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Original Article

Comparative Study of the Antioxidant, Antimicrobial and Anti-Inflammatory Activity between Essential Oil and Hydrosol Extract of the Aerial Parts of *Inula viscosa* L

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Abstract

Introduction: The main objective of this study was to determine the chemical composition of the aerial part of the essential oil and the hydrosol extract of *Inula viscosa* which has never been studied and to compare their antioxidant, anti-inflammatory, and antimicrobial properties *in vitro*. This was done in order to identify new biologically active agents.

Materials and Methods: The essential oil and hydrosol extract were analyzed by GC and GC/MS. Antimicrobial activity was tested against three bacteria and two fungi. The antioxidant activities were assessed using three different methods: radical scavenging activity (DPPH), the β-carotene bleaching test, and Ferric-Reducing Antioxidant Power (FRAP). The anti-inflammatory activity was assessed using the protein denaturation method.

Results: The essential oil of *Inula viscosa* was composed mainly of hydrocarbon sesquiterpenes (52.6%) and oxygenated sesquiterpenes (47.0%), while the hydrosol extract was mainly composed of oxygenated sesquiterpenes (86.6%). The results of the biological activities showed that the hydrosol extract exhibited an interesting antioxidant activity, nearly equivalent to the synthetic antioxidant BHT. Furthermore, the hydrosol extract displayed very good anti-inflammatory activity, with an IC_{50} of 0.51 g/L, in comparison to diclofenac sodium (IC_{50} = 0.63 g/L). The hydrosol extract also exhibited antimicrobial activity and acted as an effective inhibitor of *Bacillus cereus*, *Pseudomonas aeruginosa*, *Candida ATCC* 26960, and *Candida ATCC* 10231 microorganisms.

Conclusions: The hydrosol extract of *I. viscosa* make this specie a potential alternative natural for use in the food and pharmaceutical industries. It can be utilized in the treatment of diseases involving oxidative stress as well as in the treatment of microbial and inflammatory infections.

Keywords: Hydrosol Extract, Essential Oil, Anti-inflammatory Activity, Antioxidant Activity, Antimicrobial Activity, Natural Products

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Introduction

Antibiotics are medications used to treat infections caused by bacteria. Bacteria can develop resistance to these drugs. Resistant bacteria can lead to infections in humans that are more challenging to treat than those caused by non-resistant bacteria. Reactions of intolerance to nonsteroidal anti-inflammatory drugs (NSAIDs) may manifest during childhood, even in patients who previously tolerated NSAIDs without any issues.[1] On one hand, there are respiratory symptoms infrequently associated with digestive symptoms, which often occur after taking NSAIDs. On the other hand, there are cutaneous manifestations.[2] Furthermore, inflammation leads to the excessive production of free radicals, which are responsible for generating oxidative stress and the release of inflammatory factors that contribute to complications.[3] In an effort to discover new solutions for antibiotic and anti-inflammatory resistance, scientists have recently explored the components of essential oils and their volatile compounds. These compounds have exhibited intriguing properties, such as sesquiterpenes, which have demonstrated remarkable pharmacological activities against diseases like cancer, inflammation, and bacterial infections.[4,5] In general, the by-product formed during the extraction of essential oils, known as hydrosol, is typically considered waste and contains quantities of more polar molecules that are absent in the essential oil. Some plant hydrolats have exhibited fascinating therapeutic properties, often surpassing those of

the corresponding essential oil.[6,7] The chemical composition and biological activities of certain hydrosols have been studied. The research conducted on hydrosol extracts to date suggests that these extracts may find applications in the food industry for developing new antimicrobials, antioxidants, and anti-inflammatories. They also hold promise for addressing the issue of resistance.[8-11] Based on this data, we became interested in exploring the biological potential of the volatile compounds from the *I. viscosa* species, as the hydrosol extract had not been investigated in this field. Consequently, the present study was conducted to ascertain the chemical composition of the essential oil and hydrosol extract of *I. viscosa* and to assess their antioxidant, antimicrobial, and anti-inflammatory activities. The goal was to discover new biologically active agents.

