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Antioxidant activities and total phenol content of *Inula viscosa* extracts selected from three regions of Morocco

Naima Chahmi[1,2], Jaouad Anissi[1,3], Sanae Jennan[2], Abdellah Farah[2], Khalid Sendide[1,3], Mohammed El Hassouni[1*]

[1]*Laboratory of Biotechnology, Faculty of Sciences Dhar El Mehraz, Sidi Mohammed Ben Abdellah University, Fez, Morocco*

[2]*Laboratory of Medicinal and Aromatic Plants and Natural Substances, National Institute of Medicinal and Aromatic Plants, USMB Fez, Morocco*

[3]*Al Akhawayn university, School of Science and Engineering, Laboratoty of Biotechnology, Av. Hassan II, P. O Box 104-Ifrane.*

PEER REVIEW

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Asoss. Prof. Dr. Tülay AŞKIN ÇELİK, Department of Biology, Art and Science Faculty, Adnan Menderes University, 09010-Aydın, Turkey.
Tel: +9002562128498/1705
E-mail: tcelik@adu.edu.tr

Comments

This is a valuable research work in which authors have demonstrated the greatest antioxidant properties of *I. viscosa* extracts in all systems of assays.
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ABSTRACT

Objective: To investigate antioxidant activity, total phenolic and flavonoid content of ethanol (E) and ethyl acetate (A) extracts of *Inula viscosa* aerial parts selected from three regions of Morocco (Imouzzer, Sefrou and Taounate).

Methods: Antioxidants properties were measured by three different test systems of assay namely free radical scavenging activities against 2,2-diphenyl-1-picrylhydrazyl, total antioxidant capacity and iron-reducing capacity. Total phenolic content was measured by Folin-Ciocalteu reagent.

Results: All the extracts showed significant antioxidant activities and contained important levels of phenols. The ethanol extract (0.3 mg/mL) from Sefrou showed the greatest antioxidant capacity in the three systems of assay, which was probably due to its high content of polyphenols (274.39±6.94) mg gallic acid equivalent/g dry extract. Total flavonoid content was found equal for all extracts.

Conclusions: Our results of antioxidant assays were justified and partially supported the popular usage of the tested plants. The high antioxidant activity found in the plant from Sefrou and its great biomass in this region suggested that *Inula viscosa* is a good source of natural antioxidants compounds which might have benefits for health.

KEYWORDS

Inula viscosa, Folk medicine, Flavonoids contents, Polyphenols, DPPH, Reducing power

1. Introduction

Oxidative stress is characterized as an imbalance between the production of reactive species and antioxidant defense activity. Its enhanced state has been associated with many of the chronic diseases such as cancer, diabetes, neurodegenerative and cardiovascular diseases[1]. Health care systems are becoming more and more expensive, therefore, we have to introduce herbal medicine systems in our health care[2]. In recent years, the research of natural antioxidants[3] as alternative sources to synthesis antioxidants has emerged and the exploitation of the various secondary metabolites of the plant was highlighted. These substances are able to reduce free radicals like superoxide, peroxyl, alkoxylate and hydroxyl[4].

Plant consists of a set of organs whose growth depends on the environmental conditions in which it develops, including the intercepted light energy, water and available nutrients drawn from the soil. The genus *Inula* including more than 100 species[5] are widely distributed in the Mediterranean area. In Morocco, *Inula viscosa* (L.) Aiton (*I. viscosa*) [*Dittrichia viscosa* (L.)], locally called "Bageraman", is an herbaceous perennial Mediterranean plant of the family Asteraceae which was largely known and used topically in folk medicine to treat animal's injuries. The plant

*Corresponding author: Mohammed El Hassouni, Laboratory of Biotechnology, Faculty of Sciences Dhar El Mehraz, Sidi Mohammed Ben Abdellah University, Fez, Morocco.
Tel: (+212) 0675367364
E-mail: mohammed.elhassouni@usmba.ac.ma

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has been used to treat diabetes and inflammation in North African traditional medicine[6], to treat tuberculosis, anemia and as cataplasm for rheumatic pain in Jordan[7] and it has been used for its antiseptic, skin inflammations properties and gastroduodenal disorder treatment in Spain[8,9].

