Volutaria lippii (L.) Cass. is an indigenous perennial herb from the Tunisian flora, belonging to the medicinally important genus Volutaria Cass. (Asteraceae) which comprises eighteen species widely distributed in the Irano-Turanian and Mediterranean Basin. In this study, five different extracts from Tunisian V. lippii (L.) Cass. were evaluated for their in vitro antioxidant, antiacetylcholinesterase, antidiabetic, and antibacterial activities as well as for their total phenolic and flavonoid contents. The results indicated that the ethyl acetate and aqueous fractions have the highest levels in phenolic and flavonoid contents and showed remarkable antioxidant activities using DPPH (IC_{50} = 11.50 ± 0.57 and 28.81 ± 1.35 μg/mL, respectively), total antioxidant capacity (105.21 ± 0.01 and 98.77 ± 0.02 mg vitamin E/g extract, respectively), and reducing power (EC_{50} = 55.40 ± 2.00 and 66.65 ± 1.40 μg/mL, respectively) methods. Furthermore, they exhibited noticeable antiacetylcholinesterase and antidiabetic activities and a moderate antibacterial effect when compared to that of standards. Principal component analysis allowed highlighting the ethyl acetate extract for its interesting acetylcholinesterase enzyme (AChE) and alpha-amylase activities and the aqueous fraction for its remarkably antibacterial activity, and their richness in phytochemical content. Interestingly, the LC-ESI-MS/MS analyses of both fractions allowed the identification of ten phenolic acids and eight flavonoids. The 3-O-caffeoylquinic and 3,4-di-O-caffeoylquinic acids constituted the most abundant components in the two fractions. Taken together, these findings demonstrated, for the first time, that V. lippii (L.) Cass. is a potential source of biological active compounds which could be used in a wide range of fields, namely, nutrition and complementary pharmacological drug.

1. Introduction

The Tunisian flora has a wide plant varieties used not only in folk medicine, but also in pharmaceutical, cosmetic, and food technologies [1]. These plants represent an excellent reservoir for extracting and identifying bioactive phytochemicals, which exerted a beneficial effect on human healthiness [2] and had a preventive role against cancer and chronic diseases. Many of these natural compounds, such as polyphenols, flavonoids, and phenolic acids, are known for their various pharmacological activities including antioxidant, antimicrobial, antidiabetic, anti-inflammatory, anticancer, and anti-Alzheimer effects [3, 4].

Volutaria lippii (L.) Cass. ex Maire (syn Centaurea lippii L., Volutarella lippii (L.) Cass., Amberboa lippii (L.) DC.) is one of the Tunisian plants that belong to the genus Volutaria Cass., tribe Cardueae, subtribe Centaureinae of the Asteraceae (Compositae) [5]. The genus Volutaria comprises approximately eighteen species growing in semiarid to arid zones and widely distributed in the Irano-Turanian
and Mediterranean areas [6]. Pharmacological and phytochemical studies on several Volutaria species have reported that these plants are rich in sesquiterpene lactones and flavonoids which possess various biological activities [7, 8]. Previous study reported the isolation of one sesquiterpene flavonoids which possess various biological activities [7, 8].

The aim of actual study was to evaluate the phytochemical screening (flavonoid and total phenolic contents) and in vitro antioxidant, antiacetylcholinesterase, antiadipetic, and antibacterial potentials of V. lippii. Moreover, the LC-ESI-MS/MS technic was employed for qualitative and quantitative analyses of phytochemicals in ethyl acetate and aqueous fractions. This study was the first report depicting chemical profile and biological property evaluations of V. lippii extracts.

2. Material and Methods

2.1. Plant Material. Aerial flowering parts of V. lippii were collected in April 2014 from Sfax in the central east of Tunisia (34°44’26.02”N, 10°45’57.01”W). A voucher specimen (Number LCSN 118) was deposited in the Herbarium Laboratory of Organic Chemistry (Natural Substances Team), Faculty of Sciences, Sfax University, Tunisia.

2.2. Chemicals and Reagents. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), vitamin E (α-tocopherol), gallic acid, quercetin, potassium ferricyanide, ferric chloride, ammonium molybdate, acetylcholinesterase, tacrine, α-amylase, acarbose, penicillin, Folin-Ciocalteu phenol reagent, and HPLC grade reagents: quinic acid, caffeic acid, protocatechuic acid, 3,4-di-O-cafeoylquinic acid, 3,4-di-O-cafeoylquinic acid, 4,5-di-O-cafeoylquinic acid, apigenin-7-O-glucoside, cisirineline, and acacetin were purchased from Merck (Sigma-Aldrich, Steinheim, Germany).

2.3. Extraction Procedure. Air-dried and powdered aerial part (300 g) of V. lippii was extracted by maceration with 80% aqueous-ethanol for 24 hours three times at room temperature with regular stirring. The resulting extracts were collected, filtered, and concentrated under vacuum. The dried crude extract was solubilized in 500 mL of distilled water for fractionation. The aqueous solution was further partitioned successively with hexane, dichloromethane, ethyl acetate, and n-butanol. The hexane, dichloromethane, ethyl acetate, n-butanol, and the final aqueous fractions were filtered and evaporated to dryness under vacuum.

