SUPPLEMENTARY MATERIAL

**Inula viscosa** (L.) Aiton leaves and flower buds: Effect of extraction solvent/technique on their antioxidant ability, antimicrobial properties and phenolic profile

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Abstract

This study was designed to establish the most effective solvent/technique for extracting antioxidant phytoconstituents from leaves and flower buds of *Inula viscosa* (L.) Aiton (Asteraceae) grown wild in Morocco. Maceration and hot extraction with methanol or water and Soxhlet ethanol extraction were utilized. The antioxidant potential was evaluated *in vitro* by DPPH, reducing power, and ferrous ions chelating activity assays. *I. viscosa* leaf and flower bud extracts displayed the strongest effect in the DPPH test, being the Soxhlet ethanol the most active ones (IC\(_{50}\) = 54.24 ± 0.21 µg/mL and 39.77 ± 0.23 µg/mL); thus, they were selected for further investigations. The antimicrobial efficacy of the Soxhlet ethanol extracts against ATCC and food isolates strains was assayed; the leaf extract showed the best activity, and *Candida albicans* was the most sensitive strain (MIC = 125 µg/mL). The extracts resulted non-toxic against *Artemia salina*. Among the phenolics characterised by HPLC-PDA-ESI-MS, hispidulin hexoside, patuletin and spinacetin were identified for the first time.

**Keywords:** *Inula viscosa* (L.) Aiton; extraction solvent/technique; antioxidant activity; antimicrobial activity; *Artemia salina* Leach; phenolic compounds; HPLC-PDA-ESI-MS.
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Experimental section

**Chemicals**

FeCl$_2$ was obtained from Carlo Erba (Milan, Italy). Müeller Hinton Broth (MHB), RPMI-1640, and 3-(N-morpholino)propanesulfonic acid (MOPS) were supplied from Oxoid (Basingstoke, UK). LC-MS grade water (H$_2$O), acetonitrile (ACN), gallic acid, caffeic acid, p-coumaric acid, naringin, apigenin, luteolin, rutin, kaempferol and quercetin were obtained from Merck Life Science (Darmstadt, Germany). Unless indicated otherwise, all chemicals were purchased from Sigma-Aldrich (Milan, Italy).

**Plant material and extraction**

*Inula viscosa* (L.) Aiton was collected in Ait Ouikhalfen, near El Hajeb (Morocco). The plant was identified by Prof. Jalal el Oualidi, Institute of Scientific Research, University Mohammed V, Rabat, Morocco. A voucher specimen was deposited in the herbarium of the Institute of Scientific Research, Rabat, Morocco, under accession number n° RAB110966.

*I. viscosa* air dried and powdered leaves and flower buds (10 g) were extracted by different methods and solvents: maceration using 100 ml of methanol or distilled water under stirring for 24h (mac-H$_2$O and mac-MeOH extracts); hot extraction with 100 mL of methanol (70°C) or distilled water (100°C) for 2h (hot-H$_2$O and hot-MeOH extracts); Soxhlet extraction using 150 ml of ethanol (Sox-EtOH extract). After filtration, the extractive solutions were evaporated to dryness by rotary evaporator (45°C). The extracts yields, referred to 100 g of dried plant material, are given in Table S1.

**Antioxidant activity**

*Free radical scavenging activity*
The free radical scavenging activity of *I. viscosa* extracts was evaluated using the DPPH (1,1-diphenyl-2-picrylhydrazyl) test (Miceli et al. 2017). An aliquot (0.5 mL) of solution containing different amounts of the extracts (7.81-1000 μg/mL) was added to 3 mL of daily prepared methanol DPPH solution (0.1 mM). The optical density change at 517 nm was measured, 20 min after the initial mixing, with a model UV-1601 spectrophotometer (Shimadzu). Butylated hydroxytoluene (BHT) was used as reference standard. The scavenging activity was measured as the decrease in absorbance of the samples versus the DPPH standard solution. The results were obtained from the average of three independent experiments, and are reported as mean radical scavenging activity percentage (%) ± standard deviation (SD) and mean 50% inhibitory concentration (IC\(_{50}\)) ± SD.

