. Previous studies reported that the antifungal mechanisms of action against *B. cinerea* and *F. oxysporum* might be associated with interactions occurring at several levels in the pathogenic cell. For instance, plant extracts can cause extensive hyphal death in *B. cinerea*, which is directly responsible for membrane disruption. In *F. oxysporum*, a compromised membrane induced after exposure to antifungal compounds has been reported to cause an increase in membrane permeability and cellular leakage. Extracts might equally cause induced oxidative stress due to the rise in reactive oxygen species and high catalase and peroxidase activities, Which in turn causes membrane lipid peroxidation (Yang et al. 2020; Li et al. 2023b).

In the present study, both dichloromethane and methanol extracts demonstrated total fungal suppression, indicating potential involvement in one or more of these mechanisms (Figure 2). These extracts might interfere with membrane integrity, disrupt enzymatic functions, or inhibit crucial fungal metabolic pathways, indicating the effectiveness of the tested extracts in mitigating *B. cinerea* and *F. oxysporum* pathogenicity.

In summary, we observed the highest activity of plant extracts against pathogenic plant fungi in the impact of the crude methanolic extract on *B. cinerea*. It achieved 100% inhibition at 1.25 mg/mL; similarly, the dichloromethane extract exhibited a strong fungistatic effect at 10 mg/mL against *B. cinerea* and *F. oxysporum*. In addition, non-polar extracts obtained by *n*-hexane partition displayed a significant inhibitory effect against *B. cinerea*. Several research teams have noted the antimycotic potential of the *Lavandula* species. For instance, Hasanin et al. (2022) found that a methanol extract of *Lavandula coronopifolia* from the Western Egyptian desert was effective against *Aspergillus fumigatus* and *Aspergillus niger*.

Similarly, Silva et al. (2024) reported that *Lavandula stoechas* exhibited antifungal activity against the same fungal strains, in addition to *Aspergillus versicolor*, *Penicillium funiculosum*, *Penicillium verrucosum* var. *cyclopium*, and *Trichoderma viride*. Baptista et al. (2015) tested the fungal susceptibility of the same solvent fractions of *L. pedunculata* as those featured in our study. They concluded that non-polar extracts exert a more pronounced antimycotic activity than their polar counterparts. However, this contradicts the effect obtained by the methanolic extract in our study. This difference can be attributed to the difference in plant ecotype used in each study, resulting from the contrast in qualitative and quantitative molecular composition. Nonetheless, *L. pedunculata* extracts achieved promising results, proving their potential efficacy for the control of fungal-induced plant disease.

In that domain, they are considerably preferable to synthetic fungicides, particularly since they do not exert cytotoxicity toward non-tumor cells (Lopes et al. 2018). *Lavandula* species are typically rich in rosmarinic acid, which has been previously reported to possess antifungal properties via the suppression of enolase expression, leading to an insufficient energy flux inside the fungal cell (Xu et al. 2023). LC-MS analysis of the aqueous extract (Table 4) revealed the presence of this compound, which likely contributed to the observed effect.

Antioxidant activity (AA) and total phenolic content (TPC)

Results displayed in Table 3 detail the antioxidant activity of the tested extracts. All *L. pedunculata* extracts revealed a capacity to interact with the stable free radical 2,2-diphenyl-1-picrylhydrazyl. Compared to BHT at 2 mg/mL, dichloromethane revealed a similar activity at a rate of 91.77%. This high efficacy of the extract was prolonged at a lower concentration, with 83.78% activity at 1 mg/mL. Our results indicate that at a concentration as low as 0.031 mg/mL, a radical scavenging potential is nonetheless maintained, mainly by the aqueous extract, which achieved a 44.42% inhibition percentage, followed by the hexane extract with 12.19% (at the same concentration of 0.031 mg/mL). The data obtained for IC_{50} values show that all tested extracts are equally efficient, with values close to BHT (0.79 and 0.72 mg/mL for dichloromethane and aqueous extracts), except for the hexane fraction (2.32 mg/mL). However, in terms of IC_{50}, the methanolic extract was the most efficient among all extracts tested in this study.