Materials and Methods

Plant Materials and Extraction of Essential Oil and Hydrosol

The aerial parts of *I. viscosa* were collected in May 2017 from the Tlemcen forest in Algeria, during the flowering stage. The plant material was identified by Professor Nouri Benabadji, Department of Biology, University of Tlemcen, Algeria and deposited in the institutional herbarium. The essential oil was obtained from the fresh aerial parts (500 g) by hydrodistillation for 4-5 h using a Clevenger type apparatus.

For the isolation of the hydrosol, the first liter of water from hydrodistillation was recovered in order to obtain the corresponding hydrosols. The hydrosol was extracted three times, each time with 200 ml of diethyl ether at room temperature. The organic layer was evaporated and dried on sodium sulfate, so giving a yellowish oil. These extractions were performed in triplicate.

Analysis Conditions

Gas Chromatography (GC)

The gas chromatography analyses were performed using a Perkin Elmer Clarus Autosystem XL GC apparatus equipped with a double flame ionization, and two capillary columns made of silica (60 mx 0.22 mm ID, 0.25 µm stationary phase film thickness), Rtx-1 (polydimethylsiloxane) and Rtx-Wax (polyethylene glycol). The oven temperature was programmed for an increase from 60 °C to 230 °C at 2 °C/min and then kept isothermal for 35 min at 230 °C. For the injector and detector, the temperatures were maintained at 280 °C. The pressure at the top of the column is 25 psi with a flow rate of 1 ml/min. the sample was injected with a volume of 0.1 µl. using hydrogen as carrier gas (0.7 ml/min). Retention indices (RI) of the compounds were determined relative to the retention times of the series of n-alkanes (C_5 to C_{30}) with linear interpolation, using the Van den Dool and Kratz (2003) equation and software from Perkin–Elmer.[10]

Gas Chromatography-Mass Spectrometry (GC-MS)

The essential oil and hydrosol extract were analyzed by gas chromatography-mass spectrometry using a Perkin Elmer Turbo Mass detector (quadrupole) coupled to a Perkin Elmer Autosystem XL equipped with an automatic injector and two columns (60 mx 0.22 mm D.I., film thickness of stationary phase 0.25 µm), polar (Rtx-Wax) and non-polar (Rtx-1). The ionization energy was 70ev, the temperature of the ion source was 150 °C, and the mass range with which mass spectra were acquired was 35-350 Da. The injection of the sample was done by a divided mode with a division ratio of 1/80.[10]

Component Identification and Quantification

The components were identified by comparing the retention indices (RI) calculated on both polar and nonpolar columns with those of standard compounds found in the laboratory library "Aroma" or those reported in the literature and matching the mass spectra (electron impact) with those available in the laboratory's internal library.[11,12] The quantification of the constituents was achieved using a flame ionization detector through internal standardization. This involved using the response factor calculated in relation to tridecane (0.7 g/100 g), which was used as an internal standard.[13,14].

Antioxidant Activity

DPPH Free Radical Scavenging Assay

The antioxidant activity of samples was measured in terms of hydrogen donation or radical scavenging capacity using the DPPH (2,2-Diphenyl Picryl-Hydrazyl) method, which involves stable radicals.[11] In this assay 100 µl of various concentrations (1 to 40 g/L) of each extract were combined with 25 µl of a freshly prepared methanolic solution of DPPH (0.5 mM). After incubating the mixture in the dark for 30 min at room temperature, the absorbance was measured using a spectrophotometer at 517 nm. The IC_{50}s values were calculated graphically using a linear regression formula, relating the inhibition percentages to different concentrations of the sample being tested.

Iron Reduction Test: Ferric Reducing Antioxidant Power (FRAP)

The method used for the samples was that described by Oyaizu et al.[16] with some modifications. Specifically, 1 ml of the extract at different concentrations (from 1 to 5 g/L) was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of a 1% potassium ferricyanide $K_3Fe(CN)_6$ solution. The resulting mixture was then centrifuged at 3000 rpm for 10 min. Subsequently, 2.5 ml of the supernatant from each concentration was combined with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (0.1%). After 30 min of incubation, the absorbances were measured at 700 nm using a double-beam visible spectrophotometer, with ethanol

(80%) serving as the blank. BHT was utilized as a positive control. These analyses were conducted in triplicate.

