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Isolation, identification, and activity evaluation of antioxidant components from *Inula viscosa:* A bioguided approach

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ABSTRACT

Oxidative stress is linked to several invasive diseases which causes significant clinical and economic impact, therefore, there is a need to develop new antioxidants. The natural products could play an important role in overcoming the current need. In the present work, the antioxidant bioassay-guided fractionation of the ethanolic extract of *Inula viscosa* leaves (Asteraceae) was performed using DPPH and ABTS assays affording three known compounds, which were successfully characterized as ilicic acid (**1**), taxifolin (**2**) and quercetin (**3**) based on 1D, 2D NMR. Compounds **2** and **3** were identified as the most active, displaying similar or higher potency against ABTS (value 41.27 for quercetin and 142.58 for taxifolin) and similar activity against DPPH (value 41.27 for quercetin and 142.58 for taxifolin) than the well-known reference, ascorbic acid (value 65.36 for quercetin and 58.43 for taxifolin) but less potency than the standard gallic acid. The discussion of SAR of the antioxidant potential revealed that the type of natural product is crucial for the activity and the substitution pattern on the flavonoid skeleton modulate the antioxidant profile. Our findings show that *I. viscosa* leaves may be a natural source of antioxidants and once again the role of flavonoids health benefits is more strongly endorsed.

1. Introduction

Oxygen is essential for life. Most of the processes in our body take place in the presence of this molecule. Although useful to the body, the oxidation reaction can damage the body's cells, lead to the degradation of different oxygen substrates, fatty acids, lipids, proteins, and DNA, as well as causing different diseases in special situations such as inflammation, obesity, diabetes, arthritis, etc [12], although it is not always clear whether it is a direct cause of the pathology or its consequence [3], the oxygen paradox participates in the formation of free radicals that share the characteristic to have a single electron on an oxygen or nitrogen atom, giving them the name of reactive oxygen species (ROS) or reactive nitrogen species (RNS) [4]. These free radicals are highly unstable and play a dual role in the biological system, they can be beneficial when they are involved in physiological roles and cell signaling systems, or harmful when there is an imbalance between ROS/RNS and the defense systems or when the organism is unable to limit the damage caused by the free radicals (oxidative stress) [5]. The control of oxidation, in the various fields, is based on the use of antioxidants, which are substances that can reduce or delay the oxidation of a compound, or neutralize free radicals, even when used in very small amounts [6]. Certainly, there are some synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) that inhibit or retard oxidation. However, these synthetic antioxidants are now

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reported to be dangerous to human health and environment [78]. Consequently, the search for natural substances with antioxidant capacity present an important scientific challenge and a socio-economic interest. Recently, the efficacy of many natural antioxidants has been documented [9].

In the current work, we report a bioguided fractionation of compounds with antioxidant properties from the ethanolic extract of *I. viscosa* widely used in traditional medicine and we discuss a possible structure–activity relationship.

2. Materials and methods

2.1. General procedure

Optical rotations were determined on a Perkin-Elmer 241 automatic polarimeter. NMR spectra, ROESY (spin lock field 2500 Hz), HSQC and HMBC (optimized for $J = 7.7$ Hz) were performed on a Bruker Avance 500 and 600 spectrometers at 300 ºK. Silica gel 60 (particle size 15–40 and 63–200 µm, Macherey-Nagel) was used for column chromatography, while silica gel 60 F254 (Macherey-Nagel) was used for analytical or preparative TLC and Sephadex LH-20 (Sigma-Aldrich) were used for the column chromatographic (CC) separation. Centrifugal preparative TLC was performed using a Chromatotron (Harrison Research Inc. model 7924 T) on 4- or 1-mm silica gel 60 PF254 disks with flow rate 2–4 mL min^{-1}. The developed TLC plates were visualized by UV light and then spraying with HOAc-H_2SO_4-H_2O (80:16:4) system, followed by heating at 100 °C during 3 min. All solvents and reagents used were analytical grade from Sigma-Aldrich. EnSpire® Multimode Plate Reader (Perkin Elmer) was used.