It is well known that phenolics and flavonoids are important antioxidant substances obtained from most natural plants. Thus, this study was planned to evaluate the antioxidant properties of Moroccan *I. viscosa* extracts from three different regions. The choice of solvent was showed to have a significant influence on the concentration of antioxidants[10,11]. Thus, we investigated the ethanol and ethyl acetate extracts. As widely recommended, the antioxidant effect was assessed by three *in vitro* methods: 2,2-diphenyl-1-picrylhydrazyl (DPPH), phosphomolybdenum and reducing power assays. Further experiments were conducted to determine phenol and flavonoid contents. The results of this study will allow us to optimize the possibility of exploiting this plant to produce antioxidants agents to be used in the global effort to combat free radical damages.

2. Materials and methods

2.1. Reagents and standards

DPPH, butylated hydroxytoluene (BHT), ammonium molybdate, sodium phosphate, sulphuric acid, gallic acid, FeL_3, $K_3Fe(CN)_6$ and Folin-Ciocalteu reagent (FCR) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium carbonate was purchased from Prolabo (Paris, France). All the other chemicals and solvents used were of analytical grade.

2.2. Plants materials and preparation of extracts

Plants were collected in June to July 2012 from the regions of Imouzzer, Sefrou and Taounate, and dried for a week at ambient temperature and then ground. Identification was confirmed by professor Amina Bari, botanist (Department of Biological Sciences, Faculty of Science, Sidi Mohammed Ben Abdellah University, Fes, Morocco). Voucher specimens (ADV14101) were deposited in the Department of Biological Sciences, Sidi Mohammed Ben Abdellah University, Fes, Morocco. Ethanol and ethyl acetate extraction was performed at the ratio of 10% (w/v) for 3 h under agitation for plant powder[12], then the mixture was filtered through a filter paper (Whatman No. 1) and concentrated in vacuo at 45 ℃ to obtain an oily, dry, green paste, then stored at 4 ℃ for further use.

2.3. DPPH scavenging activity

The hydrogen atoms or electrons donation ability of the plant extracts and some pure compounds were measured from the bleaching of a purple-coloured methanol solution of DPPH[13]. Briefly, 1 mL of a 0.01 mmol/L solution of DPPH radical in methanol was added to 4 mL of the extract at different concentrations. The absorbance of the resulting solution was measured after 30 min in dark at 514 nm with a spectrophotometer (Selecta, E.U.). The percentage inhibition of activity was calculated as:

$$\%\text{Inhibition}=\frac{A_{blank}-A_{sample}}{A_{blank}}\times 100$$

BHT and ascorbic acid was used as positive control and the concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage plotted against the extract concentration.

2.4. Iron (III) to Iron (II) capacity

The reductive capacity of the extract was determined using ferric to ferrous iron reduction assay as determined spectrophotometrically from the formation of Perl's Prussian blue coloured complex[14]. Briefly, 1 mL of each sample, in methanol, was mixed with 2.5 mL of phosphate buffer (0.2 mol/L, pH 7.0) and 2.5 mL of potassium hexacyanoferrate $K_3Fe(CN)_6$ solution. After 30 min incubation at 50 ℃, aliquots (2.5 mL) of trichloroacetic acid (10%) were added to the mixture. Then, 2.5 mL of this solution was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%), and the absorbance was measured at 700 nm. BHT standard was used for comparison.

2.5. Determination of total antioxidant capacity

The assay was based on the reduction of Mo (VI) to Mo (V) and subsequent formation of a green phosphate/Mo(V) complex in acid pH[13]. A total volume of 0.3 mL extract dissolved in methanol was added to 3 mL of reagent solution (0.6 mol/L sulphuric acid, 28 mmol/L sodium phosphate and 4 mmol/L ammonium molybdate). The mixtures were incubated at 95 ℃ for 90 min then cooled to room temperature. The absorbance was measured at 695 nm. The total antioxidant activity was expressed as the number of equivalence of ascorbic acid and BHT.

