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Phytochemical profiles of *Iris unguicularis* Poir. with antioxidant, antibacterial, and anti-Alzheimer activities

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Abstract: Phytochemical investigation of medicinally important plants has gained great attention, lately. This study aims to investigate the phytochemical constituents of the partitioned extracts (ethyl acetate (AI), dichloromethane (CI) and n-butanol (BI)) derived from the 70% ethanol extract of the rhizomes (RI) of *Iris unguicularis* Poir. an Algerian-Tunisian endemic plant and to evaluate their biological activities. Seventy constituents of these partitioned extracts were qualitatively characterized by liquid chromatography ion trap time of flight mass spectrometry (LC-MS-IT-TOF) technique. Moreover, quantitative screening of phenolic acids and flavonoids in the studied extracts was accomplished by a previously validated LC-MS/MS method. It was determined that, AI and CI, which were the richest extracts in terms of phytochemical content, showed high antioxidant and antibacterial activities in parallel with their identified constituents, while AI extract was rich in protocatechuic (1080.4 µg analyte/g), 4-OH-benzoic (2218.2 µg/g), syringic (1218.6 µg/g) and p-coumaric (1071.2 µg/g) acids, CI extract has been found to be rich in naringenin (1453.4 µg/g) and ferulic acid (1088.4 µg/g). These results revealed and confirmed the importance of the species and especially the genus rhizomes which have largely been used in folk medicine for the treatment of many diseases. However,

all extracts of this endemic plant were inactive at all concentration against acetyl- and butyrylcholinesterase enzymes.

Keywords: *Iris unguicularis* Poir.; rhizomes; phenolic compounds; biological activities; LC-MS/MS; LC-MS-IT-TOF

Introduction

Iris genus, which belongs to the Iridaceae family, has a wide distribution in nature and it is found throughout the north temperate zones [1]. Rhizomes of *Iris* spp. have largely been used in traditional medicine for the treatment of several diseases. For example, *Iris dichotoma* Pall rhizomes are used for the treatment of inflammation and respiratory system disorders [2] and decoction of its roots are used as antispasmodic and emmenagogue among public [3]. Following the different applications in traditional medicine, *Iris* genus has been the object of several scientific publications which led to the isolation of many active compounds such as the twenty-one compounds isolated from *Iris marsica* I. Ricci e Colas. [4]. However, flavonoids are the major compounds with a large spectrum of biological activities [5, 6]. In Algeria this genus is mainly represented by three species: *Iris pseudoacorus* L, *Iris planifolia* (Mill.) Dur. et Sch and *Iris unguicularis* Poir. [7].

According to Quezel and Santa, *I. unguicularis*, is an Algerian-Tunisian endemic plant with creeping rhizomes producing purple or pale lilac flowers with a yellow central band on the falls. Its fragrant flowers appear in early spring and winter [8].

Based on bibliographic research, there is no use of the species in folk medicine [9]. Two scientific studies were carried out on this plant leading to the isolation of active compounds with therapeutic potential such as; 8-methoxyeriodictyol, kaempferol, and mangiferin which had good activities in the inhibition of α -glucosidase and the reduction of protein glycation [10]. In the study of Muneshiva and Naokata, a new flavone (5,4'-Dihydroxy-6,7-methylenedioxyflavone) structure was identified in the butanolic extract of *I. unguicularis* [11]. Another study demonstrated the high antioxidant activity and the strong antifungal capacities of the methanolic extract of the aerial parts of this species [12]. However, according to our literature search there are few studies on the composition of *I. unguicularis* rhizomes and no study on the antioxidant, antibacterial, and anti-Alzheimer activities were reported.

As a consequence, in this study, phytochemical composition of partitioned extracts (AI, CI, BI) derived from 70% ethanol extract of *I. unguicularis* rhizomes, was determined by using a previously developed and validated LC-MS/MS method [13] and untargeted screening of the unknown phytochemicals of the species was accomplished by LC-MS-IT-TOF. Furthermore, *in vitro* antioxidant, antibacterial and anti-Alzheimer properties of the studied extracts were also investigated.

