Comparative study of phenolic profile, antioxidant and antimicrobial properties of leaves and flower buds of *Inula viscosa* (L.) Aiton (Asteraceae)

Mohti H.[1,2], Taviano M.F.[3], Cacciola F.[4], Dugo P.[3], Mondello L.[3], Marino A.[3], Crisafi G.[3], Benameur Q.[5], Zaid A.[1,2], Miceli N.[3]

1Department of Biology, Faculty of Sciences, Moulay Ismail University, BP 11201 Zitoune, Meknes, Morocco; 2CUI-UMI-UHasselt program (Morocco-Belgium); 3Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, 98168 Messina, Italy; 4Department of Biomedical and Dental Sciences and Morphofunctional Imaging, University of Messina, 98125 Messina, Italy; 5Nursing Department, Faculty of Natural Sciences and Life, Abdelhamid Ibn Badis University, 27000 Mostaganem, Algeria.

INTRODUCTION

Morocco is a Mediterranean country having a rich and ancient tradition and historical knowledge of medicinal plants. Indeed, this country enjoys a very diverse climate with heterogeneous ecological conditions. This is reflected on its flora which is one of the most diverse in the Mediterranean region and the richest in North Africa with about 4200 taxa including 1282 subspecies [1]. *Inula viscosa* (L.) Aiton (syn. *Dittrichia viscosa* L.) is an herbaceous perennial species belonging to Asteraceae family. *I. viscosa* is reported to have many uses in traditional medicine; in Morocco it is utilized as diuretic, anti-anemic and anthielminthic and for the treatment of rheumatic pain, bronchitis, tuberculosis, cardiac disease, hypertension and diabetes mellitus [2,3]. Due to the ethnomedicinal uses, several studies are being focused on the phytochemical composition and the biological activities of *I. viscosa* [5].

In recent years, a substantial body of evidence has indicated a key role for free radicals as major contributors to aging, diseases such as cancer and cardiovascular disease, and in diabetes complications [4]. For this reason, a number of investigations have been focused on the therapeutic potential of medicinal plants as antioxidants in reducing such free radical-induced tissue injury. Most antioxidants isolated from higher plants are polyphenols, which show numerous biological effects such as antibacterial, anti-carcinogenic and anti-inflammatory [5]. Based on these statements, the present study was carried out with the main purpose of establishing the most effective solvent and technique for extracting antioxidant compounds, in particular phenolics, from two different organs, leaves and flower buds, of *I. viscosa* growing in Morocco. Besides, the best antioxidant extracts from both leaves and flower buds have been selected for further studies; particularly, the antimicrobial potential and the toxicity were evaluated and the quali-quantitative phenolic profile of the extracts was characterized.

PLANT MATERIAL AND EXTRACTION PROCEDURES

Inula viscosa (L.) Aiton was collected in Ait Ouikhalfen, near El Hajeb (Morocco). The plant was identified by professor Lhoussaine El Rhaffari, laboratory of Environment and Health, Department of Biology, Faculty of Sciences, Moulay Ismail University, Morocco. A voucher specimen was deposited in the herbarium of….

I. viscosa air dried and powdered leaves and flower buds (10 g) were extracted by different methods and solvents:

Maceration using 100 ml of distilled water or methanol under stirring for 24h (mac-H2O and mac-MeOH extracts).

Hot Extraction with 100 mL of distilled water (100 °C) or methanol (70 °C) for 2h (hot-H2O and hot-MeOH extracts).

Soxhlet Extraction using 150 ml of ethanol (Sox-EtOH extract).

All the extracts were filtered through a filter paper and concentrated in vacuo at 45 °C.

ANTIOXIDANT ACTIVITY

FREE RADICAL SCAVENGING ACTIVITY

The free radical scavenging activity of *I. viscosa* extracts has been evaluated using the DPPH test, as described by Kirby and Schmidt with some modifications [6]. Butylated Hydroxytoluene (BHT) was used as reference standard. The results were obtained from the average of three independent experiments, and are reported as mean radical scavenging activity percentage (%) ± SD and mean 50% inhibitory concentration (IC50) ± SD.

