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Innovative Process for the Recovery of Oleuropein-Rich Extract from Olive Leaves and Its Biological Activities: Encapsulation for Activity Preservation with Concentration Assessment Pre and Post Encapsulation

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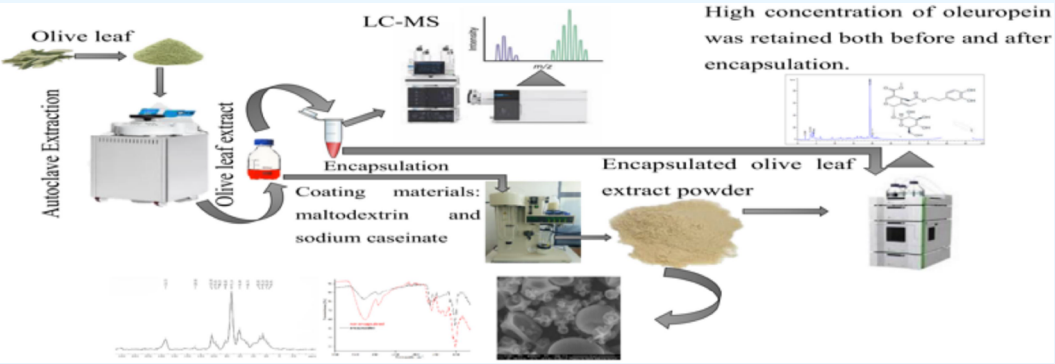

ABSTRACT: Olive leaves, often regarded as agricultural and industrial waste, hold significant potential for economic and medicinal applications. This study examines the valorization of olive leaves through the extraction of phenolic compounds, notably oleuropein and hydroxytyrosol, using autoclave extraction techniques. It also investigates encapsulation techniques employing maltodextrin and sodium caseinate as wall materials to preserve the stability and bioavailability of these compounds. The results indicate a rich phenolic profile in the nonencapsulated olive leaf extract (OLE), demonstrating high antioxidant and antibacterial activities against various pathogens. The encapsulation process achieved high efficiency with a mixture of maltodextrin and sodium caseinate. Furthermore, FTIR spectroscopy and NMR analyses confirmed the presence of functional groups in the encapsulated extract, providing insight into its molecular structure. Overall, this study underscores the potential of olive leaves as a valuable source of bioactive compounds and highlights the importance of innovative extraction and encapsulation techniques to optimize their use across different applications.

1. INTRODUCTION

Olive leaves, from *Olea europaea* L., are often seen as agricultural and industrial waste, resulting from the pruning and harvesting of olive trees. They are collected along with twigs and branches and represent around 25% of the total dry weight of pruning waste.[1] Spain, for example, generates around 1.25 million tonnes of olive leaf waste a year, equivalent to half the world's production.[2] However, instead of considering them as waste, it is essential to recognize the potential value of olive leaves to improve the profitability of the olive sector, particularly in Mediterranean countries, such as Tunisia, where they represent an important source of biomass.

Importantly, olive leaves contain phenolic compounds, such as oleuropein, offering significant health benefits thanks to their antioxidant and antimicrobial properties.[3−6]

The extraction step is critical in maximizing the yield of phenolic compounds from the samples. Traditionally, maceration has been widely used as an extraction technique for plant phenolics. However, this technique has several disadvantages, including the use of substantial solvent volumes, prolonged extraction times, limited selectivity, low yield rates, and reduced efficiency.[7]

In light of these concerns, the adoption of environmentally friendly techniques has become crucial. Companies are shifting

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toward methods that optimize yield, speed, and cost while minimizing solvent consumption. Contemporary processes such as microwave extraction, supercritical fluid extraction, and pressurized liquid extraction have proven effective for extracting phenolic compounds from olive leaves. These techniques offer advantages over traditional ones including shorter extraction times and lower solvent use. However, some of these techniques can be costly and require specific operating conditions, which may limit their practicality and scalability compared to traditional methods.[8−13]

Against this backdrop, autoclave extraction (AE) has emerged as an optimal choice. This method is distinguished by its significant extraction efficiency and relatively low cost, offering a balanced and promising solution for the recovery of phenolic compounds. AE is a technique that uses conventional solvents in a fully automated process, allowing control over various parameters such as temperature, static extraction time, extraction cycles, etc. Importantly, water can be efficiently used in AE to recover compounds ranging from polar to medium polarity, thereby maintaining the method's environmentally friendly attributes.

To maximize the benefits, it is imperative to preserve the stability, bioactivity, and enhanced bioavailability of these polyphenols. In addition, the unpleasant taste of most phenolic compounds limits their applications.[14]

To solve these two major problems, methods are required to prevent oxidation and depletion of bioactive compounds while masking the organoleptic characteristics of these compounds. Encapsulation is one of the possible solutions, with the main objective of protecting the main material, including the bioactive compounds, from adverse environmental conditions, thus ensuring a prolonged shelf life and a controlled release of bioactive compounds.[15] Encapsulation not only offers advantages such as improved functional properties, including enhanced antioxidant activity, but also addresses stability and bioavailability issues often encountered with natural compounds.[16]

Several materials have been used to encapsulate plant extracts, including chitosan, gelatin, sodium caseinate, gum arabic, and starch.[14,15,17−19]

One of the extensively employed materials for encapsulating compounds is maltodextrin. The latter, which is a partially hydrolyzed product of starch, finds versatile applications in encapsulating various substances such as food, medicine, and essential oils. Microcapsules based on maltodextrin exhibit the capability to enhance the color, aroma, and taste of products. Additionally, they contribute to the improvement in the solubility and stability of core materials. Moreover, the controlled release mechanism of core materials over an extended period allows for achieving specific and prolonged effects.[20] Sodium caseinate stands out as a natural emulsifier of considerable nutritional importance. During the emulsification process, sodium caseinate rapidly adsorbs to the oil−water interface, effectively reducing the interfacial tension and creating a substantial interfacial layer. Thanks to electrostatic repulsion and steric hindrance, sodium caseinate skillfully impedes the flocculation and condensation of newly formed droplets. This action prolongs droplet stability within the emulsion, underlining the crucial importance of sodium caseinate in preserving a long-lasting and desirable emulsifying effect.[21] Among encapsulation techniques, spray-drying is a commonly used method in the food industry due to its affordability, flexibility, ease of application, and ability to produce high-quality particles.[17,19,22,23] Previous studies have explored spray-drying of olive leaf extract (OLE).[24−26]

Like maltodextrin, sodium caseinate is used in the food industry, being biodegradable and nontoxic. So far and to the best of our knowledge, no research work has been identified using the encapsulation of the OLE by combining maltodextrin and sodium caseinate.