In fact, due to the complete saturation achieved when testing the extract at the initial concentration range, we proceeded to extend the dilution series until a valid IC_{50} value was achieved. Indeed, this step revealed that the methanolic extract was the most potent scavenger with an IC_{50} of 0.012 mg/mL, significantly lower than the values obtained with the positive reference BHT (0.6 mg/mL). In the FRAP assay (Table 3), we were able to detect a strong reduction activity in the aqueous extract, reaching OD_{700} values of 0.62 and 0.58, followed by the methanol fraction, with 0.57 and 0.5. As opposed to our observations of the DPPH system, the dichloromethane extract exhibited a low reduction capacity: OD_{700} values did not exceed the 0.3 mark even with higher doses and an IC_{50} value of 3.9 mg/mL; however, those values were still superior to the potential exhibited by the hexane extract (12.45 mg/mL). All extracts followed a dose-dependent response ($p<0.05$).

Table 3. Antioxidant potential and efficiency (IC_{50}) of *Lavandula pedunculata* extracts

Free radical scavenging assay					
Concentration	**2 mg/mL**	**1 mg/mL**	**0.0312 mg/mL**	**0.0005 mg/mL**	**IC_{50} (mg/mL)**
Hexanic	39.97±0.002[a]	38.06±0.004[a]	12.19±0.03[b]	7.95±0.0[c]	2.32
Dichloromethane	91.77±0.02[a]	83.78±0.015[b]	9.62±0.02[c]	9.79±0.0[c]	0.79
Aqueous	77.75±0.033[a]	66.01±0.017[b]	44.42±0.01[c]	15.83±0.01[d]	0.72
BHT	93.81± 0.002[a]	92.86±0.003[a]	27.48±0.01[b]	10.61±0.1[c]	0.6
Methanolic extract					
Concentration	**0.016 mg/mL**	**0.008 mg/mL**	**0.004 mg/mL**	**0.002 mg/mL**	**IC_{50} (mg/mL)**
Antioxidant potential	60±0.013[a]	38.81±0.002[b]	24.48±0.002[c]	18.41±0.004[d]	0.012
Ferric reducing capacity					
Concentration	**2 mg/mL**	**1 mg/mL**	**0.0312 mg/mL**	**0.0005 mg/mL**	**IC_{50} (mg/mL)**
Hexanic	0.112±0.004[a]	0.080±0.003[b]	0.035±0.004[c]	0.043±0.002[c]	12.45
Dichloromethane	0.264±0.006[a]	0.170±0.021[b]	0.030±0.003[c]	0.029±0.003[c]	3.9
Methanolic	0.572±0.024[a]	0.516±0.008[b]	0.180±0.004[c]	0.034±0.002[d]	1.45
Aqueous	0.625±0.043[a]	0.580±0.038[a]	0.076±0.014[b]	0.037±0.003[b]	1.31
Rutin	0.487±0.012[a]	0.296±0.020[b]	0.188±0.010[c]	0.054±0.011[d]	1.8

Note: a,b,c,d: Means presented in the same row with different letters denote a statistically significant difference according to Fisher's Least Significant Difference test ($p<0.05$)

Table 4. LC-MS analysis of the phytochemical composition of the aqueous extract of aerial parts of *Lavandula pedunculata*