**Measurement of Reducing Power**

The reducing power of *I. viscosa* extracts was evaluated by spectrophotometric detection of Fe\(^{3+}\)-Fe\(^{2+}\) transformation method (Miceli et al. 2017). Different amounts of the extracts (7.81-1000 μg/mL) in 1 mL solvent were mixed with 2.5 mL of phosphate buffer (0.2M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide \([K_3Fe(CN)_6]\). The mixture was incubated at 50°C for 20 min. The resulting solution was cooled rapidly, mixed with 2.5 mL of 10% trichloroacetic acid, and centrifuged at 1570 g for 10 min. The resulting supernatant (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% fresh ferric chloride (FeCl\(_3\)), and the absorbance was measured at 700 nm after 10 min. Ascorbic acid and BHT were used as reference; the results were obtained from the average of three independent experiments and are expressed as mean absorbance values ± SD and ascorbic acid equivalent (ASE/mL) ± SD.

*Ferrous ions (Fe\(^{2+}\)) chelating activity*
The Fe$^{2+}$ chelating activity of *I. viscosa* extracts was estimated by measuring the formation of the Fe$^{2+}$-ferrozine complex (Miceli et al. 2017). Different concentrations of the extracts (7.81-1000 μg/mL) in 1 mL solvent were mixed with 0.5 mL of methanol and 0.05 mL of 2 mM FeCl$_2$. The reaction was initiated by the addition of 0.1 mL of 5 mM ferrozine. Then, the mixture was shaken vigorously and left standing at room temperature for 10 min. The absorbance of the solution was measured spectrophotometrically at 562 nm. The control contains FeCl$_2$ and ferrozine, complex formation molecules. Ethylenediaminetetraacetic acid (EDTA) was used as reference standard. The results were obtained from the average of three independent experiments and are reported as mean inhibition of the Fe$^{2+}$-ferrozine complex formation (%) ± SD and IC$_{50}$ ± SD.

**Antimicrobial activity**

The following strains, obtained from the in-house culture collection of the Dipartimento di Scienze Chimiche, Biologiche, Farmaceutiche ed Ambientali, University of Messina (Italy), were used for the antibacterial testing: *Staphylococcus aureus* ATTC 43300, *S. aureus* ATTC 6538, *Escherichia coli* ATCC 25922, and *Candida albicans* ATCC 10231. Besides, *Escherichia coli* S4/15, *Klebsiella pneumoniae* S20/16, *Enterobacter cloacae* S16b/16, and *Salmonella* spp. S13b/16 isolated from healthy broiler chickens at the Nursing Department, Faculty of Natural Sciences and Life, Abdelhamid Ibn Badis University (Mostaganem, Algeria) were included in the experimental protocol.

Bacterial and yeast cultures for antimicrobial activity tests were grown in Mueller-Hinton Broth (MHB) at 37°C (24 h) and RPMI-1640 medium supplemented with 3-(N-morpholino)propanesulfonic acid (MOPS) at 30°C (48 h), respectively. The Minimum Inhibitory Concentration (MIC) of *I. viscosa* leaves and flower buds Sox-EtOH extracts were determined according to the standard methods (CLSI 2012; CLSI 2008). Working cultures of
bacteria and yeast were adjusted to the required concentration of $10^5$ CFU/mL and $10^3$ CFU/mL, respectively. The final concentrations of the extracts adopted were 0.49 to 500 µg/mL. Growth controls were included. Microbial growth was determined by visual readings and spectrophotometric values (550 nm) using the Microplate Reader, Model 550 (BIO-RAD Laboratories Milano, Italy). The MIC was defined as the lowest drug concentration at which there was complete inhibition of growth.

**Artemia salina lethality bioassay**

In order to investigate the potential toxicity of *I. viscosa* leaf and flower bud Sox-EtOH extracts median lethal concentration (LC$_{50}$) was determined (Taviano et al. 2018b). The extracts, opportune*ly dissolved and then diluted in artificial seawater, were tested at the final concentrations of 10, 100, 500 and 1000 µg/mL. Ten brine shrimp larvae were transferred to each sample vial, and artificial seawater was added to obtain a final volume of 5 mL. After 24 h of incubation (25-28°C), the surviving larvae were counted. The assay was carried out in triplicate, and LC$_{50}$ values were determined by the Litchfield and Wilcoxon method. Extracts are considered non-toxic if the LC$_{50}$ is higher than 1000 µg/mL.