β-carotene bleaching test

The investigation of the samples' ability to inhibit the bleaching of β-carotene was conducted following the procedure outlined by Bougatef et al.[17] To initiate this, a solution was prepared, containing 0.5 mg of β-carotene in 1 ml of chloroform, which was then combined with 25 µl of linoleic acid and 200 mg of Tween 40. The chloroform was evaporated under reduced pressure at 45 °C using a rotary evaporator. Subsequently, 100 ml of distilled water saturated with oxygen were added, and the resulting mixture was vigorously stirred. The reaction medium contains 2.5 ml of the *β*-carotene/linoleic acid emulsion and 0.5 ml of the solutions of extracts or of the antioxidant reference (BHT). The mixture was placed in a water bath and incubated at 50 °C for 2 h. The oxidation of the previous emulsion was monitored by measuring the absorbance of each sample immediately after its preparation (t = 0 min) and at 15 min intervals until the end of the experiment (t = 120 min) at 470 nm. The negative control was composed of 0.5 ml of distilled water instead of extracts and the standard antioxidant. IC_{50}s were calculated graphically by linear regression of plotted graphs; the percentages of inhibition according to the different concentrations of the extracts tested. The tests were conducted in triplicate.

Anti-inflammatory Activity

The ability of essential oil and hydrosol extract capability to inhibit the thermal denaturation of proteins was carried out in accordance with the method described by Sangita et almethod.[18] Diclofenac sodium, an anti-inflammatory drug was used as a reference. The reaction mixture was composed of 0.2 ml of egg albumin, 2.8 ml of phosphate-buffered saline solution (PBS, pH 6.4), and 2 ml of the different samples at varying concentrations (0.2 to 1.2 g/L). Distilled water was used as a negative control. Then the mixtures were incubated at 37 °C for 15 min. The mixture was incubated in a water bath at 70 °C for 45 min. The optical densities were measured at 660 nm using a UV/visible spectrophotometer every 15 min.

Antimicrobial Assays

Microorganisms and Culture Conditions

The essential oil and hydrosol extract of *I. viscosa* were tested against three microorganisms, *Staphylococcus aureus*, *Bacillus cereus*, and *Pseudomonas aeruginosa*, and two fungal microorganisms, *Candida* ATCC 26960 and *Candida* ATCC 10231. The bacteria were cultivated in brain–heart infusion broth (BHI) at 37 ± 1 °C. *Candida albicans* was cultivated in Sabouraud dextrose agar (DAS) at 27 ± 1 °C. To prepare the inoculum, colonies were directly inoculated into 1 ml of sterile saline solution and adjusted to the 0.5 standard of the McFarland scale, corresponding to 1.5×10^8 CFU/ml for the bacteria and 2.5×10^6 CFU/ml for fungal strains.[19]

Agar Disk Diffusion Test

The standard agar disk diffusion method[20] was used to evaluate the inhibitory spectrum of the essential oil against the micro-organisms. In this method, the bacterial inoculum was spread on Müller–Hinton agar solidified in Petri dishes, in such a way as to produce uniform growth throughout the dish. After preparing the dishes, 6-mm-diameter filter paper discs, each containing 10 µl of undiluted essential oil, were gently pressed against the surface of the agar. Following a 30-min incubation at room temperature, the dishes were placed in a bacteriological oven at 37 ± 1 °C for 24 h, except for the *C. albicans* cultures, which were incubated for 48 hours at 27 ± 1 °C using DAS as the substrate. At the conclusion of the testing period, the diameter of the inhibition zone formed over the agar culture was measured in millimeters. All tests were performed in triplicate and the inhibition zones in the experimental dishes were compared to those in the control dishes.

Statistical Analysis

All experiments were performed in triplicate (n = 3). Data were expressed as means ± standard deviation (SD). Statistically significant differences between experimental groups were considered when $p<0.05$. The concentration that achieved 50% inhibition (IC_{50}) was calculated by nonlinear regression with the use of Microsoft Office Excel.