2.2. Plant material identification and extraction

I. viscosa leaves have been collected in the Atlas Mountains of Imouzzer -Morocco in July 2017, then identified by botanists. The geographical coordinates for this species are 33°55′ 37 N, 5°2′50 W and a voucher specimen (RAB107342) has been deposited in the Herbarium of the Scientific Institute of Rabat, Morocco. To obtain the crude extract, cleaned leaves were shade dried at room temperature and subsequently milled with a simple commercial electric grinder. Powdered material was extracted by maceration in ethanol (1:10 w/v) for 6 h at room temperature with continuous stirring at 500 rpm (revolutions per minute). The resulting mixture was filtered through Whatman filter n°1 and the solvent concentrated under vacuum in a rotary evaporator at 45 °C, dried extract (5 g, 5%) was stored in a refrigerator at 4 °C until further use.

2.3. Bio-guided fractionation procedure

The EtOH extract (5 g, 5%) was evaluated for its antioxidant activity. After that, a bio-guided fractionation methodology was used to identify the pure compounds. As described previously [10], the active EtOH extract (5 g) was subjected to CC on silica gel eluted with mixtures of hexanes-EtOAc (100:0 to 0:100, 0.5 L each one) of increasing polarity to afford twelve fractions that were combined into seven fractions (F1-F7) based on their TLC profile. The antioxidant activity was focused on the active fraction F4 (1.7 g), which was subjected to column chromatography on sephadex LH-20 column, using a MeOH-$CHCl_3$ system (1:1, 2 L) as eluent to afford twelve sub-fractions (F4A to F4L). Indeed, the sub-fractions F4K and F4L showing a promising antioxidant activity were subjected to multiple chromatographic steps on silica gel, involving medium-pressure liquid chromatography, centrifugal planar chromatography and preparative TLC using mixtures of hexanes-EtOAc, hexanes-Et_2O, CH_2Cl_2-EtOAc and CH_2Cl_2-acetone as eluent to yield compounds **1** (ilicic acid, 8.5 mg), **2** (taxifolin, 28.5 mg) and **3** (quercetin, 5.4 mg) (Fig. 1).