**Characterisation of phenolic compounds by HPLC-PDA-ESI-MS analysis**

A quali-quantitative investigation of phenolic compounds contained in *I. viscosa* leaf and flower bud Sox-EtOH extracts has been carried out by HPLC-PDA-ESI-MS. The analyses were carried out using a Shimadzu HPLC system (Milan, Italy) equipped with a CBM-20A controller, LC-20AD pumps, a DGU-20A$_3$ degasser, a SIL-20AC autosampler, a SPD-M20A photo diode array detector (PDA) and a quadrupolar mass analyzer (LCMS-2020, Shimadzu), equipped with an ESI interface, in negative ionization mode. Data acquisition was performed by Shimadzu LabSolution software ver. 5.10.153. *I. viscosa* extracts (20 mg) were dissolved
in EtOH (1 mL). Analyses were carried out on an Ascentis Express C18, 15 cm x 4.6 mm i.d. with particle size of 2.7 μm (Supelco, Bellefonte, PA). The injection volume was 2 μL, mobile phase consisted of water/formic acid (99.9:0.1) (solvent A) and ACN/formic acid (99.9:0.1) (solvent B), the linear gradient profile was as follows: 0 min, 0% B, 5 min, 5% B, 15 min, 10% B, 30 min, 20% B, 60 min, 50% B, 70 min, 100% B, 71 min, 0% B. The flow-rate was 1 mL/min and it was split to 0.2 mL/min prior to MS detection. PDA wavelength range was 200-400 nm and the chromatograms were extracted at 280 nm. Time constant was 25 ms and sample frequency 40 Hz. The MS acquisition was performed using ESI, in negative mode, under the following conditions: mass spectral range 100-800 m/z; interval: 0.5 sec; nebulizing gas (N₂) flow: 1.5 L/min; interface temperature: 350°C Heat block: 300°C, DL temperature: 300°C; DL voltage -34 V; probe voltage 4.5 kV; Qarray voltage: 1.0 V, RF voltage: 90 V; detection gain 1.0 kV.

Quantitative determination was carried using calibration curves of nine standards, namely gallic acid, caffeic acid, p-coumaric acid, naringin, apigenin, luteolin, rutin, kaempferol and quercetin. Standard calibration curves were prepared in a concentration range 0.1-100 mg/L with five different concentration levels. Triplicate injections were made for each level, and a linear regression was generated. The calibration curves with the external standards were obtained using concentration (mg/L) with respect to the area obtained from the integration of the PDA peaks at a wavelength of 270 for benzoic acid-like compounds, 315 nm and 325 nm for cinnamic acid-like compounds, 335 and 351 for flavones-like compounds, 355 nm for flavanone like compounds, 365 and 370 for flavonol-like compounds. The amount of each compound was finally expressed in mg/g of dried extract ± percent relative standard deviation (%RSD).

References
Clinical and Laboratory Standards Institute (CLSI) 2008. Reference method for broth dilution antifungal susceptibility testing of Yeasts, 3rd ed. Approved Standard M27-A3. Wayne (PA): Clinical and Laboratory Standards Institute.

Clinical and Laboratory Standards Institute (CLSI) 2012. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 9th ed. Approved Standard M07-A9. Wayne (PA): Clinical and Laboratory Standards Institute.
Table. S1. Percentage yields of *Inula viscosa* leaf and flower bud extracts.

| *I. viscosa* extracts | Yields (w/w %) |
|-----------------------|----------------|
|                       | Leaves | Flower buds |
| mac-MeOH              | 14.9    | 10.7        |
| mac-H₂O               | 15.1    | 12.8        |
| hot-MeOH              | 17.5    | 12.5        |
| hot-H₂O               | 15.7    | 13.5        |
| Sox-EtOH              | 9.0     | 7.6         |

The yields are referred to 100 g of dried plant material.
**Figure S1.** Free radical scavenging activity of *Inula viscosa* leaf (A) and flower bud (B) extracts, measured by the DPPH method. The results were obtained from the average of three independent experiments and are expressed as the mean percentage (%) ± SD.
Figure S2. Reducing power of *Inula viscosa* leaf (A) and flower bud (B) extracts, evaluated by spectrophotometric detection of Fe$^{3+}$-Fe$^{2+}$ transformation method. The results were obtained from the average of three independent experiments and are expressed as the mean absorbance ± SD.
Figure S3. HPLC-PDA-ESI-MS (negative ionization mode) phenolic fingerprint of *Inula viscosa* leaf (A) and flower bud (B) Sox-EtOH extracts. Column: C$_{18}$, 15 cm x 4.6 mm, 2.7 µm particles (Ascentis® Express). For peak identification, see Table 2.