2.4. Determination of Total Phenolic Content. The total phenolic content in V. lippii extracts was estimated by Folin-Ciocalteu method according to Chen et al. [12]. To 100 μL of diluted sample extract, 2mL of Na2CO3 aqueous solution (2%) and 100 μL of 50% Folin-Ciocalteu reagent were added. The final mixture was incubated for 30 minutes in the dark. The absorbance of each sample was read at 750 nm with a Shimadzu UV/Vis spectrophotometer (Tenway 6320D). The results are expressed in gallic acid equivalents (mg GAE/g extract).

2.5. Determination of Total Flavonoid Content. The total flavonoid content in extracts was determined by the method described previously by Djeridane et al. [13] and expressed as quercitin equivalents (mg QE/g extract). Briefly, 1 mL of each diluted V. lippii fraction was added to 1 mL of AlCl3 methanolic solution (2%). After 15 min incubation at room temperature, the absorbance of the obtained mixture was measured at 430 nm.

2.6. DPPH Radical Scavenging Assay. The DPPH radical scavenging activity of different fractions of V. lippii was evaluated following the procedure described by Les et al. [14]. IC50 value of each fraction, i.e., concentration of sample necessary to decrease the initial DPPH concentration by 50%, is a parameter widely used to assess the antioxidant activity. Briefly, 1.5 mL of DPPH solution (10^{-4} M, in 95% Ethanol) was added to 1.5 mL of each V. lippii fraction at various concentrations (0.01-1 mg/mL). The final concentrations in the reaction's mixture were 0.005-0.5 mg/mL. Each mixture was shaken and allowed in the dark for 30 min at room temperature. The blank was prepared as above without any extract and the BHT was used as positive control. The percentage of inhibition (PI (%)) was determined spectrophotometrically by monitoring the decrease in absorbance at 517 nm against a blank. The PI was calculated using the following equation:

\[
P_{I} (\%) = \left[ \frac{A_{blank} - A_{sample}}{A_{blank}} \right] \times 100
\]

where \(A_{blank}\) is the absorbance of the blank and \(A_{sample}\) is the absorbance of the test sample.

The calibration curve for scavenging percentage against extract concentration was plotted and the IC50 (half maximal inhibitory concentration) value of each sample was established.

2.7. Total Antioxidant Capacity Assay (TAC). The total antioxidant capacity (TAC) of all fractions of V. lippii was spectrophotometrically assessed by the method of Prieto et al. [15]. A 0.1 mL of each V. lippii fraction (1 mg/mL) was mixed with 1 mL of reagent solution (28 mM sodium phosphate, 0.6 M sulfuric acid and 4 mM ammonium molybdate). The mixtures were incubated for 90 min in a boiling water bath at 95°C. After cooling the samples, the absorbance was determined at 705 nm. The antioxidant capacity was expressed as equivalents of vitamin E (μg/g of extract).

2.8. Reducing Power Assay. The reducing power of all fractions was evaluated using the procedure of Yildirim et al. [16], and BHT was used as positive control. A 1 mL of different concentrations of each V. lippii sample (5, 10, 25, 50, 100 μg/mL) was mixed with 2.5 mL of 1% potassium ferricyanide...
and 2.5 mL of sodium phosphate buffer (0.2 M, pH= 6.6). The mixture was incubated for 20 min at 50°C and then 2.5 mL of 10% trichloroacetic acid were added and centrifuged for 10 minutes. 2.5 mL of the supernatant were mixed with 0.5 mL of ferric chloride solution (0.1%) and 2.5 mL of distilled water. The absorbance of the mixture was measured at 700 nm.

2.9. Determination of Acetylcholinesterase (AChE) Inhibitory Activity. The AChE inhibitory activity was performed according to the colorimetric method described by Ellman et al. [17], with modifications. Briefly, 125 μL of DTNB (3 mM), 50 μL of sodium phosphate buffer (pH 8.0), 25 μL of AChE (0.5 U/mL), and 25 μL of each V. lippii extract dissolved in DMSO were added in a 96-well microplate and incubated for 15 min at 25°C. The reaction was then initiated by the addition of 25 μL of acetylthiocholine iodide (ATCI) and the hydrolysis of acetylthiocholine iodide was controlled by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction with thiocholine with DTNB. Plant fractions were tested for AChE inhibitory activity at the following concentrations 25, 50, 125, 250, and 500 μg/mL. The final concentrations in the reaction’s mixture were 2.5, 5, 12.5, 25, and 50 μg/mL [14]. A reaction mixture containing all the components except the V. lippii extract was used as control. Tacrine was used as positive control. The absorbance was then read three times with 3 min intervals at 405 nm by a CERES UV 900C microplate reader (Bio-Tek Instrument, USA). Any increase in absorbance due to the spontaneous hydrolysis of the substrate was revised by subtracting the absorbance before appending the enzyme. The percentage inhibition was calculated as follows:

\[
\text{PI} (%) = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100
\]

where \(A_{\text{control}}\) is the absorbance of the control and \(A_{\text{sample}}\) is the absorbance of the test sample.