This study investigates the extraction of oleuropein from olive leaves using a novel method, autoclave extraction (AE). This technique extracts the main phenolic compounds, notably oleuropein and hydroxytyrosol, at temperatures exceeding 100 °C. The study also aims to evaluate the encapsulation process using maltodextrin and sodium caseinate as wall materials, focusing on their impact on the polyphenol content of the OLE. Ultimately, the research aims to enhance the value of olive leaves by extracting these crucial phenolic compounds for beneficial applications in a variety of fields.

2. MATERIALS AND METHODS

2.1. Plant Material. Olive leaves from the Chemlali olive tree, a variety widely cultivated in Tunisia, were collected between February and April 2022 in the Sfax region. The plant material was authenticated by Professor Mohamed Bouaziz from the Department of Food Technology at the University of Sfax (ISBS). A voucher specimen has been deposited at the Laboratory of Electrochemistry and Environment, University of Sfax, Tunisia, under the reference OLTI 01. The sampling method was defined according to Bouaziz and Sayadi[27] due to the high oleuropein concentration in olive leaves. The leaves were collected in the morning immediately after evaporation of the dew. Post harvest, the leaves were transported to the laboratory and air-dried at room temperature for 1 week.

2.2. Extraction Procedure: The AE Method of Phenolic Compounds. The extraction procedure employed an autoclaving method, previously described by Bouaziz et al.[28] Briefly, the samples were finely ground for 2 min using a Nima NM-8300 electric grinder (150 W). After grinding, 50 g of the resulting powder was placed in a flask, and 750 mL of water was added as the solvent to initiate the extraction process. The vials were autoclaved for 20 min at 121 °C and subsequently filtered once cooled. The supernatant was collected, and the extraction procedure was repeated twice more using the same amount of solvent (water).

2.3. ESI-QTOF-MS and -MS/MS Analyses of OLE. The system was coupled to a 6540 Agilent Ultra-High-Definition Accurate-Mass QTOF, equipped with an ESI source (Agilent Dual Jet Stream) (Agilent Technologies). Briefly, the operating conditions were set as follows: drying nitrogen temperature at 325 °C with a flow of 10 L/min; nebulizer pressure at 20 psig; sheath gas temperature at 400 °C with a flow of 12 L/min; capillary, nozzle, fragmentor, and skimmer voltages at 4000, 500, 130, and 45 V, respectively; and octapole radiofrequency voltage at 750 V. Data acquisition (2.5 Hz) in the profile and centroid modes was obtained by MassHunter Workstation software (Agilent Technologies). The spectra were acquired in the negative ionization mode, over a mass-to-charge (m/z) range of 70−1500, and the detection window was set to 100 ppm. Reference mass correction on each sample was performed with a continuous infusion of trifluoroacetic acid ammonium salt (m/z 112.9856) and hexakis (1H,1H,3H-tetrafluoropropoxy) phosphazine (m/z 1033.9881) (Agilent Technologies). Data analysis was performed on MassHunter Qualitative Analysis B.06.00 (Agilent Technologies), which

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allowed the generation of molecular formula with a mass accuracy limit of 5 ppm and an MS score ≥95 (related to the contribution to mass accuracy, isotope abundance, and isotope spacing).

2.4. Determination of Total Phenolic Content (TPC). The TPC in OLE was determined using the Folin−Ciocalteu method, as previously detailed by Szydłowska-Czerniak and Tułodziecka.[29] Sample volumes ranging from 0.2 to 1.0 mL were taken from the extract with a concentration of 0.02 g/mL and transferred to a 10 mL calibration flask. Next, 0.5 mL of Folin−Ciocalteu reagent was added, followed by shaking for 3 min. Then, 1 mL of saturated sodium carbonate solution was added, and the mixture was diluted to the mark with redistilled water. After allowing the solutions to sit for 1 h, they were centrifuged for 15 min. The absorbance was then measured at 765 nm against a reagent blank. The total content of phenolic compounds was determined by the Folin−Ciocalteu method and expressed in milligrams of gallic acid per gram of olive leaves. All experimental tests were performed in triplicate.

2.5. Antioxidant Activity of OLE. *2.5.1. DPPH Free Radical Scavenging Activity (RSA).* The modified DPPH method was employed to determine the RSA of samples, as adapted from Szydłowska-Czerniak and Tułodziecka.[29] In this procedure, 0.1−0.5 mL of each sample was added to 1.9−1.5 mL of methanol, totaling 2.0 mL, and 0.5 mL of DPPH• methanolic solution (304 mmol/mL). Additionally, a control sample was prepared, consisting of 2.0 mL of methanol and 0.5 mL of DPPH. The mixture was vigorously shaken and left to stand in the dark for 15 min. The absorbance was then measured at 517 nm against pure methanol (used as the blank) using a Hitachi U-2900 spectrophotometer in a 1 cm quartz cell. All experimental tests were performed in triplicate.

2.5.2. FRAP Method. The AC of the studied OLE was determined by the spectrophotometric ferric reducing antioxidant power (FRAP) method according to the procedure described previously by Szydłowska-Czerniak and Tułodziecka.[29] In brief, freshly prepared FRAP reagent (2.5 mL of a 10 mmol/L TPTZ solution in 40 mmol/L HCl, 2.5 mL of 20 mmol/L FeCl3, and 25 mL of 0.1 mol/L acetate buffer, pH 3.6) was incubated at 40 °C for 15 min. Then, 0.05−0.50 mL of extracts and 2 mL of FRAP reagent were transferred into a 10 mL volumetric flask and made up to volume with redistilled water. The obtained blue solutions were kept at room temperature for 20 min. The resulting absorbance was measured at 593 nm against a reagent blank (2 mL of FRAP reagent made up to 10 mL with redistilled water) using a Hitachi U-2900 spectrophotometer in a 1 cm quartz cell. The antioxidant activity of samples was determined by the FRAP method and expressed in μmol of Trolox (TE) per mL of extract. All experimental tests were performed in triplicate.

2.5.3. CUPRAC Method. The spectrophotometric copper reducing antioxidant capacity (CUPRAC) method was used to determine the AC of OLE following the method of Szydłowska-Czerniak and Tułodziecka.[29] In this procedure, 0.1 mL of extracts, 2 mL of 0.01 mol/L Cu(II), 2 mL of neocuproine solution (0.0075 mol/L), and 2 mL of ammonium acetate aqueous buffer (ammonium buffer was prepared by dissolving 19.27 g of ammonium acetate in 250 mL of redistilled water) were transferred into a 10 mL volumetric flask and then diluted to the marked volume with redistilled water. The obtained solutions were kept in the dark at room temperature for 30 min. The resulting absorbance was measured at 450 nm against a reagent blank (2 mL of 0.01 mol/L Cu (II), 2 mL of neocuproine solution, and 2 mL of ammonium acetate aqueous buffer made up to 10 mL with redistilled water) using a Hitachi U-2900 spectrophotometer in a 1 cm quartz cell. The CUPRAC results were expressed in milliliters of TE equivalents per milliliter of extract. All experimental tests were performed in triplicate.