No. compounds	**Rt (min)**	**M-H**	**MS/MS**	**Compound names**
1	1.62	191	111	Quinic acid
2	1.64	133	115	Malic acid
3	2.49	307	133	Quinyl malic acid
4	4.98	315	153	Dihydroxybenzoic acid glucoside
5	5.10	197	179	Trihydroxy cinnamic acid
6	6.13	153	109	Dihydroxybenzoic acid
7	7.09	447	315	Dihydroxybenzoic acid caffeoyl pentoside
8	7.36	385	223	Sinapic acid glucoside
9	7.51	311	179	Caftaric acid
10	7.78	341	179	Caffeoyl glucose
11	8.92	137	108	Hydroxybenzoic acid
12	10.66	341	179	Caffeoyl glucose
13	12.74	325	178	Caffeoyl rhamnose
14	16.59	343	167	Vanillic acid glucose
15	16.91	593	353	Apigenin di-*C*-glucoside
16	17.61	325	193	Feruloyl pentose
17	18.18	167	108	Vanillic acid
18	22.18	623	285	Kaempferol glucosyl-glucuronide
19	22.84	355	193	Caffeoyl ferulic acid
20	23.86	477	301	Quercetin glucuronide
21	23.98	607	269	Apigenin glucosyl-glucuronide
22	25.15	461	285	Kaempferol glucuronide
23	25.18	447	285	Kaempferol glucoside
24	26.39	491	315	Isorhamnetin glucuronide
25	27.41	553	269	Apigenin pentosyl-gallate
26	29.39	431	269	Apigenin glucoside
27	29.63	461	285	Kaempferol glucuronide
28	29.81	445	269	Apigenin glucuronide
29	30.02	447	271	Naringenin
30	31.04	475	299	Diosmetin glucuronide
31	31.38	359	161	Rosmarinic acid
32	35.31	609	285	Kaempferol caffeoyl-glucoside
33	36.75	581	295	Unknown
34	39.08	285	227	Tetrahydroxy flavone
35	42.44	491	279	Unknown

We determined total phenolic content (TPC) by applying the Folin-Ciocalteu method. Results are shown in Figure 3. As expected, the methanolic extract was the richest in phenols (27.76 mg GAE/g), followed by the aqueous extract with 11.66 mg GAE/g, dichloromethane with 4.98 mg GAE/g, and, finally, hexane with 1.94 mg GAE/g. ANOVA analysis revealed significant differences among the four tested extracts ($p<0.05$). Notably, we also observed a close correlation between phenolic content and antioxidant activity. Generally speaking, in terms of human health, Reactive Oxygen Species (ROS) or free radicals intervene at moderate levels in beneficial processes, including a reinforcement of the body's immune defenses (Pizzino et al. 2017).

However, an inordinate increase in ROS levels leads to oxidative stress. This, in turn, harmfully affects important cell constituents, namely lipids, proteins, and DNA (Demirci-Çekiç et al. 2022). Such an imbalance can contribute, in large part, to the induction and/or exacerbation of several diseases (such as cancer, cardiovascular disease, Alzheimer's disease, and atherosclerosis) (Gong et al. 2020; Houldsworth 2024). In foods, lipid oxidation by free radicals results in the overall deterioration of the product's nutritional value, texture, and appearance. This, in turn, reduces the food's shelf life, thereby leading to substantial economic loss (Wang et al. 2023). Antioxidants are neutralizing agents: their presence at adequate levels helps restrict or delay damages prompted by free radicals (Zangeneh et al. 2019). In recent decades, the search for naturally sourced antioxidants has been at the forefront of food industry concerns due to their low toxicity and minimal side effects (Zheng et al. 2021). Antioxidant activity analysis in our study revealed a strong potential exhibited by *L. pedunculata* extracts to quench the DPPH free radical; data obtained from our screening of the methanolic extract showed a stronger effect than BHT. IC_{50} of methanol, aqueous, and BHT were 0.012, 0.72, and 0.6 mg/mL, respectively. The high potency exhibited by these two extracts can mainly be attributed to their high TPC level (Figure 3).

Several authors have reported a positive correlation between TPC and antioxidant potential (Esmaeili et al. 2015). Nonetheless, although the dichloromethane extract has a lower TP content, it achieved a similar capacity in our study (IC_{50} of 0.79 mg/mL). This could be explained by the probability of the presence of other non-phenolic compounds with strong prevention abilities that play a role in the activity we observed. Comparative analysis in similar studies supports this claim: for instance, Farooqi et al. (2024) reported that fatty acids, along with their esters, were primarily responsible for the antioxidant activity they observed. Our study is also in agreement with Baptista et al. (2015).