Results

Chemical Composition of Essential Oil and Hydrosol Extract

Hydrodistillation of the aerial parts of *I. viscosa* yielded 0.15% essential oil on a dry weight basis (See Supplementary Materials Table S1). The analysis of *I. viscosa* essential oil by GC-MS allowed the identification of 20 compounds, constituting 99.4% of the total essential oil. The compounds were identified by comparing their mass spectra (EI-MS) and retention indices (RI) with those in the mass spectral library. There were 11 hydrocarbon sesquiterpenes and 9 oxygenated sesquiterpenes. The essential oil studied consists only of oxygenated monoterpenes (52.6%) and oxygenated sesquiterpene compounds (47.0%). The main compounds that make up this

Table 1. Antioxidant Activity of Essential Oil and Hydrosol Extract by DPPH and *β*-carotene Bleaching Assays

Samples	DPPH IC_{50} (g/L)	*β*-Carotene IC_{50} (g/L)
BHT	16.4	13.2
Essential oil	31.4	20.5
Hydrosol extract	16.5	12.5

oil were alloaromadendrene (9.2%), α-bisabolol (8.9%), α-cadinol (8.6%), zingibrene (8.2%), germacrene-D (7.1%), bicyclogermacrene (6.6%), ʈ-muurolol (6.5%) β-copaene (6.1%) and (E)-β-farnesene (4.2%). The chemical composition of the hydrosol extract of *I. viscosa* was characterized only by oxygenated compounds, particularly the oxygenated sesquiterpenes (86.6%), followed by non-terpene compounds (9.9%). The oxygenated sesquiterpenes were mainly composed of caryophyllene oxide (19.3%), α-cadinol (8.5%), (E)-nerolidol (8.2%), Z-E-farnesylacetate (7.8%), α-bisabolol (6.9%), β-acoradienol (6.5%), zingiberenol (6.3%) and ledol (5.3%). The non-terpene compounds were characterized by two components, the Z-hex-3-en-1-ol (4.3%) and nonal (5.6%), while, the oxygenated monoterpenes were composed by citronellol (1.3%) only (Table S1).

Evaluation of the Antioxidant Activity of I. viscosa Essential Oil

The antioxidant properties were evaluated using three different methods i) Radical scavenging activity, reducing power and the *β*-carotene bleaching method, using BHT as positive control. Results were reported as the average of three replicates. The half maximal inhibitory concentration (IC_{50}) of the samples were shown in Table 1. The results of the study on the free radical scavenging activity $DPPH^{\bullet}$ by the essential oil and hydrosol extract of *I. viscosa* compared to that of the synthetic compound BHT are summarized in Table 1. The essential oil of *I. viscosa* exhibited the lowest free radical scavenging activity, with an IC_{50} = 31.4 ml/L. In contrast, the hydrosol extract an IC_{50} of 16.5 ml/L, nearly equivalent to the BHT control.

The result obtained with the β-carotene bleaching test showed that the hydrosol extract (IC_{50} = 12.5 g/L) indicated the strongest activity, higher than the synthetic antioxidant BHT (IC_{50} = 13.2 g/L). The essential oil also displayed notable antioxidant activity with an IC_{50} of 20.5 g/L which is approximately 1.5 times weaker than the synthetic antioxidant used as reference. Table 2 shows that the reducing power of the essential oil and hydrosol extract increased with higher concentrations. However, the best antioxidant activity was observed for the hydrosol extract with the absorbance values greater than 2.3 at the concentration of 4 g/L (Table 2).

Anti-Inflammatory Activity of Essential Oil and Hydrosol Extract

The *in vitro* anti-inflammatory activity of essential oil, hydrosol extract and diclofenac sodium was done using the protein denaturation method. Table 3 shows the variation in the percentage of protection against thermal denaturation of albumin at different concentrations (0.2 to 1.2 g/L). These results were compared to those obtained with diclofenac, which served as the reference drug in this test. The hydrosol extract of *I. viscosa* displayed protection percentages ranging from 44.1% to 98.3% at concentrations ranging from 0.2 to 1.20 g/L, surpassing diclofenac (12.4% to 86.3%) at the same concentrations, respectively. Meanwhile, the essential oil exhibited protection percentages ranging from 33.4% to 84.1% at the same concentrations (Table 3). The IC_{50} value of the hydrosol extract (IC_{50} = 0.51 g/L) was higher compared to the essential oil (IC_{50} = 0.61 g/ml) and diclofenac (IC_{50} = 0.63 g/L) (Figure 1).