2.6. Determination of Antibacterial Activity. *2.6.1. Standard Strains.* The following standard strains were tested: *E. coli*, *Enterococcus faecalis*, *Bacillus cereus*, *Salmonella Paratyphi A*, *Staphylococcus aureus*, *Micrococcus luteus*, and *Candida albicans*. The bacterial strains were cultivated in a test tube containing 10 mL of sterile nutrient broth at a temperature of 37 °C for a duration of 24 h. Following this initial cultivation, a loop of the inoculum was transferred onto a selective medium, and the plates were then incubated at 37 °C for another 24 h.

2.6.2. Preparation of Bacterial Strains. The efficacy of OLE in inhibiting bacterial growth was assessed using the agar well diffusion method, as described by Ucar,[30] with slight modifications. Subsequent to the cultivation of the bacteria in nutrient broth (Merck 1.05443, Darmstadt, Germany) at 37 °C for 24 h, inoculation was performed with a bacterial concentration of 10^8 cfu/ml on Mueller−Hinton agar (MHA, Merck 1.05437, Darmstadt, Germany). Following this, 100 μL of the sample solutions was applied to wells prepared on MHA plates. Control wells received distilled water. After incubating the plates at 37 °C for 24 h, the zones of inhibition around the wells were measured in millimeters. Each test was conducted in triplicate to ensure the reliability of the results.

2.7. Microencapsulation of OLE. Before Microencapsulation, the encapsulating agents (maltodextrin and sodium caseinate) were dispersed in the extract with a ratio of 3:3:1 (w/w/w) and homogenized using an IKA T25 Ultraturrax homogenizer (Baden-Wurttemberg, Germany) for 15 min. Subsequently, all samples were spray-dried using a Buchi Mini Spray Dryer B-290 (Switzerland) equipped with a 0.7 mm diameter spray nozzle. The samples were fed to the drying chamber at a feed flow rate of 0.5 L/h, with continuous mixing under a magnetic stirrer both before and during the drying process. The spray dryer's inlet temperature was set at 160 °C and the outlet temperature was maintained at 90 ± 5 °C. The aspirator flow rate was set at 100 m^3/h, and the pump speed was adjusted to 20%.[31] The dried samples were then stored under dark conditions at 4 °C until further analysis.

2.8. Determination of the Encapsulation Efficiency (EE). The EE was determined as described by Toprakçı and Şahin.[32] To determine the TPC of the microcapsules, 0.1 g of microcapsules was dissolved in a mixture of ethanol, acetic acid, and water (50:8:42 v/v/v), homogenized at 7000 rpm for 1 min, and then filtered through a 0.45 μm syringe filter. For surface phenolic content (SPC), 0.1 g of microcapsules was dissolved in an ethanol/methanol mixture (1:1 v/v), homogenized, and filtered in a similar manner. Phenolic compounds were quantified by using the Folin−Ciocalteu method.

The EE of the microcapsules was calculated as a function of the total and surface phenolic compound contents. It corresponds to the ratio between the encapsulated phenolic content and the TPC. The encapsulated phenolic content is determined by the difference between the TPC and the SPC. The EE of the microcapsules was calculated according to eq 1

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Table 1. Compounds Characterized in OLE Using the Negative Ionization Mode

	RT (min)	mass	$[M-H]^-$	molecular formula	main fragments via MS/MS	compound	references
Organic acid							
1	5.0	152.14	151.03	$C_8H_8O_3$	123.04;122.03	Vanillin	35
2	7.5	170.11	169.01	$C_7H_6O_5$	125.02;124.01; 97.02;73.01	Gallic acid	36
3	17.0	180.14	179.04	$C_9H_8O_4$	135.04;134.03;89.03	Caffeic acid	36
Caffe oylphenylethanoid							
4	11.8	154,13	153.05	$C_8H_{10}O_3$	123.04;109.02	Hydroxytyrosol	36
5	12.8	462.17	461.16	$C_{20}H_{30}O_{12}$	315.10;297.09;153.05;135.04;113.02	Decaffeoylverbascoside	35
6	23.0	624,20		$C_{29}H_{36}O_{15}$	461.1662; 161.0238; 113.0242 135.0451, 315.1103	Verbasoside	36
Flavonoids							
7	14.7	304.05	303.05	$C_{15}H_{12}O_7$	285.04;177.01;125.02	Taxifolin	35
8	20.4	610.14	609.14	$C_{27}H_{30}O_{16}$	463.08;301.03;300.02;178.99;151.00	Rutin	36
9	20.5	594.15	593.15	$C_{27}H_{30}O_{15}$	447.09; 285.04;133.02	Luteolin-7-O-rutinoside	35
10	21.3	448.12	447.09	$C_{21}H_{20}O_{11}$	285.03;284.03;197.06;175.03;133.02	Luteolin7-O-glucoside	36
11	23.0	432.10	431.09	$C_{28}H_{32}O_{15}$	269.04; 268.03;117.03	Apigenin7-O-glucoside	35
Secoiridoids							
12	14.7	422.17	421.17	$C_{18}H_{30}O_{11}$	371.13; 359.17; 183.06; 165.05; 151.07; 121.06; 119.03; 115.03; 113.02; 101.02; 89.02	oleoside methylester	35
13	17.7	378.15	377.14	$C_{16}H_{26}O_{10}$	197.08;153.09;113.02;101.02;89.02	oleuropein aglycone	35
14	20.2	526.16	525.16	$C_{24}H_{30}O_{13}$	481.16; 389.10; 209.04; 195.06; 183.06; 165.05; 121.06; 119.03; 113.02; 89.02	Demethyl oleuropein	35
15	20.6	556.17	555.17	$C_{25}H_{32}O_{14}$	537.16; 403.12; 393.11; 323.07; 183.06	Hydroxy oleuropein	35
16	24.2	540.18	539.17	$C_{25}H_{32}O_{13}$	403.12; 377.12; 307.07; 275.08; 223.05; 179.05; 149.02; 119.03; 89.02	oleuropein	39
17	25.4	524.18	523.18	$C_{25}H_{32}O_{12}$	361.12; 291.08; 259.09; 223.06; 127.04; 101.02		35
18	25.7	558.23	557.22	$C_{26}H_{38}O_{13}$	513.23; 345.11; 327.10; 227.12; 185.11; 183.06; 121.06	60-O-[(2E)-2.6Dimethyl-8-hydroxy-2-octenoyloxy]- secologanoside	40
19	28.4	378.13	377.12	$C_{19}H_{22}O_8$	275.05; 165.05; 149.02; 139.03; 127.0404; 111.00; 95.05	oleuropein aglycone	35
20	25.5	926.30	925.29	$C_{42}H_{54}O_{23}$	893.27; 763.24; 745.23; 693.20; 539.17; 521.16; 377.12; 307.08	Jaspolyoside	36
lignan							
21	18.8	376.15	375.14	$C_{20}H_{24}O_7$	360.12; 345.09; 327.12	Cycloolivil	35
22	20.9	376.15	375.14	$C_{20}H_{24}O_7$	360.12; 345.09; 327.12; 195.06; 179.07; 164.04; 146.03	olivil	35