Material and Methods

Plant material

The rhizomes of *Iris unguicularis* Poir. (1 Kg) were collected from the Chettaba forest (latitude 36°20.428'N, longitude 006°41.193'E) at 721 m altitude of Constantine, Algeria then air-dried at room temperature. Specimens were identified by using the key of a renowned botanist professor Gérard de Bélaire, University of Badji Mokhtar Annaba, Faculty of Nature and Life Sciences (Voucher specimen; 006_47).

Preparation of the extracts

The air-dried and powdered rhizomes (250 g) of *Iris unguicularis* Poir. were extracted with ethanol at ambient temperature and the solvent was removed using a rotary evaporator to obtain the dried extract (RI). The ethanolic extract of rhizomes was first dissolved in distilled water and then defatted with petroleum ether. Lastly, the defatted extract (aqueous) was fractionated with dichloromethane, ethyl acetate, and n-butanol (AI, CI, and BI extracts), respectively.

Instrumentation and chemicals used in bioactivity studies

Bioactivity measurements were accomplished on a 96-well microplate reader (Perkin Elmer Multimode Plate Reader EnSpire) at National Center of biotechnology Research. Potassium persulfate ($K_2S_2O_8$), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), distilled water, Ethanol, butylated hydroxyl toluene (BHT), butylated hydroxyl anisole (BHA), 1,1-diphenyl-2-picrylhydrazyl (DPPH), disodium phosphate (Na_2HPO_4), sodium bicarbonate ($NaHCO_3$), sodium (NaOH), acetylthiocholine iodide, S-Butyrylthiocholine iodide, butyrylcholinesterase from horse serum (BChE, EC 3.1.1.8, 7.8 U/mg, Sigma), acetylcholinesterase (AChE, Type-VI-S, EC 3.1.1.7, 827.84 U/mg Sigma), galantamine was obtained from Sigma Chemical Co. (Sigma-Aldrich GmbH, Stern-heim, Germany), DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)), ammonium acetate, cupric chloride, Neocuproine, Iron (III) chloride, Iron (II) chloride, ferrozine, trichloroacetic acid (TCA), ethylenediaminetetraacetic acid (EDTA), potassium ferricyanide $K_3Fe(CN)_6$, phosphate buffer, dimethylsulfoxide (DMSO), NBT (nitroblue tetrazolium), aluminum Nitrate ($Al(NO_3)_3 \cdot 9H_2O$), sodium hydroxide (NaOH), potassium acetate (CH_3COOK), quercetin, folin-ciocalteu (FCR) [Phosphotungstic acid hydrate + Molybdenum hydroxide oxide phosphate], gallic acid, sodium bicarbonate (Na_2CO_3 7.5%), Chloroform, β -carotene, linoleic acid, 3-(2-Pyridyl)-5,6-di(2-furyl)-1,2,4-triazine-5',5''-disulfonic acid disodium salt (Ferrene), polyoxyethylene sorbitan monopalmitate (Tween-40). All other chemicals and solvents were of analytical grade.

Determination of the total phenolic and flavonoids content (TPC, TFC)

Folin Ciocalteu reagent was used to determine the total phenolic contents of the studied extracts [14, 15]. Following equation was used in the total phenolic content calculations and TPC was expressed as gallic acid equivalent (GAE):

$$Y = 0.007 [\text{GAEs}] + 0.116 \quad r^2 = 0.994$$

Total flavonoids content was assessed according to the method of Türkoğlu et al [16] with slight modifications. The calculation of the content was expressed as quercetin equivalent (QE) using the following equation:

$$Y = 0.009 [\text{QEs}] + 0.063 \quad r^2 = 0.997$$

LC-MS/MS (Liquid Chromatography Tandem Mass Spectrometry) Instrument and Chromatographic Conditions