Extract concentration providing 50% inhibition (IC\(_{50}\)) was obtained by plotting the percentage inhibition against extract concentration.

2.10. Determination of α-Amylase Activity In Vitro. The in vitro α-amylase inhibitory assay of all fractions was performed by the previous method described by Gella et al. [18]. The enzyme α-amylase solution was made by blending 3.246 mg of alpha amylase (EC 3.2.1.1) in 100 mL of phosphate buffer (40 mM, pH 6.9). The assays were conducted by mixing 30μL of alpha-amylase solution, 120μL of E-PNPG, and 60μL of each V. lippii fraction in the concentration range 25, 50, and 100 μg/mL. The final concentrations in the reaction’s mixture were 7.14, 14.28, and 28.57 μg/mL. The positive control (acarbose) was prepared by dissolving 50 mg in 50 mL of phosphate buffer and diluted to get different concentrations 10, 20, and 40 μg/mL. The final concentrations in the reaction’s mixture were 2.85, 5.71, and 11.42 μg/mL. The mixture was incubated for 8 min at 37°C. The absorbance was read at 405 nm and control reaction was carried out without the V. lippii fractions. Percentage inhibition (PI) was calculated by the following expression:

\[
\text{PI} (%) = \left[ \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right] \times 100
\]

where \(A_{\text{control}}\) is the absorbance of the control and \(A_{\text{sample}}\) is the absorbance of the test sample.

2.11. Antimicrobial Activity Assay. The V. lippii fractions were assessed against a five bacterial strains: Gram-negative: Salmonella enterica (CIP 8039) and Escherichia coli (ATCC 8739), Gram-positive: Staphylococcus aureus (ATCC 6538), Bacillus thuringiensis, and Enterococcus faecalis (ATCC 29212). Bacteria not obtained from an ATCC collection were acquired from the Microbiology Department, Faculty of Science, University of Sfax (Tunisia). Bacterial strains were cultured in Mueller-Hinton agar (MHA) for 24 h at 37°C.

The disc diffusion method was employed for the determination of antibacterial activities of V. lippii fractions according to the method described by Berghé and Vlietinck [19]. Antibacterial activities were evaluated by measuring the diameters of the inhibition zones against the test organisms and compared to penicillin (10 μg per disk) as the positive control. Tests were carried out in triplicate.

2.12. Chromatographic Conditions and Apparatus. HPLC procedures chromatographic separation was performed on Aquasil C18 (Thermo Electron, Dreieich, Germany) column (150 mm × 3 mm, 3 μm particle size). The solvents used were (A) 0.1% formic acid in water and (B) 0.1% formic acid in methanol. The elution gradient established was 10-100% B, 0-45 min; 100% B, 45-55 min and reequilibration duration was 5 min between individual runs. The flow rate of the mobile phase was 0.4 mL/min, the injection volume was 5 μL, and the column temperature was maintained at 40°C. Phenolics present in the fractions were characterized according to their retention times, UV and mass spectra compared with commercial standards when available. The quantification of phenolics was determined based on DAD results, using 280 nm for the phenolic acids and 320 and 370 nm for flavonoids.

The LC-ESI-MS/MS analysis was carried out using a LCMS-8030 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan) equipped with an electrospray ionization (ESI). The mass spectrometer was operated in negative ion mode with a nebulizing gas flow of 1.5 L/min, a dry gas flow rate of 12 L/min, a block source temperature of 400°C, a DL (dissolving line) temperature of 250°C, the full scan spectra from 50 to 2000 Da, and the negative ionization mode source voltage-4500 V.

2.13. Statistical Analyses. Each calculation was accomplished using SPSS software (version 19.0; SPSS Inc., Chicago, IL, USA). All experiments were carried out as means ± standard deviations of three replicates. Analysis of variance (ANOVA) followed by Tukey’s post hoc test were used to establish the differences between means of various groups. The level of
3. Results and Discussion

3.1. Total Phenolic and Flavonoid Contents. The total phenolic (TP) and total flavonoid (TF) contents of different studied fractions from V. lippii were determined. As can be seen in Table 1, the levels of phenolic compounds varied significantly (p<0.05) depending on the influence of solvent polarity [20] and have been found to be rich in all fractions except the hexane one. The ethyl acetate fraction showed the highest (p<0.05) amount of phenolic compounds (65.22±0.03 mg GAE/g) followed by the n-butanol (35.04±0.04 mg GAE/g), whereas the lower (p>0.05) one was observed in the hexane fraction (7.46±0.23 mg GAE/g) fractions.

With regard to the TF, the highest (p<0.05) value was also found in the ethyl acetate fraction (12.50±0.04 mg QE/g), whereas the lower (p>0.05) one was observed in the hexane fraction (2.14±0.05 mg QE/g). These findings were in agreement with those revealed by Karamenderes et al. (2007) for eight different species of Centaurea (synonym of Volutaria) [21].