$$\text{EE}\ (\%) = \left(\frac{(\text{TPC} - \text{SPC})}{\text{TPC}}\right) \times 100 \quad (1)$$

2.9. Determination of the Encapsulation Yield. The yield (Y) was calculated according to eq 2, and the microparticle powder corresponded to powder recollecting into the vessel.

$$Y = \left(\frac{\text{Powder after spray drying}}{\text{Solids in the feed solution}}\right) \times 100 \quad (2)$$

2.10. High-Performance Liquid Chromatography (HPLC) Analysis. The HPLC analysis used for low-molecular-weight phenols and oleuropein was performed on a Shimadzu apparatus composed of a pump (LC-10ATvp) and a UV detector (SPD-10Avp). The column used to analyze phenols was a C-18 (4.66250 mm) Shim-pack VP-ODS. Eluates were detected at 280 nm. The temperature was maintained at 40 °C. The mobile phase used was 0.1% phosphoric acid (Prolabo, France) in water (A) versus 70% acetonitrile (Dharmadrug GmbH, Germany) in water (B) for a total running time of 50 min. The elution conditions applied for phenolic compounds were as follows: 0−25 min, 10−25% B; 25−35 min, 25−80% B; 35−37 min, 80−100% B; 37−40 min, 100% B. Finally, the washing and reconditioning steps of the column (40−50 min) included a linear gradient 100−10% B. The flow rate was 0.6 mL/min, and the injection volume was 50 mL. The identification and quantification of phenolic compounds in the *Olea europaea* L. Chemlali leaf variety were performed based on their spectral characteristics and RTs, compared with phenolic standards analyzed under identical conditions. Additionally, the method of standard addition was employed for the samples to ensure accuracy in quantification.

2.11. Microcapsule Morphology. The morphology of the microcapsules was analyzed using a scanning electron microscope (Quanta 3D FEG, FEI Company, Hillsboro, OR, USA). To enhance the quality of the images, the samples were coated with a thin layer of gold, as documented by Olewnik-Kruszkowska et al.[33]

2.12. FTIR Spectroscopy Data. Fourier transform infrared analyses were performed using an infrared spectrometer (IRAffinity-1, Shimadzu, Japan), coupled with a horizontal attenuated total reflectance accessory employing a zinc selenide crystal. All spectra were obtained in the range of 400−4000 cm^{-1}, with a resolution of 4 cm^{-1} and 60 scans.

2.13. NMR Analysis Data. In order to confirm the composition of the obtained E-OLE, a sample analysis was carried out using NMR spectroscopy. The ^{13}C CPMAS NMR spectrum of the sample in the form of powder was recorded on a BRUKER Avance III 700 Hz spectrometer (Rheinstetten, Germany).[34] As the reference of chemical shift, tetramethylsilane at 0.0 ppm was used.

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2.14. Statistical Analysis. The correlation analysis results of TPC with DPPH, FRAP, and CUPRAC were expressed as Pearson correlation coefficients using SPSS Version 20.

3. RESULTS AND DISCUSSION

3.1. ESI-QTOF-MS and -MS/MS Analyses. ESI-QTOF-MS and -MS/MS techniques were used to identify phenolic compounds that may be present in our OLE.

The HPLC-ESI-TOF-MS/IT-MS2 instrument provides high mass resolution and accuracy, making it a suitable choice for determining molecular formulas by using the Smart Formula editor. Additionally, IT-MS2 facilitates the generation of fragment ions, helping in the identification of target compounds within complex matrices. In this study, the metabolic profiling of the OLE was conducted using HPLC-DAD-MS in the negative ionization mode. The identified metabolites, including retention time (RT), experimental m/z, and proposed compounds, are detailed in Table 1. Supplementary data from UV−vis detection provided by the diode array detector (DAD) data were also used to consolidate the results. Where reference standards were available, phenolic compounds were compared with these standards in terms of the Rt, UV, and MS spectral characteristics. The study identified 22 compounds, categorized into groups such as organic acid, caffeoyl phenylethanoid, flavonoids, secoiridoids, and lignan.

The HPLC-DAD-TOF-MS analyses of the OLE revealed the following compounds already reported in olive leaf (Table 1): compound 1 (RT 5 min) was tentatively described as vanillin; it presented a fragment at m/z 151.03, which coincided with the m/z of vanillin.[35] Compound 2 (RT 7.5 min) and compound 3 (RT 17.0 min) at m/z 169.01 and 179.04, respectively, were identified as gallic and caffeic acid, consistent with literature reports.[36] The molecular formula of compound 4, which elutes at an RT of 11.4 min, along with the detection of a fragment ion at m/z 153, led to its tentative identification as hydroxytyrosol. This identification is supported by the findings reported by Grabska-Zielińska et al.[36]

Flavonoid peaks were observed between 14.7 and 23 min in the chromatogram, and our findings identified five flavonoids. Compound 7 at 14.7 min and m/z at 303.05 was described as taxifolin based on these data and literature references.[35]

Compound 8 (RT 20.4) was tentatively identified as rutin, according to the molecular formula provided for its mass and, corroborated by its fragment ion at m/z 609.14, corresponding to rutin[36,37] Compound 9 at 20.5 min and m/z 593.15 was tentatively identified as luteolin-rutinoside, which accords well with previous findings. Compound 10 (at m/z 447.09) was assigned as luteolin glucoside,[36] while compound 11 at 23 min and m/z 431.09 was tentatively identified as apigenin glucoside.[35]