For this analysis the extracts were dissolved in methanol at concentration of 1g/L and filtrated with microfilter (0.2 µm) special for LC-MS/MS analysis into vial of 2 mL of volume. The phytochemical quantification of the extracts was performed by a previously developed and validated LC-MS/MS method [13]. The LC-MS/MS system consisted of Shimadzu model U-HPLC combined with Shimadzu LCMS 8040 model triple quadrupole mass spectrometer. The U-HPLC comprised a gradient pump (LC-30 AD model), a degasser (DGU-20A3R model), a column oven (CTO-10ASvp model) and an autosampler (SIL-30AC model). The chromatographic separation was performed on a RPC18 Inertsil ODS-4 model reversed phase column (100 mm×2,1 mm, 2 µm). The column temperature was kept at 35°C throughout the analysis. The mobile phases consisted of A (water; 10 mM ammonium formate, 0.1% formic acid) and B (acetonitrile; % 0.1 formic acid). The applied gradient profile was optimized as 5-20% B (0-10 min), 20% B (10-22 min), 20-50% B (22-36 min), 95% B (36–40 min), 5% B (40–50 min). The flow rate of the mobile phase and the injection volume were optimized as 0.25 mL/min and 4 µL, respectively. The optimum ESI parameters for the mass spectrometer were determined as; 350°C interface temperature, 250°C DL temperature, 400°C heat block temperature, 3 L/min and 15 L/min were nebulizer and drying gas (N₂) flow rates, respectively.

Liquid chromatography Ion Trap Time of Flight Mass Spectrometry (LC-MS-IT-TOF) conditions

The qualitative identification of phytochemical constituents was carried out using a Shimadzu LC-MS-IT-TOF instrument. The instrument is a combination of ultra-high performance liquid chromatograph (UHPLC) and a high-resolution mass spectrometer (IT-TOF-MS). The U-HPLC system composed of an autosampler (SIL-20AC), a gradient pump, (LC-20AD), a degasser (DGU-20A3), a communication bus module (CBM-20A), and a column oven (CTO-20AC). An Agilent Eclipse XDB model reversed phase chromatography column (150 mm (length), 4.6 mm (Internal diameter), 3.5 µm (particle size)) was used for the separation of analytes. The mobile phase flow rate, the column temperature, and the injection volume were optimized as 35C, 0.35 mL/min, and 4 µL. The mobile phase consisted of A (aqueous 5 mM ammonium formate with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid). The following gradient elution profile was used: 7.5% B (0-5 min), 7.5-95% B (5-45 min), 95% B (7 min), 7.5% B (13 min). The hybrid high resolution mass spectrometer used (IT-TOF-MS) possessed an ESI (electrospray ionization) source

operating in both negative and positive ionization modes. Mass spectrometer (MS) conditions were optimized as follows: positive and negative ionization modes; drying gas (N₂) pressure, 100 kPa; nebulizer gas (N₂) flow rate, 1.5 L/min; heater block temperature, 200° C; CDL (curved desolvation line) temperature, 200 C; electrospray voltage, 4.5 kV; detector voltage, 1.63 kV; mass range, m/z 100–1000 (MS1); IT pressure, 1.8×10^4 Pa; TOF pressure, 1.4×10^4 Pa; ion accumulation time, 30 msec. LC-MS Solution Version 3.4.1 (Shimadzu, Japan) software and Formula Predictor Software were used to analyze the obtained data.

Antibacterial activity

The antibacterial effect was evaluated by disk diffusion method on Mueller-Hinton agar medium [17] for two Gram (+) bacteria; *Staphylococcus aureus* (ATCC 43300) and *Bacillus subtilis* (ATCC 6633) and for five Gram (-) bacteria; *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Proteus vulgaris* (ATCC 29905), *Citrobacter freundii* (ATCC 8090) and *Morganella morganii* (ATCC 25830). The negative control was DMSO and Ox:oxacillin (1 µg/disc), Pi: pipemicide (20 µg/disc), E: erythromycin (15 µg/disc), PRL: piperacillin (100 µg/disc), C: chloramphenicol (30 µg/disc), CD: clindamycin (2 µg/disc), AK: amikacin (30 µg/disc) and CIP: ciprofloxacin (5 µg/disc) discs were used as positive controls.