Results

I. viscosa extracts have a noticeable effect on scavenging free radicals, with IC50 values ranging from 54.24 ± 0.21 µg/mL (Sox-EtOH) to 148.79 ± 0.11 µg/mL (mac-MeOH) for the leaves and from 39.77 ± 0.23 µg/mL (Sox-EtOH) to 86.06 ± 0.25 µg/mL (mac-MeOH) for flower buds.

REDUCING POWER ASSAY

The reducing power of I. viscosa extracts was determined according to the method of Oyaizu [7]. Ascorbic acid and BHT were used as reference standards. 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.

Results

The extracts exhibited reducing power, with ASE/mL values ranging from 5.05 ± 0.17 (hot-MeOH) to 8.20 ± 0.63 (mac-H2O) for the leaves and from 4.65 ± 0.45 (hot-H2O) to 9.03 ± 0.64 (mac-H2O) for flower buds.

FERROUS IONS (Fe^{2+}) CHELATING ACTIVITY

The chelating activity of ferrous ions by I. viscosa extracts was estimated by the method of Decker and Welch [8]. Ethylenediaminetetraacetic acid (EDTA) was used as reference standard. The results were obtained from the average of three independent experiments, and are expressed as mean chelating activity percentage (%) ± SD and IC50 ± SD.

Results

In the Fe2+ chelating activity assay, the extracts were found to be not effective, except for mac-H2O from both leaves and flower buds and hot-H2O from flower buds.

IDENTIFICATION OF PHENOLIC COMPOUNDS BY HPLC-PDA-ESI-MS

A quali-quantitative investigation of phenolic compounds contained in *I. viscosa* leaves and flower buds Sox-EtOH extracts has been carried out by HPLC-PDA-ESI-MS analysis. 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 mobile phase flow rate was 1.0 mL/min, and it was splitted to 0.2 mL/min prior to MS detection. PDA wavelength range was 200-400 nm and the chromatograms were extracted at 280 nm. MS acquisition was performed using ESI, in negative mode, with mass spectral range 100-800 m/z.

Phenolics identification was carried out by the complementary information provided by chromatographic retention times, PDA and mass spectra, and further supported by comparison to existing literature data [9-16].

Quantitative determination was carried using calibration curves of eight standards, namely gallic acid, caffeic acid, p-coumaric acid, apigenin, luteolin, rutin, kaempferol and quercetin. 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 different wavelengths. The results were obtained from the average of three determinations and are expressed as mg/g dried extract ± percent relative standard deviation (%RSD).

Results

A total of 31 and 28 different compounds were positively identified in the leaves and flower buds extracts Sox-EtOH extracts, respectively. Among them, 9 were phenolic acid derivatives while the rest was composed of flavonoids and one lactone. Interestingly, three polyphenols have been identified for the first time as constituents of *I. viscosa* leaves and flower buds, namely Hispidulin hexoside, Patuletin and Spinacetin. From a quantitative point of view, *I. viscosa* leaves extract presented the highest amount in terms of bioactive compounds (258.66 mg/g), more than double with respect to the flower buds (118.99 mg/g).

ANTIMICROBIAL ACTIVITY

The antimicrobial properties of *I. viscosa* Sox-EtOH extracts (500 to 0.49 µg/mL) were tested against a representative set of ATCC and food isolates bacterial strains and the yeast Candida albicans ATCC 10231. The minimum inhibitory concentration (MIC) values were determined using the in broth microdilution method according to the protocols recommended by the Clinical and Laboratory Standards Institute [17,18]. The results were obtained from the average of three independent experiments. Positive and negative controls were also included.

Results

I. viscosa Sox-EtOH extracts exhibited antimicrobial activity; the leaves extract was found to be more active than flower buds extract, displaying the best efficacy against Candida albicans ATCC 10231. Among bacteria Staphylococcus aureus ATCC 6538, Escherichia coli ATCC 25922 and Klebsiella pneumoniae S20/16 food isolate were the most sensitive strains.