Secoiridoids and their derivatives make up a class of compounds found in *Olea europaea* that are derived from the iridoid monoterpene deoxyloganic acid, which is a common intermediate of these compounds. These molecules are produced by the opening of the iridoid cyclopentane ring.[38] Our analysis confirmed the presence of several secoiridoids previously identified in olive leaves.[35,36,39] These include oleoside methyl ester (compound 12, RT 14.7 min, m/z 422.17), oleuropein aglycone (compound 13, RT 17.7 min, m/z 377.14), demethyloleuropein (compound 14, RT 20.2 min, m/z 525.16), hydroxyoleuropein (compound 15, RT 20.6 min, m/z 555.17), oleuropein (compound 16 and m/z 539.17), and ligustroside (compound 17 at 25.4 min and m/z 523.18), and compound 18 was tentatively identified as secologanoside (an isomer of oleoside) based on its molecular formula, obtained fragments, and order of elution in the literature.[35−37,39,40] Another secoiridoid, identified as compound 20, was detected with an RT of 25.5 and m/z at 925.29. This compound was tentatively identified as jaspolyosideh. It is trustworthy to mention that the secoiridoid glucoside was previously reported in olive leaves by Grabska-Zielińska et al.[36]

These results reveal that RP-HPLC-DAD-QTOF-MS and -MS/MS are effective tools for detecting and characterizing new chemical structures whose existence in other matrices is still poorly documented. In this study, lignans, a significant group of compounds from *Olea europaea*, were analyzed. Specifically, two lignans in the OLE, cycloolivil and olivil, were characterized. Although these compounds share the same molecular formula and mass-to-charge ratio (m/z), they can be distinguished by their RTs. These results corroborate similar findings reported by Ammar et al.[35]

In conclusion, many phenolic compounds were identified in Chemlali olives from Tunisia using HPLC-DAD-TOF-MS. These phenolic compounds of olive leaves are of great interest as they may be involved in biochemical and pharmacological effects, including anticarcinogenic and antioxidant properties.[41,42] The phenolic composition represents a useful contribution to the biochemical characterization of the Chemlali olive cultivar.

3.2. TPC and Antioxidative Properties of Non-encapsulated OLE. It is worthwhile to mention that phenolic substances extracted from plants are endowed with antioxidant activities. Indeed, they can act as reducing agents, hydrogen donors, oxygen scavengers, or chelators of metal ions. The total content of phenolic compounds and the antioxidant activity of the extract obtained from powdered olive leaves were measured, and the results are presented in Table 2,

Table 2. TPC and Antioxidant Activity of the OLE

method	OLE
TPC (mg EAG/g)	395.45 ± 8.21
DPPH (μmol TE/100 g)	322.54 ± 1.73
FRAP (μmol TE/100 mL)	7.2 ± 0. 3
CUPRAC (μmol TE/ml)	14.83 ± 0.63

indicating high phenolic content (395.45 ± 8.21 mg GAE/g) of the extract. Our results confirmed that olive leaves represent a potential source of phenolic compounds, which is in harmony with those reported in the literature.[43] The phenolic compound contents of olive leaf results obtained in the current study were broadly comparable to those of González-Ortega et al.,[43] higher than those of Abdellah[44] and Zayed et al.[45]

The phenolic compound content of OLE depends on the extraction method used and the olive variety.[46] For example, González-Ortega et al.[43] obtained a total phenolic compound (TPC) content of approximately 403 mg GAE/g using the maceration method. In comparison, Debs et al.[47] reported that with ultrasound-assisted extraction, the phenolic compound content of the extracts reached 187.31 mg GAE/g.

The antiradical activity involves reducing DPPH, a violet-colored radical with a maximum absorbance at 517 nm, endowed with the ability to accept an electron or a hydrogen atom, thus forming a stable nonradical molecule of pale-yellow color. This reduction results from the attachment of a

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hydrogen atom from the extract to the DPPH molecule.[48] In terms of antioxidant activity, the extract exhibited a notable antioxidant capacity (322.54 ± 1.73 μmol TE/100 g) (Table 2).

The high antioxidant activity of OLE has been demonstrated in numerous previous studies.[49−52] Mansour et al.[49] found that OLE from the Chemlali variety exhibited significant free RSA, with an IC50 of 56.00 ± 0.13 μg/mL. The reasonable and effective antioxidant power of OLE could be explained by the diversity and nature of the phenolic compounds and flavonoids, which have a great capacity to neutralize free radicals and prevent oxidation processes. The CUPRAC and FRAP methods also gave significant values for the extract obtained by autoclaving. The method FRAP reached 7.2 ± 0.3 μmol TE/100 mL. This trend is followed by the CUPRAC method, according to which the measured value in the extract is 14.83 ± 0.63 μmol TE/mL (Table 2). In general, the different methods used to assess antioxidant activity are based on distinct reaction mechanisms, which often lead to variable results. For example, CUPRAC tests quantify the reducing power of the antioxidant, capable of reducing metal ions (Cu^{2+}) to lower oxidation states.[53] The FRAP test is based on an electron transfer mechanism and assesses the ability of antioxidant molecules to reduce ferric ions in the Fe-$(TPTZ)_2Cl_3$ complex, resulting in the formation of a blue ferrous complex (Fe^{2+}) in an acidic environment.[54] These methods use various reaction mechanisms,[53] which may specifically favor the activity of particular antioxidants, hence the variations observed in the antioxidant capacity measured.

The correlation between TPC and antioxidant activity (measured by the DPPH, FRAP, and CUPRAC methods) was studied (Table 3). Significant correlations were observed between the TPC of OLE and its antioxidant activity (FRAP method), with a correlation coefficient of 0.998 and a *P*-value of 0.012.

Table 3. Correlation Coefficients between TPC Antioxidant Activity (DPPH and CUPRAC)

		DPPH	FRAP	CUPRAC
TPC	*R*	−0.082	0.998[a]	0.980
	P-value	0.948	0.012	0.129

[a]Correlation is significant at the 0.05 level (2-tailed).

However, for the CUPRAC and DPPH methods, the correlation is not significant.

In summary, the high correlations highlight the importance of phenolic compounds in the activity of the OLE. However, the lack of correlation between TPC and antioxidant activity measured by the DPPH and CUPRAC method indicates that there is no direct link observed between the total amount of phenolic compounds in an extract and its ability to neutralize free radicals.

In other words, even if an extract contains a high quantity of phenolic compounds (measured by TPC), this does not necessarily guarantee that it will have high antioxidant activity measured by the DPPH method. The variability in antioxidant activity of OLE could therefore be explained by the presence of various phenolic compounds such as oleuropein, hydroxytyrosol, and caffeic acid, indicating that other factors or specific types of phenolic compounds present in the extract may have a greater influence on its antioxidant activity, independent of the total amount of phenols. According to Terpinc et al.,[55] a negative correlation between TPC and DPPH radical scavenging efficiency suggests that TPC may not be a reliable indicator of antioxidant capacity. These results highlight the potential nonspecificity of the Folin−Ciocalteu method for comprehensively assessing the antioxidant activity of phenolic extracts.