2.6. Determination of total phenolic compounds content

The total phenolic content was determined using the FCR[15]. The reaction mixture contained 100 μL of methanolic solution (1 mg/mL) of extract, 0.5 mL of FCR, 1.5 mL of 20% (w/v) sodium carbonate and 10 mL of distilled water. After 2h of reaction at ambient temperature, the absorbance was measured at 765 nm and used to calculate the phenolic contents, using gallic acid as a standard. Then the total phenolic contents were expressed in term of gallic acid equivalents (mg GAE/g dry extract).

2.7. Total flavonoids contents

The flavonoid contents of the ethanolic and ethyl acetate extracts of *I. viscosa* were assessed using the method of Lamaison and Carnat based on the formation of a complex flavonoid-aluminium[16]. Briefly, 1 mL of diluted sample (20 μg/mL) was mixed with 1 mL of 2% aluminium chloride methanolic solution, after incubation for 10 min at room temperature. The absorbance was read at 430 nm and the flavonoids content was expressed in μg quercetin equivalent (QE) per mg of dry extract.

2.8. Statistical analysis

The measurements of total phenolic compounds, total flavonoids and DPPH radical-scavenging activity, total antioxidant and reducing capacity were carried out for three replicates. The results are expressed as mean±SD.

3. Results

3.1. Extraction yield

As shown in Table 1, the most extractive solvent was ethanol. The highest extraction yield of *I. viscosa* aerial part was the extract from

Sefrou with 23.90% (percentage of dry matter w/w), followed by the extract from Imouzzer (20.80%) then Taounate (13.35%).

Table 1

Residues yields (% of dry matter) of *I. viscosa* in the organic solvents.

Regions	Samples	Yields (%)
Sefrou	A	21.30
	E	23.90
Imouzzer	A	10.50
	E	13.35
Taounate	A	18.50
	E	20.80

A: Ethyl acetate extract; E: Ethanol extract.

3.2. DPPH scavenging activity

In this study, ethanol and ethyl acetate extract of *I. viscosa* from three regions were investigated for their antioxidant activity with DPPH scavenging assay. The results were shown in Figure 1.

Results showed an important antioxidant power of *I. viscosa* extracts compared to the standard product and the ethanol was more effective than the ethyl acetate as organic solvent. The IC_{50} value was defined as the concentration of sample that scavenged 50% of the DPPH. The results (Table 2) showed that antioxidant activity of the ethanol extract from Taounate was superior to all samples tested with an IC_{50} value of 0.18 g/L which was near to the inhibition capacity of the positives controls BHT (IC_{50}=0.15 g/L) and ascorbic acid (IC_{50}=0.12 g/L), followed by ethanol extract from Sefrou (IC_{50}=0.20 g/L) then Imouzzer (IC_{50}=0.27 g/L). While with the ethyl acetate extracts we found that the extract from Sefrou had the greatest capacity with an IC_{50} value of 0.28 g/L, followed by the extract from Taounate (IC_{50}=0. 63 g/L) then Imouzzer (IC_{50}=1.86 g/L).

Table 2

IC_{50} (g/L) values of ethanol and ethyl acetate extracts of *I. viscosa* according to DPPH assay.

Regions	Samples	IC_{50} (g/L)
Immouzer	A	1.86
	E	0.27
Sefrou	A	0.28
	E	0.20
Taounate	A	0.63
	E	0.18
	BHT	0.15
	A Asc	0.12

A: Ethyl acetate extract; E: Ethanol extract; A Asc: ascorbic acid.

Up to date, there is no universal and simple method to evaluate qualitatively and quantitatively the antioxidant activity[17]. This property has been evidenced by a large number of tests, measuring the antioxidant activity *in vitro*[18]. It is not always a simple task to choose the most appropriate method to determine antioxidant capacity[19]. In fact, we have adopted an approach combining three complementary techniques.

3.3. Determination of total antioxidant capacity

The phosphomolybdenum was a quantitative assay[20]. Since the antioxidant activity was expressed as number of ascorbic acid and BHT, the method was based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate Mo (V) complex at acid pH. We found that the acetate ethyl extract had a higher capacity than ethanol, and the three regions had different levels of antioxidant capacity (Table 3). The region of Sefrou had the most important value with (139.31±3.47) and (155.42±3.54) mg/g equivalent to ascorbic acid and equivalent to BHT (mg)/g dry extract respectively, followed by the regions of Taounate then Imouzzer.