ARTEMIA SALINA LETHALITY BIOASSAY

The potential toxicity of *I. viscosa* Sox-EtOH extracts was tested using brine shrimp (Artemia salina Leach) lethality bioassay. Median lethal concentration (LC50) determination was carried out according to the method of Meyer et al. [19]. The extracts were tested at different concentrations (10, 100, 500 and 1000 µg/ml). The assay was carried out in triplicate, and LC50 values were determined by the Litchfield and Wilcoxon method. Extracts are considered non-toxic if the LC50 is higher than 1000 µg/mL [20].

Results

The Sox-EtOH extracts did not display any toxicity against brine shrimp larvae.

Fig.1 : Extraction Yields of different extracts

Extract	Yields (w/w %)	
	Leaves	Flower buds
mac-H_2O	15.1	**12.8**
mac-MeOH	14.9	**10.7**
hot-H_2O	15.7	**13.5**
hot-MeOH	17.5	**12.5**
Sox-EtOH	**9**	**7.6**

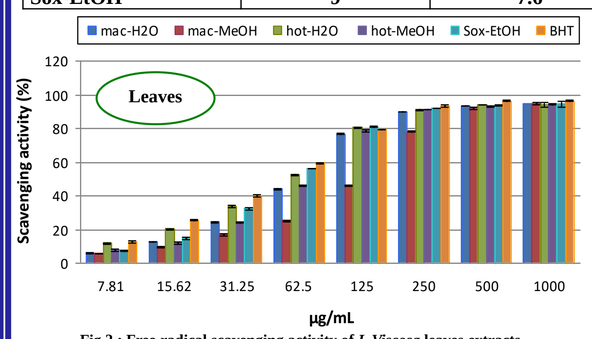


Fig.2 : Free radical scavenging activity of *I. Viscosa* leaves extracts

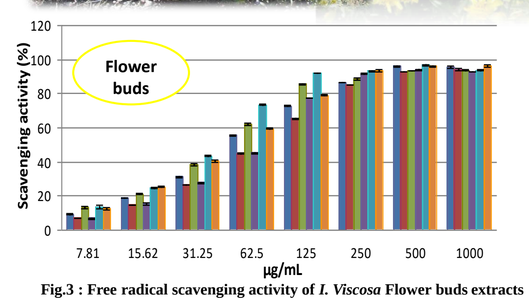


Fig.3 : Free radical scavenging activity of *I. Viscosa* Flower buds extracts

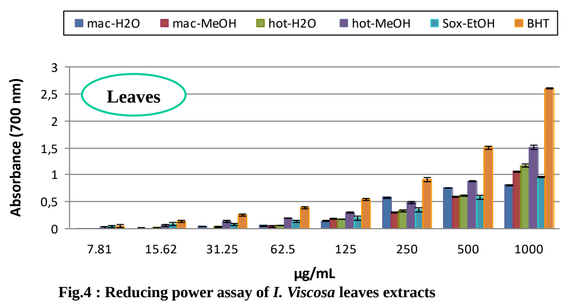


Fig.4 : Reducing power assay of *I. Viscosa* leaves extracts

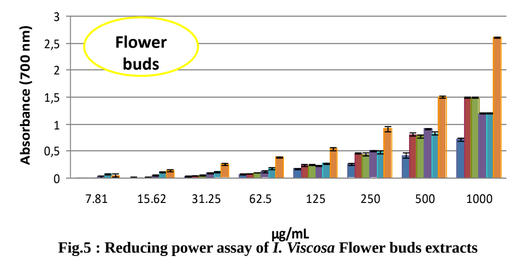


Fig.5 : Reducing power assay of *I. Viscosa* Flower buds extracts

Fig.6 : Free radical scavenging activity IC_{50}

Extract	IC_{50} (µg/mL)	
	Leaves	Flower buds
mac-H_2O	77.48 ± 0.16	**54.63 ± 0.85**
mac-MeOH	148.79 ± 0.11	**86.06 ± 0.25**
hot-H_2O	59.65 ± 0.68	**47.45 ± 0.62**
hot-MeOH	75.17 ± 0.60	**74.43 ± 0.32**
Sox-EtOH	54.24 ± 0.21	**39.77 ± 0.23**
BHT	**48.47 ± 0.44**	