3.3. Antibacterial Activity: Determination of Inhibition Diameters of the Extract. The results of the antibacterial activity of OLE are summarized in Table 4

Table 4. Antibacterial Activity of Extract (Agar Diffusion Method), Expressed as a Zone of Inhibition (mm) [Mean ± Standard Deviation]

type of bacteria	inhibition zone (mm)
Enterococcus faecalis	7.13 ± 0.11
Bacillus cereus	8.07 ± 0.47
Salmonella Paratyphi A	7.38 ± 0.08
Staphylococcus aureus	7.21 ± 0.20
Candida albicans	7.7 ± 0.14
Escherichia coli	11.0 ± 0.10
Micrococcus luteus	11.5 ± 0.75
Listeria monocytogenes	no zone

(inhibition diameter is given in (mm)). These results indicate that bioactive compounds from olive leaves have inhibitory effects on various tested bacterial strains. The antimicrobial efficacy of the OLE against different pathogenic strains was evaluated qualitatively by the presence or absence of inhibition zones. According to the results displayed in Table 3, the extracts show strong antibacterial activity. The obtained inhibition diameter against *Enterococcus faecalis* was 7.13 ± 0.11 mm, the inhibition diameter toward *Bacillus cereus* was 8.07 ± 0.47 mm, and the inhibition diameter toward *Salmonella Paratyphi A* was 7.38 ± 0.08 mm. The sensitivity of *Bacillus cereus* to OLE was investigated by Palmeri et al.[56] and Coşansu & Kıymetli.[57] The authors attributed the antibacterial effect of the OLE against *Bacillus cereus* to its phenolic compounds, such as oleuropein, hydroxytyrosol, and tyrosol.

Besides, the inhibition zone of *Candida albicans* was 7.7 ± 0.14 mm. This inhibitory effect has also been reported by several authors. For example, Ghaffaripour et al.[58] observed a minimum inhibitory concentration (MIC) of OLE against *Candida albicans* of 4 mg/mL, while Nasrollahi & Abolhasannejad[59] found an MIC of 24 mg/mL. This inhibitory effect is mainly attributed to the presence of phenolic compounds such as hydroxytyrosol and oleuropein in the extract.

An antimicrobial activity was also detected against *Escherichia coli* and *Micrococcus luteus* with an inhibition zone of about 11 ± 0.1 and 11.5 ± 0.75 mm, respectively. The susceptibility of *Escherichia coli* to OLE was investigated by Coşansu & Kıymetli,[57] Fazeli-Nasab et al.,[60] and Liu et al.[61] Similarly, the sensitivity of *Micrococcus luteus* to OLE was analyzed by Khemakhem et al.[62]

The results also show that the studied extract has no activity against *Listeria monocytogenes*.

According to Pinto et al.,[63] polyphenols are the main antimicrobial compounds in plants, with diverse modes of action and inhibitory and lethal activities against a large class of microorganisms. In most cases, the antimicrobial effects observed in this work are comparable to those reported by Lee & Lee.[64] In a previous study, Lee & Lee[64] had reported

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that olive leaves have a high natural biological capacity. Indeed, these two authors tested individually and in combination the antioxidant and antimicrobial effects of two phenolic compounds specific to olive leaves (oleuropein and caffeic acid). The results showed that these two phenolic compounds have potential antiradical activity. In terms of antimicrobial activity, both compounds are also effective. However, the antimicrobial effect observed is significantly greater when the two compounds are used in combination rather than applied individually. These observations are in good agreement with those obtained by Djenane et al.,[65] which suggest that the extracts of olive leaves have a synergistic action due to the presence of oleuropein and other phenolic compounds. Many studies have shown that oleuropein, found in olive leaves, is one of the most powerful phenolic compounds due to its antimicrobial properties.[65,66]

Furthermore, the antimicrobial activities of these extracts are difficult to correlate to a specific compound due to their complexity and variability. Nevertheless, some researchers have reported a close relationship between the chemical composition of the most abundant elements and the antimicrobial activity.

3.4. Microencapsulation of OLE and Its Efficiency. The effectiveness of Microencapsulation is a critical factor in evaluating the potential degradation and oxidation of polyphenols during spray-drying. The ability of carriers to preserve encapsulated molecules is related to their chemical structure. Maltodextrin, which is a short-chain hydrolyzed starch, serves as a barrier against oxygen, although it possesses a low emulsification and film-forming capacity.[25] Furthermore, sodium caseinate, a soluble caseinate, not only offers effective emulsifying properties and excellent heat stability but also features a neutral taste and a low lactose content.[67] Murugesan and Orsat[68] have affirmed that since a single carrier cannot always meet all of the requirements for successful drying, a mixture of carriers is often used.

The EE depends on the used encapsulation method, the chemical characteristics of the wall material, and the active compound, together with their concentrations. Using maltodextrin and sodium caseinate as wall materials, we obtained a high EE of about 71.92 ± 0.10%.

González-Ortega et al.[43] evaluated the EE of olive (*Olea europaea* L.) leaf extract particles coated with maltodextrin and trehalose alone or in mixtures, using freeze-drying as an encapsulation technique. The authors obtained higher values of EE (74.96%) using 50% maltodextrin, which is comparable to our results. The efficiency of the spray-drying process can be better evaluated by the powder production yield.[69] In fact, the encapsulated OLE (E-OLE) was found to attain a production yield of approximately 53.51 ± 1.81%. Similarly, Sarabandi et al.[69] have reported a comparable encapsulation yield of about 52.03 ± 2.47 when using maltodextrin as the wall material at an inlet temperature of 140 °C.

Sarabandi et al.[69] further demonstrated that increasing the drying temperature from 140 to 170 °C led to enhanced powder recovery, particularly with MD, which increased from 52.03 to 66.47%. This improvement can be attributed to the boosted efficiency of heat and mass transfer, accelerated water removal, and reduced particle stickiness on the dryer walls resulting from the higher inlet temperature.