Antioxidant activity

1. DPPH free radical scavenging activity

DPPH assay was performed according to the method described by Blois [18]. The BHA and BHT were used as positive control. The following equation was used in order to calculate the scavenging activity of DPPH radical:

$$A_{\text{Control}} - A_{\text{Sample}} / A_{\text{Control}} \times 100$$

Where A_{Control} represents the initial DPPH concentration and A_{Sample} represents the absorbance of the remaining DPPH concentration in samples. Results were presented as 50% inhibition concentration in µg/mL (IC₅₀).

2. β-Carotene/linoleic acid bleaching assay

Antioxidant activity was determined by the method described by Marco and Öztürk [19, 20]. BHA and BHT were used as positive control. The solution was prepared as follow: 0.5 mg of β-carotene dissolved in 1mL of chloroform was mixed together with 200 µL of Tween 40, and 25 µL of linolic acid, the chloroform was evaporated and the solution was diluted with 50mL of oxygenated water. The absorbance of β-carotene is between 0.8-0.9 nm, if the value is higher such as 1 or 2 we add the oxygenated water. 160 µL of the β-carotene solution was added to 40 µL of each concentration of the samples (3.125 µg/mL, 6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL, 200 µg/mL in methanol). The absorbance was measured at 470 nm

in the beginning and after every 30 min after incubation of the sample at 45°C (120 min). Results were given as 50% inhibition concentration $\mu\text{m/mL}$ (IC_{50}). Antioxidant activity was calculated by the following formula:

$$\text{AA (\%)} = [1 - (\text{A}_{\text{H}_0} - \text{A}_{\text{H}_t}) / (\text{A}_{\text{C}_0} - \text{A}_{\text{C}_t})] \times 100,$$

where:

AA (%): antioxidant activity

A_{H_0} : The absorbance of β -carotene in the presence of sample at $t=0\text{min}$

A_{C_0} : The absorbance of β -carotene in the presence of negative control at $t=0\text{min}$

A_{H_t} : The absorbance of β -carotene in the presence of sample at $t=120\text{min}$

A_{C_t} : The absorbance of the β -carotene in the presence of the negative control at $t=120\text{min}$.

3. Ferrous ions Chelating activity

The method of Decker and Welch was used to determine ferrous ions chelating activity [21]. Two solutions were prepared: 4 mg (Fe^{+2}) [$0,2 \text{ Mm FeCl}_2, 2\text{H}_2\text{O}$] + 100 mL (H_2O) $\rightarrow$ [S1] and 2,5 mg (0,5 Mm Ferrozinin) + 10 mL (H_2O) $\rightarrow$ [S2]. The concentrations of the extracts were as follow: 40 μL of MeOH were mixed with 40 μL Fe^{+2} (0,2 mM) (S1) and 80 μL Ferene (S2). The absorbance was measured after 10 min at 593 nm. EDTA was used as standard. The results were given as 50% inhibition concentration $\mu\text{m/mL}$ (IC_{50}).

4. Cupric reducing antioxidant capacity (Cuprac assay)

The CUPRAC assay was determined by the method of Apack et al [22]. Three solutions were prepared: 1.927 g of (ACNH_4) + 25 mL (H_2O) $\rightarrow$ [S1]; 0.042625 g of ($\text{CuCl}_2, 2\text{H}_2\text{O}$) + 25 mL (H_2O) $\rightarrow$ [S2]; 0.039 g of (Neocupronin) + 25 mL (MeOH) $\rightarrow$ [S3]. Then, 40 μL of the samples at different concentration (3.125 $\mu\text{g/mL}$, 6.25 $\mu\text{g/mL}$, 12.5 $\mu\text{g/mL}$, 25 $\mu\text{g/mL}$, 50 $\mu\text{g/mL}$, 100 $\mu\text{g/mL}$, 200 $\mu\text{g/mL}$) were mixed with 60 μL of (S1), 50 μL of (S3) and 50 μL of (S2). After one hour the absorbance was measured at 450 nm. This method is based on the follow-up of the decrease in the increased absorbance of the complex Neocuproine (Nc) and copper Cu^{2+} , in fact, in the presence of an antioxidant agent the complex is reduced. Results were given as $\text{A}_{0.50}$ which corresponds to the concentration providing 0.500 absorbance.