Fig.7 : Reducing power assay IC_{50}

Extract	ASE/mL	
	Leaves	Flower buds
mac-H_2O	8.20 ± 0.63	**5.51 ± 0.17**
mac-MeOH	7.21 ± 0.19	**9.03 ± 2.64**
hot-H_2O	5.20 ± 1.27	**4.65 ± 0.45**
hot-MeOH	5.05 ± 0.17	**5.02 ± 0.12**
Sox-EtOH	7.56 ± 0.72	**5.45 ± 0.12**
BHT	**1.97 ± 0.08**	

Fig.8 : Ferrous ions (Fe^{2+}) chelating activity IC_{50}

Extract	IC_{50} (µg/mL)	
	Leaves	Flower buds
mac-H_2O	450.85 ± 5.23	**199.08 ± 2.14**
mac-MeOH	–	–
hot-H_2O	–	**549.57 ± 0.31**
hot-MeOH	–	–
Sox-EtOH	–	–
EDTA	**6.68 ± 0.04**	

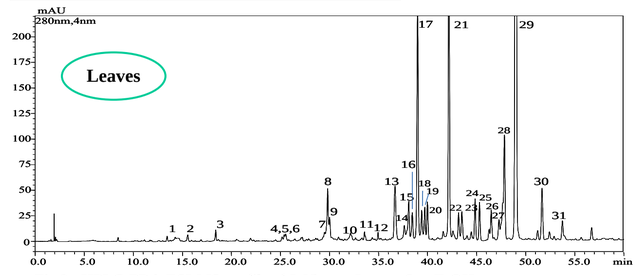


Fig.9 : HPLC-PDA-ESI-MS profile of *I. Viscosa* Leaves Sox-EtOH extract

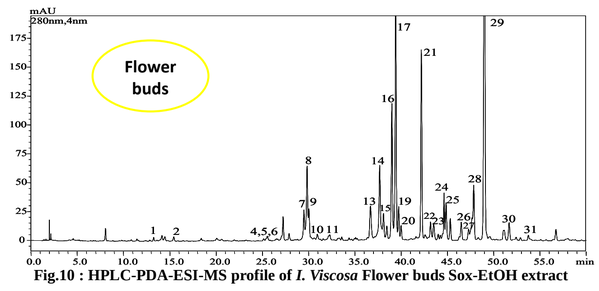
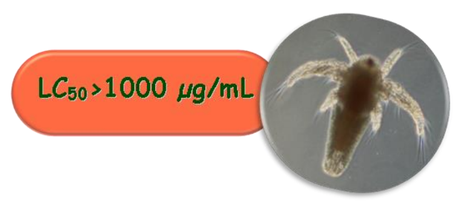