To compare the two methods, the results from the FRAP assay revealed a profile that contrasted with those we had obtained in the radical scavenging assay; this can be mainly attributed to the existing difference between testing parameters. The FRAP assay, on the one hand, relies on a compound's capacity to reduce Fe^{3+} to Fe^{2+} at low pH; this reaction can be traced through a change in coloration due to the presence of an electron-donating antioxidant (Kubalová et al. 2022). On the other hand, DPPH applies a mixed system relying on a compound's ability to transfer hydrogen atoms or single electrons (Rumpf et al. 2023). Since the FRAP assay cannot detect compounds that act by radical quenching, the two methods can be regarded as complementary. Together, they provide a comprehensive understanding of a substance's antioxidant capacity, demonstrating the thoroughness of our research.

LC/MS profile of the aqueous extract

Data from our LC-MS analysis of the aqueous extract of *L. pedunculata* are represented in Table 4 and Figure 4 (chromatogram). The obtained phytochemical profile indicates that the plant extract is made up of 35 compounds appearing in a retention period ranging from 1.62 to 42.44 min. Quinic acid was the first eluted compound, closely followed by malic acid at 1.64 min. Caftaric acid, a benzene derivative phenol, was obtained at 7.51 min. Vanillic acid, a benzoic acid derivative, peaked just afterward at 18.18 min. The aqueous extract revealed the presence of a variety of flavonoid compounds such as the flavonol quercetin glucuronide (23.86 min), the flavonoid glucoside kaempferol glucoside (25.18 min), apigenin glucoside and diosmetin glucuronide (flavones) obtained at 29.39 min and 31.04 min, as well as the flavanone naringenin at 30.02 min. Other eluted compounds include sinapic acid glucoside (7.36 min), caffeoyl glucose and caffeoyl rhamnose (7.78 min and 12.74 min, respectively), rosmarinic acid, which is classified as a hydroxybenzoate polyphenol (31.38 min), and tetrahydroxy flavone (39.08 min). Compounds 33 and 35 were not determined.

The profile obtained in our study featured several similarities in comparison with previous investigations. Rosmarinic acid is present in all similar studies we have consulted on this topic (Costa et al. 2013; Lopes et al. 2018; Mansinhos et al. 2021; Boutahiri et al. 2022). Further similarities we detected in the chemotypic profile include naringenin, apigenin, and vanillic acid.

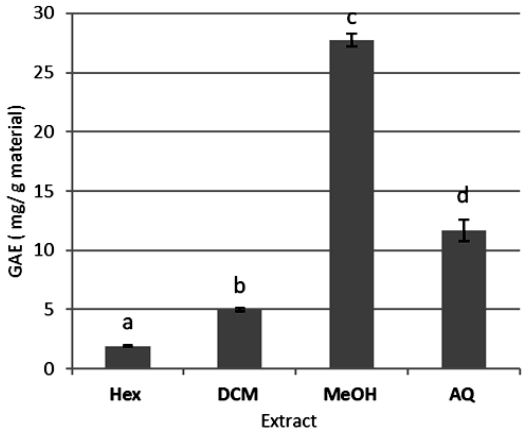

Figure 3. The total polyphenol content of *Lavandula pedunculata* extracts in mg of gallic acid equivalents per 1g of dry material (mg GAE/g material). Different superscript letters indicate statistically significant differences between extracts ($p<0.05$)