2.4. Antioxidant assay

2.4.1. DPPH assay

As described in [11], the reduction of the free radical DPPH was

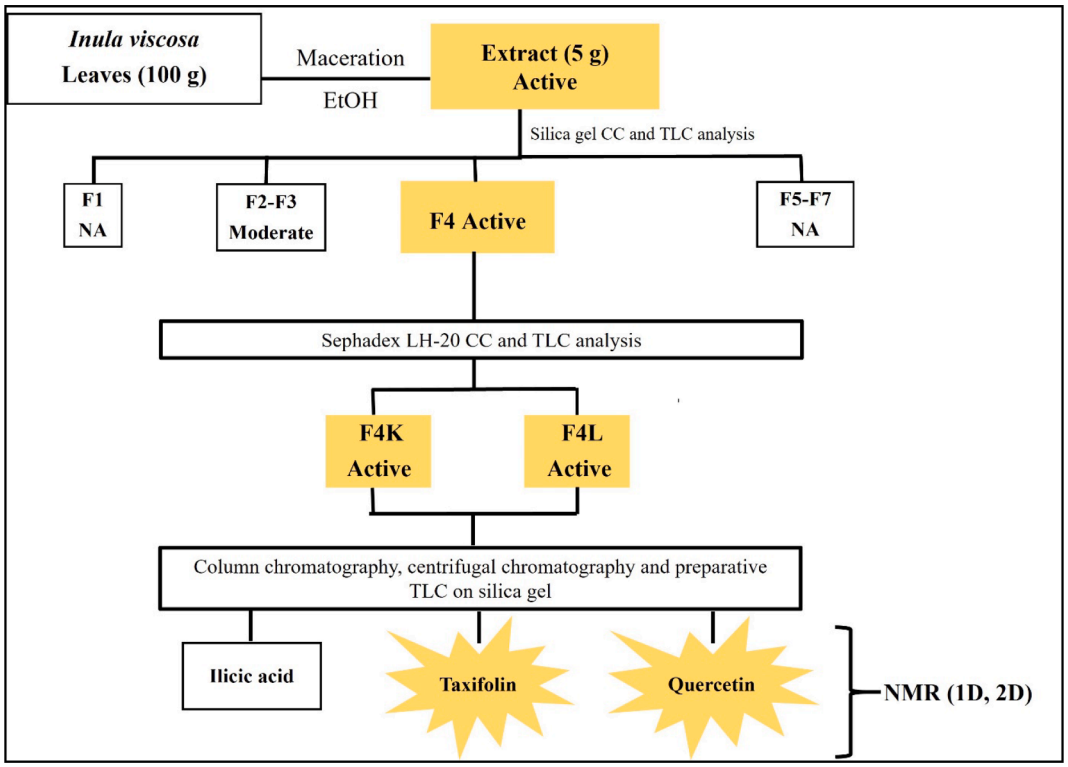

Fig. 1. Flowchart of antioxidant bio-guided fractionation of *I. viscosa* leaves using DPPH and ABTS assays.

highlighted by mixing 180 µl of DPPH (60 µM) with 20 µl of the diluted samples (extract, fractions, sub-fractions, and pure compounds) in 96-well plates. After 2 h, the absorbance was measured using an EnSpire multimode plate reader (PerkinElmer, MA, USA) at a test wavelength of 517 nm. The IC_{50} (the concentration necessary to reduce 50% of the ABTS radical) was determined based on the obtained curves using the Sigma Plot 12.0 software program (Systat Software Inc.). All assays were carried out in triplicate. The results were defined as the mean values of three experiments.

2.4.2. ABTS assay

As previously described in [12], the reduction of the radical cation ABTS + was highlighted by reaction of ABTS water solution (7 mM) with potassium persulphate (2.45 mM) (1:1). Technically, 180 µl of ABTS + solution was added to 20 µl of the sample (extract, fractions, sub-fractions, and pure compounds) at a dilution of 1:50 in 96-well plates. After 15 min of reaction time, the color change was measured using an EnSpire multimode plate reader (PerkinElmer, MA, USA) at a test wavelength of 73. The IC_{50} was determined using the Sigma Plot 12.0 software program (Systat Software Inc.). Samples were analyzed Triplicate.

2.5. Statistical analysis

All assays were carried out in triplicate. The results were defined as the mean values of three experiments. The obtained inhibition curves were performed using the Sigma Plot 12.0 software program (Systat Software Inc.).

3. Results

3.1. Antioxidant activity

The antioxidant activity of the extract of *I. viscosa* and the pure compounds was measured following the reduction of both DPPH and ABTS. The analysis of the results obtained revealed that the extract of *I. viscosa* showed a significant antioxidant activity with an IC_{50} of 768,04 ± 0.5 µg/ml (Table 1). Furthermore, the purified active compounds, taxifolin (**2**) and quercetin (**3**), were more active. However, ilicic acid (**1**) was not active and taxifolin (**2**) was less active than the subfractions F4/11 and F4/12. Using the ABTS test, same results were obtained with more sensitivity towards this reagent.

3.2. Chemical structures characterization

The chemical structures of the known isolated compounds (**1, 2** and **3**) were identified as ilicic acid (**1**) [13], taxifolin (**2**) [14], and quercetin (**3**) [15] (Fig. 2) using spectrometric and spectroscopic data, including 1D and 2D NMR experiments (S1-S3). In addition, the obtained spectrums were compared with those previously reported in literature.

Table 1
Antioxidant activity of natural compounds isolated from *I. viscosa* using DPPH and ABTS assays.

***I. viscosa* Leaves**	**IC_{50} (µg/mL)**	
	ABTS	**DPPH**
EtOH extract	452.08 ± 0.5	768.06 ± 0.5
F1	NA	NA
F2	>1000	>1000
F3	>1000	>1000
F4	172.89 ± 5.82	343.50 ± 0.5
F4K	109.86 ± 3.37	103.45 ± 2.51
F4L	43.31 ± 1.38	72.95 ± 1.16
Ilicic acid (**1**)	NA	NA
Taxifolin (**2**)	142.58 ± 3.52	103.46 ± 1.02
Quercetin (**3**)	41.27 ± 1.56	62.53 ± 1.73
F5-F7	NA	NA
Ascorbic acid	65.36 ± 2.34	58.43 ± 1.74
Gallic acid	2.33 ± 0.76	4.42 ± 0.41

IC_{50} (µg/mL): Means ± Standard deviation.