Fig.10 : HPLC-PDA-ESI-MS profile of *I. Viscosa* Flower buds Sox-EtOH extract

Peak	Compound	t_R (min)	Molecular Formula	[M-H]$^-$	PDA (nm)	Leaves (mg/g±%RSD)	Flower buds (mg/g±%RSD)
1	Caffeic acid-O-hexoside	13.5	-	341, 179	325	<LoQ	<LoQ
2	Caffeic acid	15.6	$C_9H_8O_4$	179	325	0.90±3.10	0.56±2.52
3	p-Coumaric acid	18.4	$C_9H_8O_3$	163	310	0.88±3.21	-
4	Taxifolin hexoside	25.2	$C_{21}H_{22}O_{12}$	465	337	0.16±2.81	0.15±3.21
5	Hydroxybenzoic acid hexoside	25.4	$C_{13}H_{16}O_8$	299	255	0.97±3.25	0.48±2.9
6	Isorhamnetin-O-hexoside	25.7	$C_{22}H_{22}O_{12}$	477	355	0.19±3.57	<LoQ
7	3.4-Dicaffeoylquinic acid	29.5	$C_{22}H_{24}O_{12}$	515, 353	325	1.45±2.59	3.11±2.21
8	3.5-Dicaffeoylquinic acid	29.8	$C_{22}H_{24}O_{12}$	515, 353	325	10.46±1.87	8.50±0.96
9	4.5-Dicaffeoylquinic acid	30.1	$C_{22}H_{24}O_{12}$	515, 353	325	3.73±2.18	5.12±1.25
10	Coumaryl caffeoylquinic acid	32.1	-	499, 353	325	2.28±1.94	0.79±1.56
11	Caffeic acid-O-hexoside dimer	33.4	-	683, 179	325	1.68±2.55	0.65±2.54
12	Luteolin	35.0	$C_{15}H_{10}O_6$	285	351	0.69±2.71	-
13	Isorhamnetin-3-O-(6-O-feruloyl)-glucoside	36.7	-	653	355	11.24±0.64	5.43±1.26
14	Hispidulin hexoside	37.6	$C_{22}H_{22}O_{11}$	461	335	1.15±1.65	8.33±1.56
15	Patuletin	38.1	$C_{16}H_{12}O_8$	331	370	2.88±1.68	2.00±2.36
16	7-O-Methylaromadendrin	38.5	$C_{16}H_{14}O_6$	301	365	1.34±2.31	10.24±1.11
17	Padmatin	38.9	$C_{16}H_{14}O_7$	317	355	38.82±0.32	12.61±1.32
18	3-O-Acetylaromadendrin	39.4	-	329	365	4.14±0.68	-
19	Spinacetin	39.7	$C_{16}H_{12}O_8$	345	370	4.21±0.72	3.10±1.26
20	Apigenin	39.9	$C_{15}H_{10}O_5$	269	335	2.14±1.23	0.34±3.21
21	Hispidulin	42.1	$C_{16}H_{12}O_6$	299	335	46.48±0.54	11.38±1.23
22	3.3‘ Di-O-methylquercetin	43.1	$C_{17}H_{14}O_7$	329	370	1.59±1.35	1.91±1.25
23	3-O-methylquercetin	43.5	$C_{16}H_{12}O_7$	315	370	1.04±1.33	1.12±1.26
24	Rhamnocitrin	44.9	$C_{16}H_{12}O_6$	299	365	10.00±0.65	7.81±0.98
25	Isorhamnetin	45.3	$C_{16}H_{12}O_7$	315	355	11.84±0.72	8.66±0.99
26	Helenin	46.4	$C_{15}H_{20}O_2$	231	288	n.q.	n.q.
27	Quercetin dihydrate	47.5	$C_{15}H_{14}O_9$	337	370	0.69±1.58	0.39±3.12
28	Nepetin	47.8	$C_{16}H_{12}O_7$	315	351	19.01±0.72	6.04±0.99
29	3-O-Acetylpadmatin	49.0	$C_{18}H_{16}O_8$	359	355	60.45±0.23	17.50±1.25
30	Sakuranetin	51.7	$C_{16}H_{14}O_5$	285	355	16.80±0.83	2.29±1.12
31	Genkwanin	53.8	$C_{16}H_{12}O_5$	283	335	1.45±1.35	0.48±2.65
	Total amount					258.66	118.99

Fig.11 : Antimicrobial properties of *I. viscosa* Sox-EtOH extracts

Gram positive bacteria	MIC (µg/mL)	
	Leaves	Flower buds
Staphylococcus aureus ATTC 403300	500	**500**
Staphylococcus aureus ATTC 6538	250	**>500**
Gram negative bacteria		
Escherichia coli ATCC 25922	250	**500**
Escherichia coli S4/15	>500	**>500**
Klebsiella pneumoniae S20/16	250	**500**
Enterobacter cloacae S16b/16	>500	**>500**
Salmonella spp S13b/16	>500	**>500**
Yeast		
Candida albicans ATCC 10231	**125**	**250**



CONCLUSION

The results obtained from the comparative study indicated that Soxhlet extraction with ethanol represents the most efficient method of extracting antioxidant components from *I. viscosa* leaves and flower buds.