3.2. Antioxidant Activity. Several studies were devoted to finding natural antioxidants such as phenolic compounds and flavonoids, which are principally responsible for the antioxidant properties of plant [22, 23]. The antioxidant capacity of different plant extracts cannot be evaluated by a single testing method due to the complex nature of phytochemicals [24]. For this reason, three complementary in vitro chemical assays, in terms of DPPH radical scavenging, reducing power, and total antioxidant capacity, were applied in order to screen the potential antioxidant properties of V. lippii extracts.

3.2.1. DPPH Radical Scavenging Assay. Antioxidant activity in food can be expressed in terms of radical scavenging ability using free radicals. DPPH assay is extensively used to determine the antioxidant property of many plant extracts [25, 26]. It is well known that free radicals have an important role in the autoxidation of unsaturated lipids in foodstuffs and in oxidative cell damage in the human organism resulting in a variety of pathological diseases [27]. Antioxidants can intercept the chain autoxidation of lipids and donate hydrogen to free radicals, particularly to the lipid peroxides radicals, thereby forming stable free radicals, which do not initiate or propagate further lipid oxidation [28]. The DPPH scavenging activity of V. lippii fractions, expressed as IC₅₀ (µg/mL), were illustrated in Table 1. Obtained results demonstrated that IC₅₀ of different fractions ranged from 11.50 ±0.57 to 43.77 ±2.09 µg/mL, indicating their antioxidant potentials compared with the standard BHT (13.00 ±0.57 µg/mL). The order of DPPH scavenging ability of fractions and BHT was as follows: ethyl acetate (11.50 ±0.57 µg/mL) > BHT (13.00 ±0.57 µg/mL) > aqueous (28.81 ±1.35 µg/mL) > dichloromethane (34.25 ±1.69 µg/mL) > n-butanol (43.77 ±2.09 µg/mL). The hexane fraction was found inactive.

These results proposed that the tested fractions, especially ethyl acetate and aqueous ones, have a good capability to donate electrons to reactive free radicals converting them into more stable forms [22]. The raised free radical scavenging activity of V. lippii is in favour of the implication of phenolic compounds which have been quantified in all its fractions (Table 1).

3.2.2. Total Antioxidant Capacity Assay (TAC). CAT method offers a broader view of the antioxidant potential of plant extracts and expresses different aspects of antioxidant action [25]. The results depicted in Table 1 showed that the ethyl acetate fraction has the highest (P<0.05) antioxidant capacity (105.21±0.01mg vitamin E/g extract) that can be related to its high levels in TP and TF. The TAC of V. lippii extracts was found to increase in the following order: hexane < dichloromethane < n-butanol < aqueous < ethyl acetate. This result is in good accordance with the phenolic content variation in all fractions.

3.2.3. Reducing Power Assay (FRAP). The reducing powers of testing fractions and BHT were also determined (Table 1). In this test, all fractions presented dose-dependent activity whose results are lower than that of BHT (EC₅₀ = 43.35±0.95 µg/mL). In fact, reducing power of V. lippii fractions increased and was well correlated when concentration increased. The reducing power was expressed as effective concentration EC₅₀ at which the absorbance is 0.5. As indicated in Table 1, the results revealed that the ethyl acetate fraction exhibited the highest (P<0.05) activity (EC₅₀ = 55.40±2.00 µg/mL), followed by aqueous (EC₅₀ = 66.65±1.40 µg/mL), n-butanol (EC₅₀ = 150.0±3.12 µg/mL), dichloromethane (EC₅₀ = 216.66±2.49 µg/mL), and hexane (EC₅₀ = 383.33±2.85 µg/mL) fractions.

These reducing properties are often due to the presence of reductones capable of exerting an antioxidant effect by breaking the free radicals chain and by donating a hydrogen atom. It was reported that reductones respond to various precursors of peroxides and therefore prevent their generation [29].

The results obtained with the three antioxidant activity tests revealed that the different plant extracts contained a considerable amount of antioxidant components. Differences in solvent polarities and thus different extractability of the antioxidant components may explain the differences in the antioxidant activity of V. lippii extracts. Both ethyl acetate and aqueous fractions have the strongest antioxidant effects that are related to their high levels of phenolic and flavonoid contents (Table 1). Thus, V. lippii extracts, especially ethyl acetate, could be used as natural antioxidant agents.
Table 1: Total phenolic (TP) and flavonoid (TF) contents, DPPH radical scavenging, reducing power and total antioxidant capacity (TAC) activities of extracts from *V. lippii*.