Determination of acetyl-and butyrylcholinesterase inhibition activity

The activity was determined by the method of Ellman et al [23]. AChE and BChE were used as enzymes, acetylthiocholine iodide and butyrylthiocholine chloride were used as their substrates. DTNB was used as a coloring agent for measurement of the activity. Galantamine was used as positive standard drug in this assay. Sodium phosphate buffer (150 μL , 100 mM, $\text{pH}=8.0$) was mixed with 10 μL of the extract dissolved in methanol at different concentrations (3.125 $\mu\text{g/mL}$, 6.25 $\mu\text{g/mL}$, 12.5 $\mu\text{g/mL}$, 25 $\mu\text{g/mL}$, 50 $\mu\text{g/mL}$, 100 $\mu\text{g/mL}$, 200 $\mu\text{g/mL}$). 20 μL of AChE ($5.32 \times 10^{-3} \text{ U}$) or BChE ($6.85 \times 10^{-3} \text{ U}$) solutions were added, then incubated at 25°C for 15 min. Thereafter, 10 μL of DTNB (0.5 mM) and 10 μL of acetylthiocholine iodide (0.71 mM) or 10 μL of butyrylthiocholine chloride (0.2 mM) were added and the absorbance was

measured at 412 nm in the beginning and after 5min, 10min and 15min (incubation was done every 5 minutes). Results were given as IC₅₀ value (µg/mL).

Statistical analysis

The results were conducted in triplicate. The data were recorded as mean ± SD. Statistical analyses were performed using package GraphPadPrism7. Significance was determined by ANOVA variance analysis.

Results and Discussion

Total phenolic and flavonoid contents

It's already known that secondary metabolites and especially phenolic acids and flavonoids compounds are responsible for treating various ailments and exert significant pharmacological effects [24]. The TPC and TPF flavonoids of various extracts are given in Table 1 in the Supplementary File. The results for TPC are expressed as gallic acid equivalent, whereas TFC are expressed as quercetin equivalent. Results showed that the crude ethanolic extract (RI) contains a good amount of phenolics and flavonoids (TPC: 71.99 ± 0.01 µgAcG/mg extract, TPF: 43.86 ± 2.63 µgQEs/mg extract). Compared with CI and BI, AI extract had the highest TPC and TPF [213.77 ± 0.22 (µgAcG/mg extract) and 92.59±2.92 (µgQEs/mg extract)] respectively, followed by CI and BI extracts. The good TPC and TFC of *I.unguicularis* were coherent with some research that focused on *Iris ssp.* and revealed their high TPC and TPF [25].

Elucidation of phytochemical profiles by LC-MS-IT-TOF

The fact that the effects of medicinal plants are based on their chemical constituents led to a critical demand for powerful analytical instrumentation techniques performing the chemical analysis [26]. Phytochemical profiles of AI, CI and BI extracts were determined by LC-MS-IT-TOF method (Figure 1) and the results are given in Table 4 in the Supplementary File, with retention times, forms of detected ions, measured and predicted masses *m/z* (with errors) and efficiency (DBE). Only the analyte peaks having intensities higher than 2,500,000 were evaluated. A total of 70 phytochemical constituents were identified in *I. unguicularis* (Figures S5-S70 in the Supplementary File). However, screening of secondary metabolites by LC-MS-IT-TOF is very important and interesting especially for qualitative applications and this is due to its accurate mass measurements and high resolution characteristics, so this analysis is well-suited to this field but also limited and insufficient this is why identification, isolation and NMR studies are necessary.