Table 2. Reducing-power Activities of *I. viscosa* Essential Oil and Hydrosol Extract by FRAP Assay

Concentrations (g/L)		1	2	3	4
Absorbances	BHT	2.469	2.495	2.613	2.698
	Essential oil	1.088	1.216	1.376	1.536
	Hydrosol extract	1.863	19.68	2.156	2.356

Table 3. Percentages of Inhibition of Protein Denaturation of *I. viscosa* and Hydrosol Extract

Concentrations (g/L)	Percentage of inhibition (%)		
	Diclofenac Sodium (%)	Essential oil	Hydrosol extract
0.2	12.4 ± 0.1	33.4 ± 0.2	44.1 ± 0.4
0.4	32.4 ± 0.2	42.4 ± 0.2	54.1 ± 0.6
0.6	56.5 ± 0.8	58.3 ± 0.4	66.1 ± 0.8
0.8	72.3 ± 0.1	76.4 ± 0.8	88.2 ± 1.2
1.0	78.3 ± 0.1	76.8 ± 0.6	94.7 ± 0.8
1.2	86.3 ± 0.1	84.1 ± 0.9	98.3 ± 1.1

Samples and positive control were done in triplicates (n=3). SD= standard deviation

Antimicrobial Activity

The *in vitro* antimicrobial activity of the essential oil and hydrosol extract from *I. viscosa* species was assessed against three (*B. cereus, S. aureus,* and *P. aeruginosa*) and two fungus (*Candida* ATCC 26960 and *Candida* ATCC 10231) using the filter paper disc agar-diffusion technique. The results showed variation in the antimicrobial properties of essential oil and hydrosol extract (Table 4). The assessment of antimicrobial activity demonstrated that both the hydrosol extract and essential oil exhibited significant activity against *P. aeruginosa* (36.3 and 31.5 mm respectively) and *B. cereus* (28.4 and 25.4 mm, respectively) activity, while the lowest activity was demonstrated against *S. aureus* with diameters zones of inhibition of 11.3 mm and 10.4 mm by essential oil and hydrosol extract respectively, compared to Gentamycin. However, *Candida* ATCC 26960 and *Candida* ATCC 10231 were also prone to growth inhibition, with diameter zones of inhibition of 25.4 mm and 18.8 mm for hydrosol extract and 20.1 mm and 15.5 mm for essential oil (Table 4).

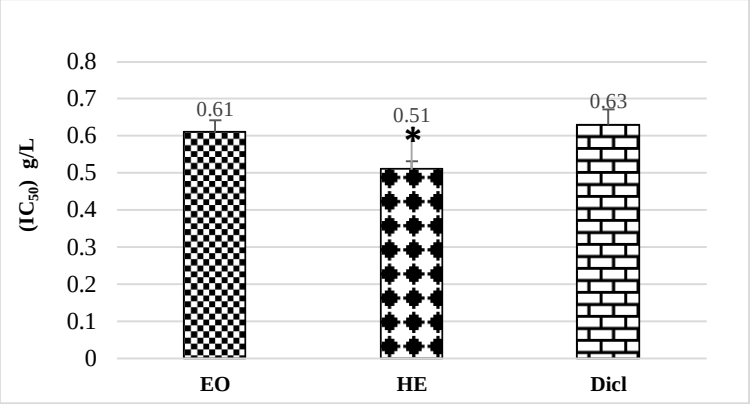

Figure 1. Median Inhibitory Concentration IC_{50} of Protein Denaturation of *I. viscosa* essential oil (EO) Hydrosol Extract (HE), and Diclofenac (Dicl) at Different Concentrations.

Table 4. Inhibition Zone Diameters of *I viscosa* Essential Oil, Hydrosol Extract and References Using the Disc Diffusion Assays

Microorganisms	Essential oil	Hydrosol extract	Gent	Amph
B. cereus	25.4 ± 0.1	28.4 ± 0.2	33 ± 0.0	/
S. aureus	11.3 ± 0.2	10.4 ± 0.2	23 ± 0.0	/
P. aeruginosa	31.5 ± 0.8	36.3 ± 0.4	25 ± 0.0	/
Candida ATCC 26960	20.1 ± 0.1	25.4 ± 0.8	/	30 ± 0.0
Candida ATCC 10231	15.5 ± 0.1	18.8 ± 0.6	/	30 ± 0.0

Samples and positive control were done in triplicates (n=3). SD= standard deviation