Table 3

Total antioxidant capacities of *I. viscosa* from different regions.

Regions	Samples	Equivalent to ascorbic acid (mg)/g dry extract	Equivalent to BHT (mg)/g dry extract
Immouzer	A	91.84±1.52	99.79±1.49
	E	13.61±0.09	8.16±0.02
Sefrou	A	139.31±3.47	155.42±3.54
	E	103.33±3.17	113.25±3.22
Taounate	A	103.71±2.78	113.70±3.31
	E	84.85±1.38	91.61±1.36

Assay performed in three replicates and the data were reported as means±SEM. A: Ethyl acetate extract; E: Ethanol extract.

3.4. Reductive capacity

Generally, the reducing properties are associated with the presence of compounds which exert their action by breaking the free radical chain via donating a hydrogen atom[21,22]. In the ferric to ferrous iron reduction assay, the electron donation capacity of the extracts was assessed and compared to that of BHT, which was known to be a strong reducing agent. The reducing power of all extracts increasesd with the increasing of their concentrations. Extracts from Sefrou were the most active ones among the three

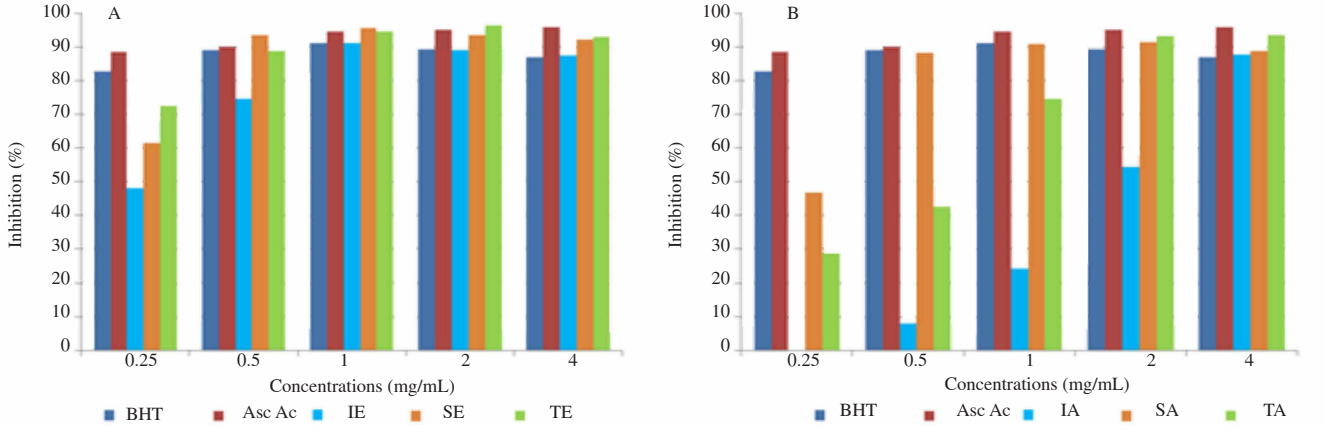

Figure 1. Scavenging activities of different concentrations of *I. viscosa* ethanol and ethyl acetate extracts on the DPPH radical.
A: Scavenging activities of different concentrations of *I. viscosa* ethanol extracts on the DPPH radical; B: Scavenging activities of different concentrations of *I. viscosa* ethyl acetate extracts on the DPPH radical. Asc Ac: Ascorbic acid; IE: Immouzer ethanol extract; SE: Sefrou ethanol extract; TE: Taounate ethanol extract; IA: Immouzer ethyl acetate extract; SA: Sefrou ethyl acetate extract; TA: Taounate ethyl acetate extract.