Figure 1. LC-MS-IT-TOF chromatograms of AI, CI and BI

Quantitative analysis of phenolic and flavonoids by LC-MS/MS

Phytochemical screening of the studied extracts by LC-MS/MS (Figure 2) revealed the presence of 17 phenolic compounds and this is the first time that the examined compounds were identified by this method in *I. unguicularis* (Table 2 in the Supplementary File). Moreover, there is only one study about chemical profiling of this species by HPLC [10]. AI and CI were the most abundant extracts in terms of both flavonoids and phenolic acids. Indeed, the BI extract was quite poor in phenolic compounds, it only contained low amounts of quinic acid, nicotiflorin, and apigetrin (95.1, 58.3, and 6.1 μg analyte/g extract respectively). Additionally, the AI extract was richer than the CI extract in terms of phenolic acids. 4-OH-benzoic, syringic, protocatechuic, and *p*-coumaric acids (2218.2, 1218.6, 1080.4, and 1071.2, respectively) were the most abundant phenolic acids in the AI extract. Besides, the AI extract contained good amounts of caffeic and ferulic acids (272.0 and 348.2 μg analyte/g extract, respectively) and low amounts of quinic and sinapinic acids (43.2 and 99.1 μg analyte/g extract, respectively). On the other hand, syringic, *p*-coumaric, ferulic, and sinapinic acids and vanillin (aldehyde) (518.3, 394.7, 1088.4, and 202.3 μg analyte/g extract, respectively) were the most phenolic acids present in the CI extract. When it comes to flavonoids, the AI extract contained

high amounts of apigenin, kaempferol, and luteolin (425.5, 404.8, and 326.7 μg analyte/g extract, respectively) and low amounts of nicotiflorin, apigetrin, fisetin, quercetin, and naringenin (76.7, 37.3, 143.3, 112.1, and 114.2 μg analyte/g extract, respectively). The CI extract was quite rich in terms of naringenin flavonoid (1453.4 μg analyte/g extract). Moreover, it contained good amounts of quercetin and kaempferol (352.6 and 253.9 μg analyte/g extract, respectively) and medium amount of apigenin (146.2 μg analyte/g extract) and low amounts of fisetin and luteolin (29.2 and 10.1 μg analyte/g extract, respectively). Referring to the literature, compounds: quercetin, luteolin, naringenin, apigenin, and kaempferol identified in this study are parallel to those identified in other species of *Iris* genus [27]. However, quinic acide, 4-OH-benzoic acid, sinapinic acid, nicotiflorin and and apigetrin were reported for the first time in this genus.

Figure 2. LC-MS/MS TIC chromatograms of AI, BI and CI extracts

Antioxidant activity

Many studies explored and reported the advantages of phenolic compounds mostly for their antioxidant activity. In the current study, DPPH, β -carotene, CUPRAC, and Metal-chelate assays were

evaluated and represented in Table 1 in the Supplementary File. As shown, RI extract has a positive results for all methods using, but the strong activity was recorded for β -carotene assay ($IC_{50} = 1.03 \pm 0.35 \mu\text{g/mL}$) which is close to the IC_{50} of the BHA [$IC_{50}=1.05 \pm 0.01 \mu\text{g/}$]. Nevertheless, AI extract displayed the highest activity against metal-chelate ($IC_{50}=4.52 \pm 3.47 \mu\text{g/mL}$ compared with EDTA [$IC_{50}=3.47 \pm 0.35 \mu\text{g/}$] used as positive control), DPPH ($IC_{50}=6.12 \pm 2.42 \mu\text{g/mL}$ compared with BHT [$IC_{50}=22.32 \pm 1.19 \mu\text{g/mL}$] and BHA [$IC_{50}=5.73 \pm 0.41 \mu\text{g/mL}$] used as positive controls) and finally against CUPRAC ($IC_{50}=8.05 \pm 0.10 \mu\text{g/mL}$ compared with BHT [$IC_{50}=9.62 \pm 0.87 \mu\text{g/mL}$] and BHA [$IC_{50}=3.64 \pm 0.19 \mu\text{g/mL}$]. A good antioxidant activity was also recorded for CI extract against β -carotene and Metal-chelate assays with an IC_{50} value of $4.64 \pm 3.44 \mu\text{g/mL}$ which is better than the IC_{50} value of the Ascorbic acid ($IC_{50} = 9.01 \pm 1.46 \mu\text{g/mL}$) for the β -carotene assay and an IC_{50} value of $2.28 \pm 0.36 \mu\text{g/mL}$ for Metal-chelate assays compared with EDTA [$IC_{50}=3.47 \pm 0.35 \mu\text{g/}$]. Results showed that high amount of TPC and TPF in extract are coherent with the good activity [28]. However, antioxidant properties were previously attributed to apigenin, quercetin, luteolin and naringenin and could explain the good antioxidant activity [29, 30].