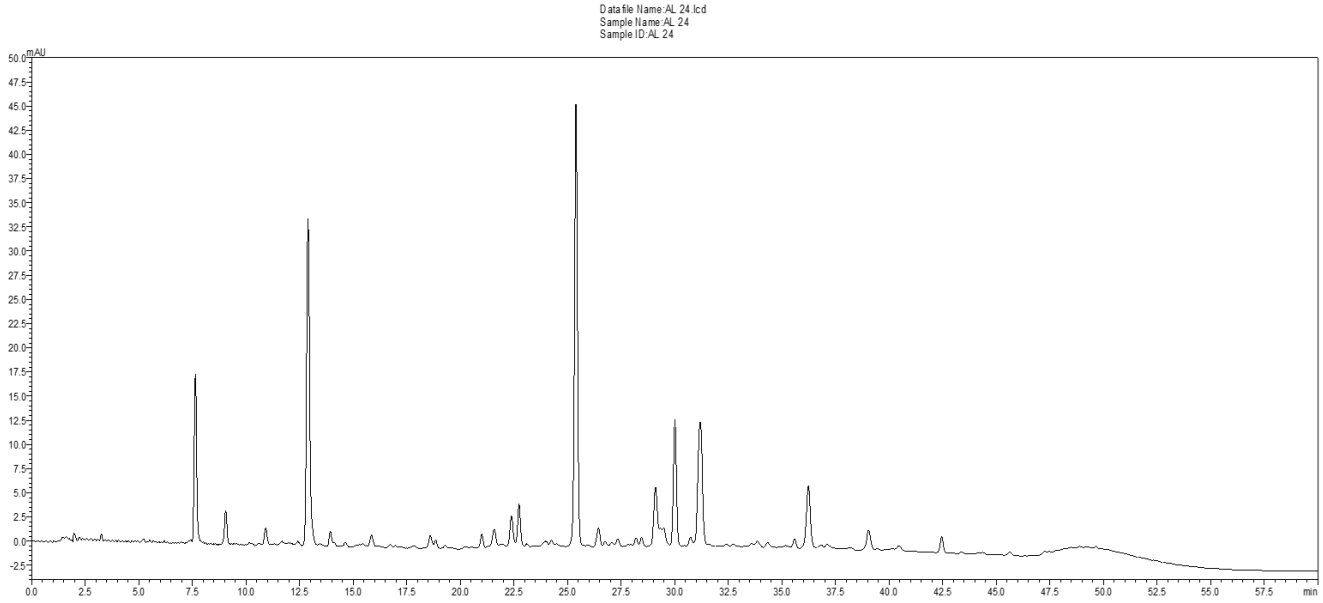

Figure 4. LC-MS chromatogram obtained from the chromatographic characterization of *Lavandula pedunculata* aqueous extract

Other compounds detected in our study have been previously shown to have bioactive potentials: isorhamnetin, for instance, has been found to successfully inhibit H_2O_2-induced oxidative damage in HaCaT cells (Hu et al. 2023), caftaric acid was shown to have a positive effect on parameters related to nephrotoxicity (Koriem et al. 2017), and the pharmacological properties of the glucuronide conjugates of quercetin were recently investigated, namely their anti-inflammatory, antioxidant, moisturizing, and anti-melanogenesis properties (Ha et al. 2022).

The aqueous fractions of *L. pedunculata* featured in our study had a profile rich in phenolics and flavonoids (kaempferol glucoside, apigenin glucoside, naringenin, diosmetin glucuronide, vanillic acid glucose, apigenin di-C-glucoside). We conclude that these compounds are closely associated with the observed antioxidant effect. Previous studies have reported that quinic acid and rosmarinic acid possess the capacity to inhibit fungal strains, including *Candida* spp. biofilms and planktonic cells, which suggests that these same compounds, detected in our aqueous extract of *L. pedunculata*, might be implicated in the observed inhibitory effect (Muthamil et al. 2018; Fialová et al. 2019).

In conclusion, this study aimed to highlight the importance of organic extracts acquired from *L. pedunculata*, a medicinal plant endemic in Morocco featuring a great number of biological properties. The extracts investigated in our study were shown to be pertinent and reliable as safer alternatives for multi-domain applications. The anti-yeast effect exerted by the aqueous extract against *C. albicans* points to potential use in the development of therapeutic agents, particularly since they offer higher resistance to prevent biofilm formation, as well as EPS and protein release. Their antifungal and antioxidant activities can be applied to prevent food deterioration caused by oxidation and contamination, offering a promising solution for food science; moreover, they can be used as additives for a healthier and lower-risk source of phenols and other antioxidants. They equally offer an alternative solution capable of limiting reliance on potentially harmful synthetic fungicides for crop control. However, the mechanism of action of each fraction needs to be further investigated, and their general safety needs to be confirmed in future research.

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A. Laglaoui contributed to the study's design, critical revision, and approval of the final version for publication. H. Bakrim conducted the sample screenings, data analysis, and interpretation, while M. Mabchour assisted in sample preparation and data analysis. O. El Galiou, M. Bakkali, and A. Arakrak contributed to the study design and critical revision of the manuscript. All authors were involved in the final approval of the version to be published. All authors declare no conflict of interest.

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