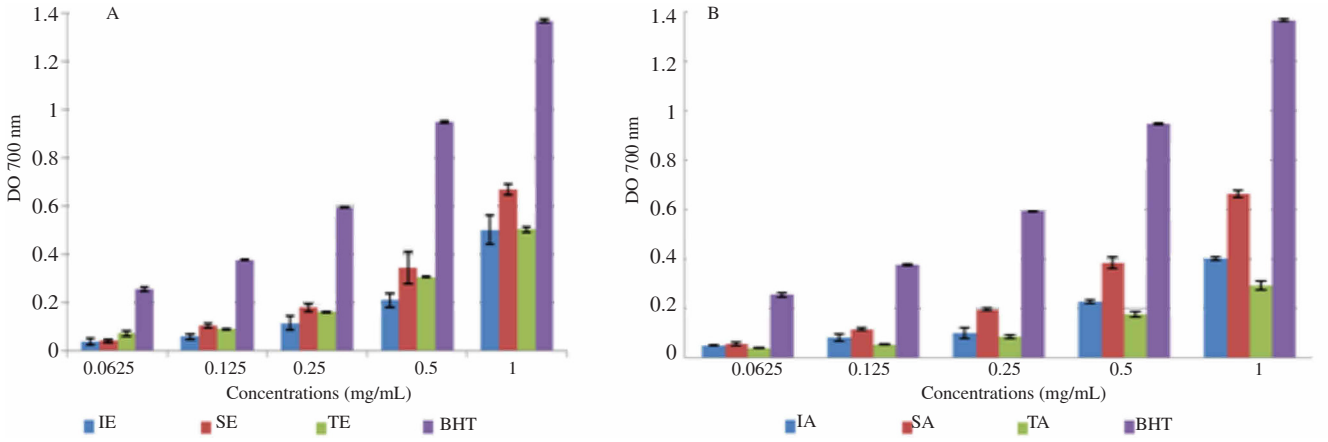

Figure 2. The iron (III) to iron (II) reductive activities for the ethanol and ethyl acetate extract of *I.viscosa* compared to that of BHT. The vertical bars represent±SEM (n=3).
A: The iron (III) to iron (II) reductive activities for the ethanol extract of *I.viscosa* compared to that of BHT; B: The iron (III) to iron (II) reductive activities for the ethyl acetate extract of *I.viscosa* compared to that of BHT.
IE: Immouzer ethanol extract; SE: Sefrou ethanol extract; TE: Taounate ethanol extract; IA: Immouzer ethyl acetate extract; SA: Sefrou ethyl acetate extract; TA: Taounate ethyl acetate extract; DO: Dissolved oxygen.

regions, Taounate showed the weakest reductive power in the assay (Figure 2).

3.5. Total phenolic compounds and flavonoids contents

Total phenol content was determined in comparison with standard gallic acid and the results were expressed in terms of mg GAE/g dry extract. The organic extracts of *I. viscosa* had an important charge of phenols and their values varied widely for both organic solvent used and in the different origin area ranging from 140 to 274 mg GAE/g dry extract. The most extractible solvent of phenols was the ethanol and the highest amount of total phenolics was found in *I. viscosa* from Sefrou with (274.39±6.94) mg GAE/g dry extract, followed by Taounate (176.49±5.49) mg GAE/g dry extract and Imouzzer (170.41±2.04) mg GAE/g dry extract (Figure 3). On the other hand, total flavonoid content has been found equal for the extract of *I. viscosa* from the three regions (Table 4).

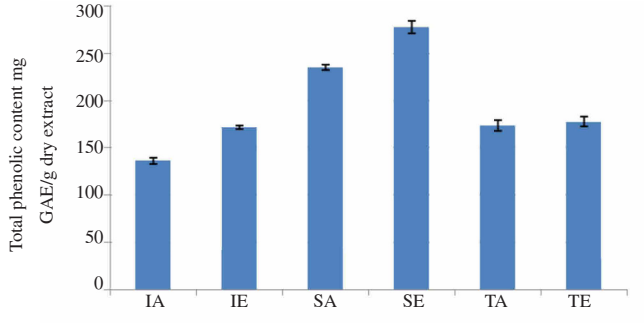

Figure 3. Total phenolic compounds of the different extracts of *I. viscosa*. Concentration of sample was 0.3 mg/mL. Results were expressed as mg GAE/g dry extract. The vertical bars represent±SEM (n=3).
IE: Immouzer ethanol extract; SE: Sefrou ethanol extract; TE: Taounate ethanol extract; IA: Immouzer ethyl acetate extract; SA: Sefrou ethyl acetate extract; TA: Taounate ethyl acetate extract.