| Sample       | Total phenolic (mg GAE/g extract) | Total flavonoid (mg QE/g extract) | DPPH IC<sub>50</sub> (μg/mL) | TAC (mg vitamin E/g extract) | Reducing power EC<sub>50</sub> (μg/mL) |
|--------------|-----------------------------------|----------------------------------|------------------------------|-----------------------------|--------------------------------------|
| Hexane       | 7.46±0.23<sup>a</sup>             | 2.14±0.05<sup>a</sup>            | inactive                     | 45.97±0.05<sup>a</sup>      | 383.33±2.85<sup>b</sup>             |
| Dichloromethane | 24.13±0.04<sup>b</sup>            | 10.49±0.02<sup>cd</sup>          | 34.25±1.69<sup>c</sup>       | 64.37±0.03<sup>b</sup>      | 216.66±2.49<sup>d</sup>             |
| Ethyl acetate | 65.22±0.03<sup>d</sup>            | 12.50±0.04<sup>d</sup>           | 11.50±0.57<sup>a</sup>       | 105.21±0.01<sup>e</sup>     | 55.40±2.00<sup>b</sup>              |
| n-Butanol    | 38.83±0.07<sup>c</sup>            | 5.00±0.03<sup>b</sup>            | 47.72±2.09<sup>d</sup>       | 81.68±0.01<sup>c</sup>      | 150.00±3.12<sup>e</sup>             |
| Aqueous      | 35.04±0.05<sup>c</sup>            | 8.90±0.04<sup>c</sup>            | 28.81±1.35<sup>b</sup>       | 98.77±0.02<sup>d</sup>      | 66.65±1.40<sup>b</sup>              |
| BHT          | -                                 | -                                | 13.00±0.57<sup>a</sup>       | -                           | 43.35±0.95<sup>d</sup>             |

±: standard deviation of three replicates.
a–e: averages with different letters in the same column are different (P<0.05).
3.3. Acetylcholinesterase Enzyme (AChE) Inhibitory Activity. The results related to the AChE inhibitory activity of V. lippii extracts are given in Table 2. Only aqueous extract gave a strong AChE inhibition (P<0.05) with IC\textsubscript{50} value of 3.91 ± 0.19µg/mL when compared to the tacrine (IC\textsubscript{50} = 3.50 ± 0.17 µg/mL) used as a standard of AChE inhibition. The n-butanol (IC\textsubscript{50} = 9.97 ± 0.48µg/mL), dichloromethane (IC\textsubscript{50} = 15.78 ± 0.78µg/mL), and ethyl acetate (IC\textsubscript{50} = 17.76 ± 0.88µg/mL) extracts showed moderate AChE inhibitory activity. However, the hexane fraction exhibited no inhibition, and its activity was same as that of control. These results reveal that the active fractions were those obtained by extraction with polar solvents (aqueous and n-butanol). In comparison with other studies, the aqueous extract of V. lippii exhibited AChE inhibitory activity higher than that obtained from aqueous extracts of some plants from Argentina [30] and some Centaurea species such as C. antalyense, C. polypodifolia var. pseudobehen, and C. pyrrhophepha [31].

AChE inhibitors have been extensively used in the treatment of mild to moderate Alzheimer disease. There are several researchers who focused on the quest of new AChE inhibitors from the herbal resources to replace synthetic drugs such as donepezil and tacrine having any adverse effects [32]. For this purpose, the obtained results indicate that V. lippii could serve as an inhibitor against the cholinesterase enzyme family and used as complement for the treatment of some Alzheimer diseases. The AChE inhibitory activity of V. lippii extracts has never been reported before and their anticholinesterase activity could be attributed to their TP and TF contents (Table 1). Indeed, several authors reported previously a strong relationship between anticholinesterase activity and phenolic content of some Centaurea species such as C. depressa, C. drabifolia subsp. Detonsa, C. kotschyi var. persica, C. patula, C. pulchella, C. tchihatcheffi, C. triumfettii, and C. urvillei subsp. Hayekiana [31, 33].

3.4. In Vitro Alpha-Amylase Inhibitory Assay. This assay evaluated the ability of V. lippii extracts to inhibit α-amylase activity. The α-amylase, a digestive enzyme secreted from the pancreas and salivary gland, is engaged in important biological processes such as digestion of carbohydrates, reducing postprandial hyperglycaemia. The inhibition of this digestive enzyme is therefore used as one of the diabetic treatments [23]. As indicated in Table 3, the IC\textsubscript{50} values of aqueous, n-butanol, ethyl acetate, and dichloromethane fractions were 7.48 ± 0.34, 11.07 ± 0.56, 13.28 ± 0.65, and 16.99 ± 0.81µg/mL, respectively, indicating their promising inhibitory activity against the pancreatic α-amylase enzyme. It should be noted that acarbose (standard antidiabetic agent) showed more potent inhibition of α-amylase (IC\textsubscript{50} = 5.54 ± 0.27µg/mL) than all tested fractions.

Moreover, the appreciable α-amylase inhibitory capacities of these fractions might be attributed to their TP and flavonoids could prevent the activity of carbohydrate-hydrolyzing enzymes, related to their capacity to bind with proteins [25, 29]. Therefore, V. lippii can be considered a new natural source able to fight against type 2 diabetes.