Besides, the Sox-EtOH extracts exhibited antimicrobial activity, as well as absence of toxicity against *A. salina*. From a pharmaceutical point of view, it is an advantage when antibacterial drugs are selectively toxic to the microbe but non toxic to eukaryotic cells [21].

Flavonoids and phenolic acids represent the largest classes of plant phenolics; phytochemicals from these classes were found to have excellent antioxidant activity and antimicrobial efficacy against a wide array of microorganisms [20]. Hence, it can be hypothesized that the observed effects could depend, almost in part, on the presence of these compounds.

The results of our investigation provide additional information for a feasible use of *Inula viscosa* as a safe source of antioxidant and antimicrobial agents.

Acknowledgements: This work was carried out within the program Erasmus+/KA107 Higher Education Agreement between the University of Messina (Italy) and the Moulay Ismail University, Meknes (Morocco).

REFERENCES

[1] Rankou H., Culham A., Jury S.L., et al. Phytotaxa 78(1), 1–69, 2013. [2] Eddouks M., Ouahidi M.L., Farid O., et al. Phytothérapie 5(4), 194–203, 2007. [3] Seca A.M.L., Grigore A., Pinto D.C.G.A., et al. J. Ethnopharmacol. 154(2), 286–310, 2014. [4] Pham-Huy L.A., He H., Pham-Huy C. Int. J. Biomed. Sci. 4(2), 89-96, 2008. [5] Srivastava T., Mishra S.K. Res. J. Phytochem. 9(3), 116-126, 2015. [6] Kirby A.J., Schmidt R.J. J. Ethnopharmacol. 56, 103-110, 1997. [7] Oyaizu M. Jpn. J. Nutr. 44, 307-315, 1986. [8] Decker E.A., Welch B. J. Agric. Food Chem. 38, 674-677, 1990. [9] Rhimi W., Salem I.B., Immediato D., et al. Molecules 22, 942, 1-13, 2017. [10] Talib W.H., Zarga M.H., Mahasneh A.M. Molecules 17(3), 3291-3203, 2012. [11] Kheyar-Kraouche N., da Silva A.B., Serra A.T., et al. Pharm. Biomed. Anal. 156297-306, 2018. [12] Mahmoudi H., Hosni K., Zaouali W., et al. Food Saf. 36, 77-88, 2016. [13] Grande M., Piera F., Cuenca A., et al. Planta Med. 51, 414-419, 1985. [14] Hernández V., Recio M.C., Máñez S., et al. Life Sci. 81, 480-488, 2007. [15] Orhan N., Gökbulut A., Orhan D.D. S. Afr. J. Bot. 111, 86-92, 2017. [16] Gökbulut A., Ozhan O., Satilmiş B., et al. Nat. Prod. Commun. 8, 475-478, 2013. [17] Clinical and Laboratory Standards Institute (CLSI), 9th ed. Wayne, PA, USA; 2012. [18] Clinical and Laboratory Standards Institute (CLSI), 3rd ed. Wayne, PA, USA; 2008. [19] Meyer B.N., Ferrigni N.R., Putnam J.E., et al. Planta Med. 45(5), 31–34, 1982. [20] Taviano M.F., Rashed K., Filocamo A., et al. BMC Compl. Alt. Med. 18(1), 161, 1-11, 2018. [21] Shivanand S., Vidya G.D., Vidya C.S., et al. Int. J. Appl. Eng. Res. 13(5), 2959-2964, 2018.