4. Discussion

The results obtained regarding the antiradical capacity towards DPPH and ABTS revealed that the ethanolic extract of *I. viscosa* exhibited remarkable antioxidant activity. Our findings corroborate numerous investigations regarding the antioxidant activity of *I. viscosa* crude extract [1617 18]. Recently, Asraoui et al. have evaluated the antioxidant activity of different extracts from *I. viscosa* using DPPH, ABTS and FRAP assays. They have reported that the extracts showed an important antioxidant activity, especially the EtOAc extract [19]. Additional studies have assessed the antioxidant activity of the genus *Inula,* Silinsil et al. have described the significant antioxidant capacity of ethanol and water extracts of *I. graveolens* (L.) Desf. prepared from the leaves, and they have linked this activity to the abundance of phenolic compounds such as chlorogenic acid, quinic acid, hyperoside, protocatechuic acid and quercetin [20]. Furthermore, Bursal et al. have studied the phenolic constituent, enzyme inhibitory and antioxidant activities of the aqueous and methanol extracts of *I. discoidea* showing better results than the standard antioxidants namely BHA, BHT, ascorbic acid, and α-tocopherol using ABTS and CUPRAC methods in addition to the abundance of phenolic compounds, among which the main ones were quinic acid, protocatechuic acid and gallic acid [21].

These results are to be associated with the phytochemical composition of *I. viscosa* because the purified compounds showed a more interesting potential than the crude extract. These compounds are generally belonging to the flavonoid family (quercetin and taxifolin). Indeed, among natural antioxidants, phenolic compounds especially flavonoids are known to possess an interesting antioxidant activity due to their chemical structure suitable for scavenging the free radicals and their conversion into more stable compounds through electron and proton transfer mechanisms [22]. The most studied compounds of this family are quercetin and taxifolin [2324], which corroborates our result. According to several studies, the antioxidant capacity of these compounds is linked to the molecular structure and to different physicochemical parameters. Thus, the preliminary structure–activity relationship (SAR) analysis revealed the following trends: The type of natural product is crucial for the activity, therefore, comparison between the activities of ilicic acid (**1**), taxifolin (**2**) and quercetin (**3**), whose only structural difference is the classe of secondary metabolites, showed that the active compounds were the flavonoids. Furthermore, the influence of the substitution pattern of the tested flavonoids on their antioxidant activity was analyzed considering three regions the two-aromatic moiety (A and B rings) and the propane unit of the 1,3-diarylpropane (C6-C3-C6) flavonoids skeleton. This analysis revealed that the presence of α,β-unsaturated or saturated carbonyl group at C ring causes changes in the antioxidant activity, the conjugated double bond at C-2/C-3 of propane unit increases the activity as revealed by comparison of potency of quercetin (**3**) with flavonol skeleton with taxifolin (**2**) with flavanonol skeleton on ABTS and DPPH assays, suggesting that the flavonoids skeleton is important for the antioxidant effect. It was reported that the ring B of these compounds was largely involved in the antioxidant capacity, especially when this ring includes a catechol moiety like in quercetin and taxifolin [25] (Fig. 3).

Furthermore, it has noticed that the presence of 5- and 7-OH groups with 4-oxo function in rings A and C is involved in the radical scavenging effect while stability is provided by the O-dihydroxy structure in ring B [26]. The only structural difference between these two compounds is the presence of a 2,3-double bond in quercetin which has been implicated in its higher antioxidant power than taxifolin [27]. It has demonstrated that the antioxidant activity of taxifolin is related to the electrochemical redox potentials, especially that of the hydroxyl groups of the ring A assessed by a deoxyribose degradation test [28]. Additional authors