3.5. Antibacterial Activity Assay. In the present study, the antibacterial activity of V. lippii fractions was evaluated through a set of pathogenic bacteria. The relevant results of this assay were depicted in Table 4. The V. lippii extracts inhibited the growth of bacterial strains producing a zone diameter of inhibition from 5.0 to 17.6 mm for Gram-negative bacteria and from 5.25 to 15.5 mm for Gram-positive bacteria. The standard antibiotic, penicillin, showed a strong antibacterial inhibition against practically all bacteria strains. Among Gram-negative bacteria, the strongest activity was observed (p<0.05) for aqueous and n-butanol fractions against Salmonella enterica (17.6±0.5 mm and 14.0±0.3 mm, respectively). These values are higher (p<0.05) than that of penicillin, which is sensitive to the bacteria. Generally, plant extracts were usually more active against Gram-positive than Gram-negative bacteria [25]. This could be explained by the fact that Gram-positive strains have only a peptidoglycan layer which is not a selective barrier to plant extracts [26]. The antibacterial activity of V. lippii could be assigned to the presence of a high concentration of phenolic compounds which were reported in previous works to exhibit a powerful antimicrobial effect [22, 26].

3.6. Activities Discrimination of Ethyl Acetate and Water Fractions by Combining Phytochemical Contents within a Multivariate Analysis: Principal Component Analysis. Principal Component Analysis (PCA) was conducted to get a general overview of the data distribution, thus a new set of latent factors or principal components (PCs) was generated. In this part, PCA based on the corresponding data of ethyl acetate and water fractions set including chemical (total phenolic and flavonoid contents), antioxidant (DPPH radical scavenging assay, total antioxidant capacity assay (TAC) and reducing power assay (FRAP)), alpha-amylase inhibitory assay, antimicrobial and acetylcholinesterase enzyme (AChE) inhibitory values of ethyl acetate and water fractions values was carried out (Figure 1).

The first principal component (PC1) had the highest eigenvalue of 5.89 and accounted for 54.36% of the variability in the data set. The second, third, and fourth PCs (PC2, PC3, and PC4) had eigenvalues of 2.851, 1.328, and 0.841 and explained 25.92%, 13.28%, and 7.64% of the variance in the data, respectively. Subsequently, plotting the scores

| Sample       | IC\textsubscript{50} (µg/mL) |
|--------------|-----------------------------|
| Hexane       | inactive                    |
| Dichloromethane | 15.78 ± 0.78\textsuperscript{g} |
| Ethyl acetate | 17.76 ± 0.88\textsuperscript{d} |
| n-Butanol    | 9.97 ± 0.48\textsuperscript{b} |
| Aqueous      | 3.91 ± 0.19\textsuperscript{a} |
| Tacrine      | 3.50 ± 0.17\textsuperscript{a} |

±: standard deviation of three replicates.
a-d: averages with different letters in the same column are different (P<0.05).
and antibacterial (anti-waterfraction which correlate with DPPH radical scavenging activities. The group G2 mainly constituted by the positive the group G1 with ethyl acetate fraction correlating with TAC, acetylcholinesterase enzyme (AChE), and alpha-amylase activities (Figure 1). These axes' components selected two groups around the PC1 and PC2 axes' components of the previous observations, allowed the discrimination of the samples in the subspaces PC1 vs. PC2 (Figure 1) (80.28% of the total variance of the data) a clear grouping of samples was observable based on solvent extraction (ethyl acetate and water). Moreover, the PCA, which confirms the previous observations, allowed the discrimination of two groups around the PC1 and PC2 axes' components and activities (Figure 1). These axes' components selected positively the group G1 with ethyl acetate fraction correlating with TAC, acetylcholinesterase enzyme (AChE), and alpha-amylase activities. The group G2 mainly constituted by the water fraction which correlate with DPPH radical scavenging and antibacterial (anti-Salmonella enterica, anti-Escherichia coli, and anti-Staphylococcus aureus) activities.

3.7. Qualitative Phytochemical Analyses of Ethyl Acetate and Aqueous Fractions. LC-ESI-MS/MS analyses were performed to determine for the first time the chemical profiles of ethyl acetate and aqueous fractions exhibiting remarkable biological activities. The HPLC chromatograms at 254 nm of these two fractions were illustrated in Figure 2. Eighteen constituents, numbered 1-18, were detected and tentatively identified as belonging to both phenolic acid and flavonoid groups. The structures of all identified compounds are shown in Figure 3. The identification of the phenolic compounds was carried out by mass spectra, comparison with reference compounds and with literature data. Table 5 summarized all the identified peaks with retention times (tR), UV values (λmax), pseudomolecular ions, molecular formula, and main fragment ions.