During spray-drying, powder stickiness and deposition on the walls of the drying chamber are the main problems that lead to an undesirable decrease in the product yield. However, losses can be reduced by selecting the proper substrate and optimizing the process parameters.[70] The viscosity of the mixture and the dry matter content increase with an increase in the concentration of a carrier. Consequently, more solid particles are available to come into contact with the wall of the drying chamber, thus increasing the possibility of adherence. This can ultimately result in a decrease in product yield.[71]

Yousefi et al.[71] have also confirmed that using numerous carriers (MD, GA, waxy starch) leads to more effective drying. However, Krishnaiah et al.[22] have reported that increasing the number of carriers (MD and κ-carrageenan) does not always yield positive effects. The variable impact of carrier quantity on product yield can likely be attributed to differences in the chemical structure, which may explain the negative results reported in the literature. In addition, a higher amount of carrier may increase the production cost and negatively affect the product's taste, potentially leading to consumer dissatisfaction. According to Dobrinčić et al.,[25] the highest yield was generally obtained at 150 °C. Similarly, Akcicek et al.[72] observed that while temperatures below 130 °C could result in moisture accumulation on the walls of the drying chamber, those above 160 °C might cause significant degradation of polyphenols.

3.5. HPLC Analysis of Nonencapsulated and Encapsulated Extracts. The phenolic composition of E-OLE and OLE was studied by HPLC. Peaks were identified by comparing the chromatographic RTs and UV absorbance spectra of olive leaf extract compounds with those of authentic standards. Figure 1a,b shows each of the phenolic compounds identified in the order of their elution.

The results show that oleuropein predominates in the OLE as well as in the E-OLE. Its concentration reaches 16.58 and 29.69 mg/g, respectively. Notably, the concentration of oleuropein in E-OLE is significantly higher than that in OLE.

This observation may be attributed to the hypothesis that direct exposure of oleuropein to temperature in the case of the nonencapsulated extract results in a reduction in its concentration. Conversely, in the case of the encapsulated extract, the molecule is protected from the direct impact of temperature due to its encapsulation. This hypothesis underlines the importance of the encapsulation process in maintaining the stability of phenolic compounds, particularly under adverse environmental conditions, such as temperature variations.

3.6. Morphology of Microcapsules. Scanning electron microscopy images of spray-dried microcapsules obtained under the optimal conditions are shown in Figure 2, with images taken at different magnification ratios. The spray-dried E-OLE forms generally spherical shapes that differ in size. Moreover, it was observed that the irregularly shaped spherical microcapsules had numerous indentations and bumps on the surface. This shape is similar to that observed for the spray-dried plum extract powder by Li et al.[73] It should be stressed that wrinkles can be observed on the surface of the microcapsules. This indicates that the formed materials are characterized by poor fluidity. The mentioned phenomenon was described in the research work of Fernandes et al.,[74] in which gum arabic/starch/maltodextrin/inuline-based microcapsules containing rosemary essential oil were produced.

3.7. FTIR Spectroscopy Data. Infrared spectroscopy is utilized to identify the presence of functional groups in organic molecules and to determine the structures of some simple molecules. Bond vibrations in molecules occur at specific

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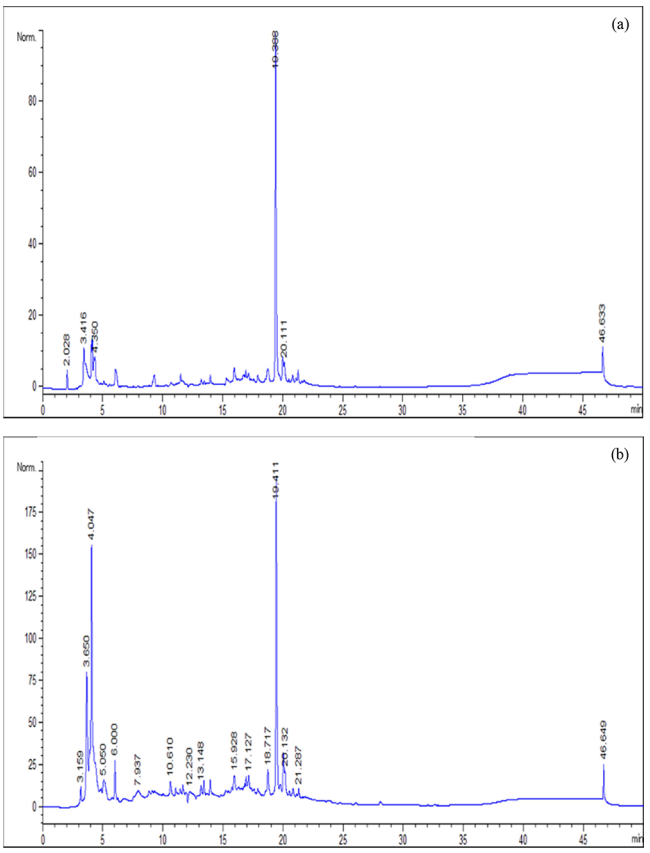

Figure 1. HPLC chromatograms of nonencapsulated (a) (tR (oleuropein) = 19.38 min) and encapsulated (b) (tR(oleuropein) = 19.41 min) OLEs; detection at 280 nm.

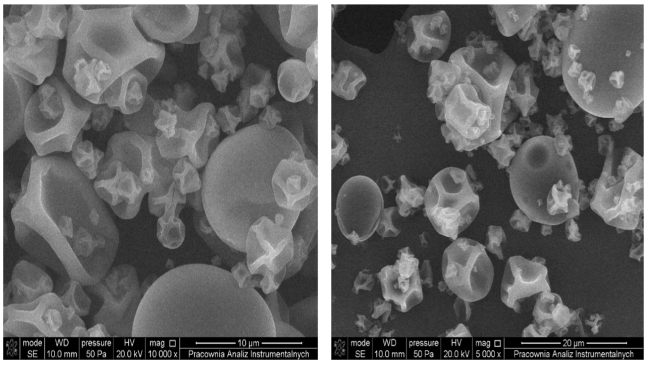

Figure 2. Scanning electron microscope images of the E-OLE.

frequencies influenced by the types of atoms involved and their surrounding environment. At certain frequencies, these bonds enter resonance, and the energy supplied is absorbed, leading to a reduction in the transmission. When we plot the transmission against frequency or more commonly against the wavenumber, which is the frequency divided by the speed of light in the medium, we observe distinct variations. Each absorption peak or band on this graph is characteristic of a specific type of bond.