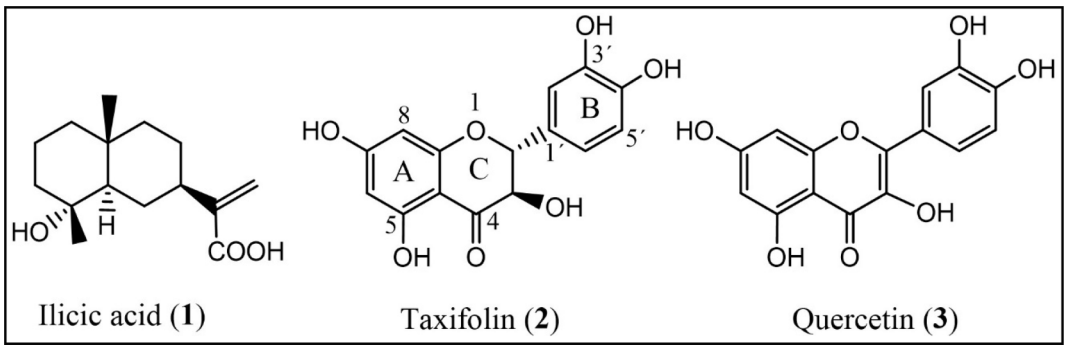

Fig. 2. Chemical structure of natural compounds 1–3 isolated from leaves. of *I. viscosa*.

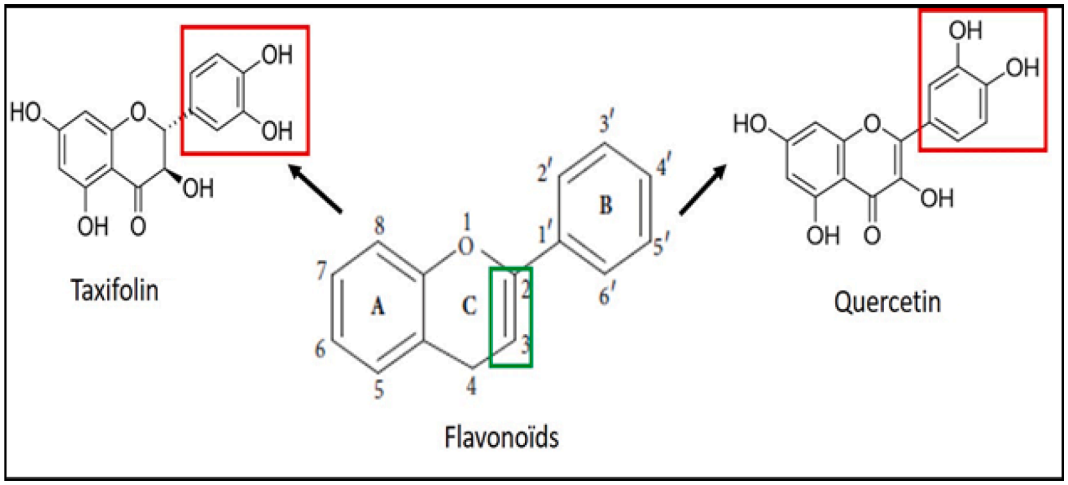

Fig. 3. Chemical structures of active flavonoids isolated from I. viscosa leaves.

have compared the antioxidant activity of quercetin and taxifolin and proposed that the radical from quercetin is more persistent in the environment and able to interact with a second radical by transferring hydrogen, protons and electrons which may be involved in its more significant antioxidant capacity [29]. In addition, other authors have summarized the essential structural features for the antioxidant effect namely the number of hydroxyl groups, hydroxyl configuration, C-2-C-3 double bond and ketone functional group at C-4 [30] which could explain the inactivity of ilicic acid isolated from *I. viscosa* in the present study.

Our results are also in agreement with many studies that have reported positive and negative interactions between different compounds regarding taxifolin (**2**) that was less active than the subfractions F4K and F4L. Synergistic effects between synthetic antioxidants such as tocopherol with ascorbic acid and BHA with BHT against DPPH have been illustrated [31 32]. Furthermore, the synergistic and antagonistic effects between phenolic antioxidants in a binary mixture have been evaluated showing that the effectiveness of the mixture increases with the number of hydroxyl and methoxyl groups, which may increase the chances to provide multiple interaction and/or reaction sites [33]. Meanwhile, it has shown that antioxidant interactions can have not only positive but also negative effects suggesting the importance of strategically selecting compounds to maximize synergies and minimize antagonisms of antioxidant activity [34].

5. Conclusion

This study aimed to investigate the antioxidant bioassay-guided fractionation of *I. viscosa*, a Moroccan species used in traditional medicine. The results reported herein indicated that the fractionation of the crude ethanolic extract enhanced the antioxidant activity. Pure compounds were generally more active than the fractions and sub-fractions that were more active than the crude extract.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2021.105551.

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