3.7.1. Quinic Acid Derivatives. The quinic acid derivatives exhibited characteristic UV spectra with an absorption maxima at λmax around 320-325 nm [34]. Peak 1 (tR 6.21 min) was identified as quinic acid by comparison with a
### Table 5: Qualitative and quantitative phytochemical analyses of ethyl acetate and aqueous extracts of *V. lippii*.

| Peak | $t_r$ (min) | $\lambda_{max}$ (nm) | [M-H]$^-$(m/z) | Main fragment ions MS$^2$(m/z) | Molecular formula | Tentative identification | Content ($\mu$g/g of Dried material) |
|------|-------------|----------------------|-----------------|-------------------------------|-------------------|---------------------------|-----------------------------------|
| 1    | 6.21        | 321                  | 891             | 173, 135                      | $C_7H_8O_3$       | Quinic acid$^a$           | 1265.104                          |
| 2    | 7.86        | 280, 216             | 169             | 125                          | $C_9H_8O_5$       | Gallic acid               | 899.40                            |
| 3    | 11.21       | 294                  | 153             | 109                          | $C_7H_8O_3$       | Protocatechuic acid$^a$   | 136.743                           |
| 4    | 16.93       | 324, 218             | 353             | 191, 179                      | $C_9H_6O_4$       | Chlorogenic acid$^a$      | 5868.61                           |
| 5    | 21.22       | 332, 270             | 179             | 161, 135, 143                 | $C_7H_8O_3$       | Caffeic acid$^a$          | 57.846                            |
| 6    | 23.77       | 280, 210             | 197             | 182, 167, 153, 138           | $C_7H_{12}O_6$    | Syringic acid             | 106.743                           |
| 7    | 26.03       | 325                  | 515             | 353, 191, 173                 | $C_{16}H_{13}O_9$ | 1,3-di-O-caffeoylquinic acid$^a$ | 318.634                           |
| 8    | 42.30       | 332, 234             | 193             | 178, 149, 134                 | $C_{10}H_{12}O_4$ | Trans ferulic acid        | 61.567                            |
| 9    | 59.01       | 355, 254             | 609             | 463, 301, 271                 | $C_{27}H_{30}O_{16}$ | Rutin$^a$                 | 38.684                            |
| 10   | 61.03       | 338, 270             | 447             | 285                          | $C_{21}H_{20}O_{11}$ | Luteolin-7-O-glucoside   | 115.247                           |
| 11   | 63.49       | 324                  | 515             | 353, 191, 173                 | $C_{16}H_{17}O_9$ | 3,4-di-O-caffeoylquinic acid$^a$ | 98.49.464                          |
| 12   | 67.22       | 353, 256             | 447             | 301                          | $C_{21}H_{20}O_{11}$ | Quercetin                 | 53.336                            |
| 13   | 68.63       | 334, 217             | 431             | 269                          | $C_{21}H_{20}O_{10}$ | Apigenin-7-O-glucoside$^a$ | 7.350                             |
| 14   | 70.36       | 323                  | 515             | 353, 191, 179, 173           | $C_{16}H_{17}O_9$ | 4,5-di-O-caffeoylquinic acid$^a$ | 149.012                           |
| 15   | 80.84       | 365, 264             | 285             | 269, 179                      | $C_{15}H_{10}O_6$ | Kaempferol                | 56.397                            |
| 16   | 89.16       | 334, 268             | 269             | 179, 151, 119                 | $C_{15}H_{10}O_6$ | Apigenin                  | 2.408                             |
| 17   | 99.73       | 340, 270             | 343             | 297, 255, 241                 | $C_{16}H_{14}O_7$ | Cirsilineol$^a$           | 2.255                             |
| 18   | 102.51      | 332, 269             | 283             | 240, 151, 131                 | $C_{16}H_{12}O_5$ | Acacetin$^a$              | 10.893                            |

$^a$Identity confirmed by standards; (-): absence in extract.
standard and also by its pseudomolecular ion [M-H]− at m/z 191 and fragmentation pattern at m/z 173 [quinic acid-H-H2O]− [35]. Peak 4 (tR 16.93 min) was identified as 3-O-caffeoylquinic acid that was assigned according to the MS2 fragment ions at m/z 191 [quinic acid-H]− and at m/z 179 [caffeic acid-H]− and by comparison with an authentic compound [36].

Additionally, three di-O-caffeoylquinic acid isomers were characterized by their deprotonated parent ion [M-H]− at m/z 515 and by similar fragmentation patterns at m/z 191 [M-H-cafeoyl-cafeoyl]−, 173 [quinic acid-H-H2O]−, and 353 [M-H-cafeoyl]− characteristics of dicaffeoylquinic acid. However, by comparing the tR of the standard compounds the peaks 7 (tR 26.03 min), 11 (tR 63.49 min), and 14 (tR
70.36 min) were identified as 1,3-di-O-caffeoylquinic; 3,4-di-O-caffeoylquinic; and 4,5-di-O-caffeoylquinic acids, respectively [35, 36]. It should be noted that 1,3-di-O-caffeoylquinic acid was only detected in the aqueous fraction.