Antibacterial activity

The *in vitro* antibacterial potential was evaluated in terms of inhibition zone of bacterial growth depending on strains sensibility and fractions concentrations. AI extract exhibited a notable antibacterial activity against *S. aureus* with inhibition diameters ranging from 11mm to 23mm in concentrations of 25, 50, and 100 $\mu\text{g/mL}$, followed by *B. subtilis* with inhibition diameters ranging from 8mm to 13mm in concentrations of 25, 50, and 100 $\mu\text{g/mL}$ and finally *M. morgani* with inhibition diameter of 10 ± 0.86 in concentration of 100 $\mu\text{g/mL}$. CI extract had also a good antibacterial activity against *B. subtilis* with inhibition diameters ranging from 8mm to 19mm in concentrations of 6.25, 12.5, 25, 50, and 100 $\mu\text{g/mL}$ and against *S. aureus* with inhibition diameters of 10 mm, 11 mm, and 12 mm in concentrations of 25, 50, and 100 $\mu\text{g/mL}$ respectively (Table 3 in the Supplementary File). In addition, a study reported that *S. aureus* and *B. subtilis* were susceptible against quercetin [31] while another one reported that *P. aeruginosa* and *P. vulgaris* were susceptible against naringenin [32]. Apigenin is considered as a very effective bacteriostatic and bactericidal compound [33].

Anticholinesterase activity

Samples were assessed at 12.5 to 200 $\mu\text{g/mL}$ towards both AChE and BChE enzymes. Samples with an $IC_{50} > 200 \mu\text{g/mL}$ were considered as inactive. All extracts of *L. unguicularis* Poir. were inactive at all concentration against acetyl- and butyrylcholinesterase enzymes (Table 1 in the Supplementary File). Indeed, the BI extract was quite poor in phenolic compounds. It only contained low amounts of quinic acid, nicotiflorin, and apigetrin which may explain the absence of activity. For AI and CI extracts, the absence of activity could be explained by the effect of antagonism between the active compounds which causes the

partial or complete inhibition of the action of one of the active compounds. However, a study that aimed to compare the anticholinesterase activities of selected phenolic acids singly, in pairs or combined with more than two phenolic acids and in combination with flavonoids in order to study interactions between these compounds, had demonstrated that phenolic compounds were effective cholinesterase inhibitors singly but combined phenolic acids (with phenolic acids or flavonoids) were less efficient inhibitors or inactive [34]. This is why for further studies it will be important to do purification of these different compounds and test them singly for this activity in order to confirm this hypothesis.

Conclusions

This is the first report on the antioxidant, antibacterial, and anti-Alzheimer activities of *Iris unguicularis* rhizomes extracts with phytochemical study by using LC-MS/MS and LC-MS-IT-TOF methods. From the phenolic profile of AI and BI extracts, seven phenolic acids and ten flavonoids were identified by LC-MS/MS and these compounds are known for their several biological activities, especially that many studies demonstrated that phenolic acids and flavonoids are pharmacologically active as antioxidant and bactericidal agents [35, 36], AI extract was the richest one in phenolic acids and flavonoids with good antioxidant and antibacterial activities and may have potential industrial applications. Also, 70 compounds were identified by LC-MS-IT-TOF with their exact masses and molecular formulas. These preliminary data are promising and this is why in perspective it would be important to deepen this research by the isolation of the secondary metabolites to find the molecule or molecules responsible for each activity.

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