The FTIR spectra of OLE and E-OLE are shown in Figure 3. The obtained FTIR spectra for both samples appeared similar upon visual inspection, indicating no noticeable qualitative differences between the leaf samples. When the infrared spectra of the leaf extract without and with

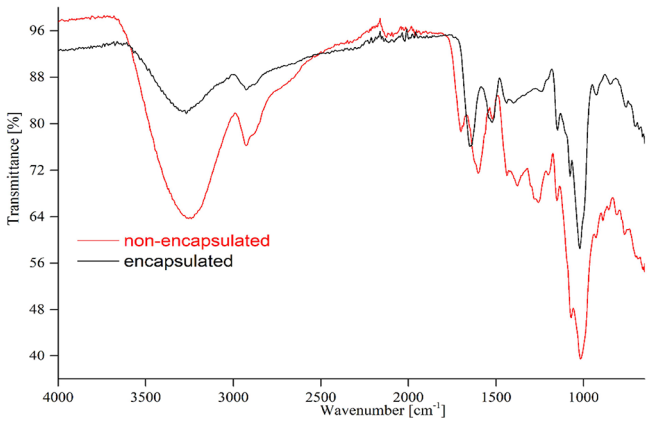

Figure 3. FTIR spectra of the encapsulated and nonencapsulated extracts.

encapsulation were compared, it was observed that the spectral bands were aligned at the same frequencies, although with variations in intensity. This consistency in frequency suggests that the atomic bonds remained unchanged post encapsulation. Given that the mass of the sample analyzed was constant, variations in the height or intensity of the bands could signify either an increase or a decrease in the concentration of the detected groups, allowing for quantitative analysis of these spectra. Nevertheless, in this case, the spectra were evaluated only qualitatively, and the primary information derived pertained to the presence or absence of specific types of atomic bonds, as indicated by the location of the peaks.

Upon analyzing the spectra, we can identify the presence of alcohol hydroxyl groups (OH). This is evident from a broad band in the range from 3269 to 3239 cm^{-1} for both encapsulated and nonencapsulated extracts. The absorption around 2926 cm^{-1} indicates the valence vibrations associated with the C−H bonds of the methyl and methylene groups found in any carbon chain present in both extracts. Additionally, a distinct band at 1700 cm^{-1} is observed exclusively in the nonencapsulated extract, indicating a carbonyl function's C═O stretching vibration. In contrast, two broad bands observed at 1601 and 1649 cm^{-1} correspond to the C═O stretching vibration of an ester in the nonencapsulated and encapsulated extracts, respectively.

Notably, the nonencapsulated extract demonstrates a medium-intensity peak at 1437 cm^{-1}, while the encapsulated extract exhibits a similar peak at 1439 cm^{-1}. These peaks signify the symmetric deformation vibration of the CH_3 groups. The valence C−O stretching is represented by the bands observed at 1151 cm^{-1} for the nonencapsulated extract and at 1149 cm^{-1} for the encapsulated extract. Furthermore, the peaks at 1117 and 1121 cm^{-1} correspond to the C−O stretching vibration of ether in the nonencapsulated and encapsulated extracts, respectively. Finally, we observe two additional medium-intensity bands at 766 and 757 cm^{-1}, indicative of an aromatic ═C−H vibration in the nonencapsulated and encapsulated extracts, respectively.

Overall, these findings demonstrate the presence of alcohol hydroxyl groups, methyl and methylene groups, carbonyl functions, esters, CH_3 groups, valence C−O stretching, ethers, and aromatic ═C−H bonds in the analyzed extract.

3.8. Nuclear Magnetic Resonance (NMR) Analysis. High-resolution NMR is an exceptional tool to determine the structures of both natural and synthetic molecules. Its

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capability to analyze a wide range of measurable parameters makes it particularly effective for studying molecules in solution. Unlike other spectroscopic methods, NMR provides detailed and specific information about a majority of the constituent atoms of the molecule. It can identify interatomic bonds within various entities, outline functional groups, and determine their spatial relationships.[75]

The structure of the obtained extract was investigated using C^{13}-NMR analysis. Figure 4 presents the chemical shifts of the

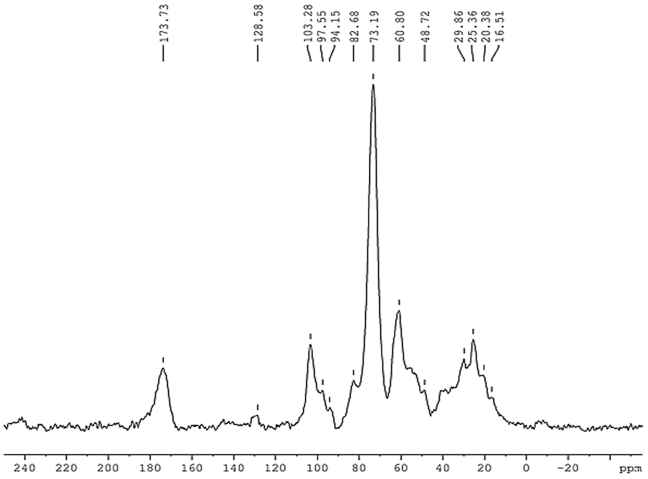

Figure 4. ^{13}C spectral pattern of E-OLE.

particular carbons and atom groups from the encapsulated olive leaf extract. A functional ester was detected from a C^{13}-NMR signal at δC 173.6 ppm, linked to an RCH_2O group at δC73.29 ppm. An additional RCH_2O group was noted at δC60.73. The C^{13}-NMR spectrum displayed two methyl group resonances for squalene at 29.81 and 25.33 ppm, aligning with findings reported in previous studies by Aggul et al.[76] and Ghanem et al.[77]

4. CONCLUSIONS

The present study has exhaustively characterized the OLE, highlighting rich phenolic content, which correlates with their pronounced antioxidant activity and antibacterial properties. These findings validate the effectiveness of the AE method employed in this research.

The use of maltodextrin and sodium caseinate for encapsulation achieved an efficiency rate of 71.92%, indicating that a substantial proportion of the phenolic compounds was successfully retained. Moreover, microscopic analysis revealed the formation of microcapsules, further evidencing the success of our technique.

These results underline the reliability and efficiency of our encapsulation techniques, which have significant implications for various industries, including pharmaceuticals and food. In these sectors, the controlled release and efficient use of encapsulated materials are crucial.

Overall, this research provides valuable insights into the antioxidant and antimicrobial properties of the OLE and showcases an effective encapsulation strategy, underscoring the potential health benefits of these compounds.

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I.T.: Conceptualization, methodology; I.T., E.O.-K.: software; I.T., E.O.-K., A.A.-K., and M.B.: validation; I.T. and E.O.-K.: formal analysis; I.T. and E.O.-K.: investigation; I.T.: resources; I.T.: data curation; I.T.: writing—original draft preparation; I.T., E.O.-K., A.A.-K., and M.B.: writing—review and editing; I.T. and E.O.-K.: visualization; I.T., E.O.-K., A.A.-K., and M.B.: supervision; I.T.: project administration. All authors have read and agreed to the published version of the manuscript.

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Notes

The authors declare no competing financial interest.

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