3.7.2. Phenolic Acid Derivatives. The MS spectra of compounds 2, 3, 5, 6, and 8 indicated specific fragments which prove the presence of free phenolic acids (Table 5). The UV spectra of peaks 5 and 8 showed the same absorption maximum at 332 nm typical of cinnamic acid derivatives [34]. Peak 5 (t_R 21.22 min) was identified as caffeic acid by comparing with a reference compound and according to its deprotonated molecular ion [M-H]^- at m/z 179 and MS^2 ions at m/z 161 [caffeic acid-H-H_2O]^-. Ferulic acid (peak 8, t_R 42.30 min) was easily identified by its MS ions at m/z 191 [M-H]^-, and at m/z 149 [ferulic acid-H-CO_2]^-[37, 38]. Moreover, peaks 2 (t_R 7.86 min), 3 (t_R 11.21 min), and 6 (t_R 23.77 min) were tentatively assigned as gallic, protocatechuic, and syringic acids, respectively, based on the UV spectra, MS^2 fragmentation pattern and previous report [38]. Gallic and syringic acids were only detected in the aqueous fraction.

3.7.3. Flavonoids. In ethyl acetate and aqueous fractions, flavonoids were represented with kaempferol and quercetin derivatives that exhibited typical UV spectra with band II in the 252-257 nm range and band I in the 350-359 nm range [34].

The assignment of peak 15 (t_R 80.84 min) as kaempferol was done via its pseudomolecular ion [M-H]^-, at m/z 285 and MS^2 ions at m/z 179 and 115 [37]. Compounds 9 (t_R 59.01 min) and 12 (t_R 67.22 min) produced characteristic MS^2 ions at m/z 463 [M-H-rhamnosyl-Glucose]^-, therefore, it was identified as rutin [37]. While peak 12, having MS ions at m/z 447 [M-H]^-, and 301 [M-H-rhamnosyl-Glucose]^-, was associated with quercetrin (quercetin-3-O-rhamnoside) [39].

Besides flavonols, five flavonones, possessing similar UV spectra, were discerned in the ethyl acetate and aqueous fractions. Apigenin (peak 16, t_R 89.16 min) was easily identified by its MS fragmentation ions at m/z 269 [M-H]^-, and at m/z 179, 151, and 119 [35]. On the basis of literature data [36], peak 10 (t_R 61.03 min) was tentatively assigned as luteolin-7-O-glucoside (MS ions at m/z 447 [M-H]^-, and 285 [luteolin-H]^-). Compounds 13 (t_R 68.63 min), 17 (t_R 99.73 min), and 18 (t_R 102.51 min) were identified as apiogenin-7-O-glucoside, cirsimarinol, and acacetin, respectively, by comparison with standard compounds and with literature data [40].

3.8. Quantitative Phytochemical Analyses of Ethyl Acetate and Aqueous Fractions. The quantification analyses data of the identification constituents were achieved by HPLC. The amounts of the compounds, detected in the samples and expressed in µg/g of dry material, were reported in Table 5.
The results showed that quinic acid derivatives were the dominant components in ethyl acetate and aqueous fractions with percentages 95.93 and 96.96 %, respectively. On the other hand, phenolic acids and flavonoids were found for the two fractions in lower levels of contents which are less than 200 μg/g of dry material. Quinic, 3-O-caffeoylquinic, and 3,4-di-O-caffeoylquinic acids were the most abundant acids in the two samples. The major quinic acid derivative in the ethyl acetate fraction was 3,4-di-O-caffeoylquinic acid (9849.46 μg/g of dry material) while 3-O-caffeoylquinic acid was found the dominant compound in the aqueous fraction (11721.58 μg/g of dry material). Several works reported that the 3,4-di-O-caffeoylquinic acid exhibited antioxidant [41], antidiabetic [42], antibacterial [43], cytotoxic [42], and anti-HVS-1 effects [44]. Also, 3-O-caffeoylquinic acid was mentioned possessing antioxidant [45], antidiabetic [46], antimicrobial [47], and anti-Alzheimer activities [48]. Thus, the biological activities of V. lippii fractions could be related to the high amounts of quinic acid derivatives and specially 3-O-caffeoylquinic and 3,4-di-O-caffeoylquinic acids.

4. Conclusions

This paper is the first report that highlights the antioxidant, antiacetylcholinesterase, antidiabetic, and antibacterial properties of V. lippii. Among all the extracts, ethyl acetate and aqueous fractions exhibited the greater biological activities and the highest levels in total phenolic and flavonoid contents. LC-ESI-MS/MS analyses of these two fractions led to the identification of eighteen compounds belonging to phenolic acids and flavonoids, where quinic, 3-O-caffeoylquinic and 3,4-di-O-caffeoylquinic acids were present in higher amounts. Therefore, the qualitative and quantitative analyses of major constituents in V. lippii samples could be helpful for understanding the relationship between the total phenolic and flavonoid contents and their biological activities. However, further studies are required to isolate new bioactive components in the ethyl acetate and aqueous fractions and to evaluate their in vivo biological capacities.

Data Availability

All data used to support the findings of this study are included within the article.

Conflicts of Interest

Authors declare that there are no conflicts of interest regarding the publication of this paper.

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