Discussion

The chemical composition of our essential oil of *I. viscosa* harvested in western Algeria was found to be notably distinct. Previous studies documented in the literature have indicated that the essential oil from the leaves of *I. viscosa* originating from the village of Sidi Rzine (North West of Algeria) was characterized by Eudesma-3,11, dien-12-oic acid and 1,2-Benzenedicarboxylic acid, diisooctyl ester.[21] The main components obtained by hydrodistillation of the leaves collected of South of Algiers were the following: 12-carboxyeudesma-3,11 (13) diene, linolenic acid and palmitic acid.[22] Steam-distilled essential oil from the leaves of *I. viscosa* (L.) Aiton from Algeria consisted of isocostic acid and fokienol.[23] Essential oils from the Island region have been characterized by 10-epi-γeudesmol, α-eudesmol, β-caryophyllene and limonene.[24] While globulol, valerianol and caryophyllene oxide were the main constituents of oil from Italy.[25,26] Turkey essential oil was composed of borneol, bornyl acetate and iso-bornyl acetate.[27] The essential oil from France and Spain consisted mainly of fokienol, nerolidol-(E) and α-eudesm-6-en-4-ol.[28] The essential oils of *I. viscosa* from Syria and Tunisia were rich in 11-hydroxy-6-ememopheryl (7)-9-(10)-dien-8-one, veridiflorol, cedr-8-en-13-ol, caryophyllene oxide, β-elinene, terpineol and β-bisabolene,[29] and neryl-Z isovalerate, 1,10-di-epicubenol and 2,5-dimethoxy-p-cymene, respectively.[30]

The analysis of the hydrosol extract of *I. viscosa* by GC and GC-MS allowed the identification of 18 compounds, representing 97.8% of the total extract. Nine (09) compounds were identified in the both essential oil and hydrosol extract, (E)-nerolidol, caryophyllene oxide, globulol, ledol, zingiberenol, ʈ-muurolol, α-cadinol, α-bisabolol and (*E-Z*)-farnesylacetone, while the other ten components (10), Z-hex-3-en-1-ol, nonal, citronellol, curcumen-15-al, endesma-7(11)-en-4-α-ol, shyabunol, β-acoradienol, Z-E-farnesylacetate and heptadec-.11.14. trien-2-one were not been identified in the essential oil. The hydrosol extract showed a more pronounced antioxidant activity than the essential oil, this activity can be explained by the high content of oxygenated compounds (97.8%) and the synergistic effect resulting from the different constituents in the extract. On the other hand, various therapeutic and biological properties of terpenes in the prevention of oxidative stress have been reported.[31] α-bisabolol has attracted a lot of interest due to its therapeutic potential and its multiple pharmacological properties. α-bisabolol showed concentration-dependent antioxidant activity with significant effects even at very low concentrations in neutrophils and human cell-free systems.[32]

Constituents of essential oils and plant extracts are emerging as promising sources of anti-inflammatory agents. It has been shown that sesquiterpenes have very interesting anti-inflammatory properties.[33] The notable inflammatory activity of the hydrosol extract of *I. viscosa* can be partly explained by the presence of compounds such as caryophyllene oxide and bisabolol. Indeed, caryophyllene oxide has been shown to demonstrate anti-inflammatory activity in carrageenan -induced paw edema in rats and mice. However, bisabolol exhibited anti-inflammatory action in acute models of dermatitis induced by croton oil, arachidonic acid, phenol and capsaicin in mouse ear.[34,35] The antimicrobial and antioxidant activities of hydrosol extract of *I. viscosa* can also be partly explained by the presence of oxygenated

sesquiterpene components that exhibit a wide range of biological effects.[36] Previous studies had shown that oxygenated sesquiterpenes, exhibited significant antimicrobial activity than the other constituents,[37,38] thus, suggesting the good activities evaluated in the present study.

Conclusion

Within the scope of our research program, we aimed to assess the biological properties of the hydrosol extract of *I. viscosa* with the aim of discovering new natural products. This study revealed that the hydrosol extract of *I. viscosa* contains various components and exhibits the most substantial antioxidant, anti-inflammatory and antimicrobial activities. Nevertheless, further investigations are required to validate the therapeutic potential of the hydrosol extract of *I. viscosa*.

Authors' Contributions

AN and DMA design of activity tests and experiments; SA and DMA performed the experiments; DN and SA analyzed and interpreted the data; AN and DMA wrote and edited the manuscript. The manuscript has been reviewed and approved by all authors.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

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