Table 4
Total flavonoids contents of *I. viscosa* extracts.

Regions	Samples	QE μg/mg extract
Immouzer	A	44.80±0.14
	E	44.70±0.16
Sefrou	A	44.60±0.07
	E	44.62±0.15
Taounate	A	44.14±0.28
	E	44.70±0.17

A: Ethyl acetate extract; E: Ethanol extract. Assay performed in three replicates and the data were reported as means±SEM. Results are expressed in μg QE per mg of dry extract.

4. Discussion

In order to show the antioxidant potentials of *I. viscosa* and to ascertain the conditions of growth effect in its chemical constituents, the present work is the first comprehensive study of phenolic, flavonoid, and antioxidant activity of selected *I. viscosa* from Morocco. In the studies of selected *Inula* species from Turkey, they found that total phenolic content of three *Inula* species were ranging from (21.1±0.8) to (190.9±6.1) mg GAE/g extract[23], while our study showed the highest value of (274.39±6.94) mg GAE/g extract. It is extremely important to point out that, there was a correlation between antioxidant activity potential and amount of phenolic compounds in all extracts, in agreement with the previous investigation[13,24,25]. The phenolic content estimated in our results was probably responsible for the free radical-scavenging activity of *I. viscosa*. As reported, phenolic compounds were attributed to the overall antioxidant activities[26,27] and they have reserved considered attention because of their physiological function, including cardio protective action[28] and hepatoprotective activity which was mainly attributed to the antioxidant potential that might occur by reduction of lipid peroxidation and cellular damage[29]. Many contributions concerning the chemistry of numerous species of *Inula* have been reported. The members of this genus contain terpenic compounds, especially sesquiterpene lactones, flavonoids, glycolipids and anthranilic acid derivatives[30-33]. In previous studies, chemical analysis showed that *I. viscosa* contained many biologically active compounds, fourteen known and four new compounds were isolated from Jordanian *I. viscosa*[34]. The antioxidant property of *I. viscosa*It has also been reported[35]. Scavenging of different types of reactive oxygen and nitrogen species, mostly free radicals, was exhibited by phenolic phytochemicals. According to work performed by Ortal *et al*[33], they isolated and identified several polyphenolic antioxidants from *I. viscosa* leaves. Therefore, most of our investigated phenolics could be responsible for the potent antioxidant activity of Moroccan *I. viscosa*.

Another way of body defense mechanisms against free radicals was reducing these molecules by the antioxidant substance. Results of reducing power assay were similar as given in the first two systems. The antioxidative effectiveness in natural sources has been reported to be mostly due to phenolic compounds. A strong correlation was found between phenolic contents and antioxidant activities[36]. This study showed that the flavonoid content did not vary relative to the

location of *I. viscosa*. Flavonoids, phenolic compounds and plant phenolics or polyphenols were important components which could be used for the free radical-scavenging activity[37].

Comparing the plant from the three regions, we found that total phenolic contents exhibited the descending order: *I. viscosa* extract from Sefrou followed by *I. viscosa* extract from Imouzzer then *I. viscosa* extract from Taounate. These results showed that the total phenolic contents have an obvious variation according to region. This study also showed that *I. viscosa* extract from Sefrou has the greatest antioxidant properties in all systems of assays, and the highest total phenol content, suggested, as reported previously, that phenolics have been considered powerful antioxidants *in vitro* and proved to be more potent antioxidants. The plant *I. viscosa* is a good source of phenols and natural antioxidants that might have benefits for health. The results of the present work on antioxidant assays justified and partially supported the previous literature data and the popular usage of the tested plants. Further studies are required to investigate the *in vivo* efficacy of these extracts.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

The genus *Inula* has been used in North African traditional medicine to treat diabetes and inflammation, in Jordan to treat tuberculosis, anemia and as cataplasm for rheumatic pain, and it has been used for its antiseptic, skin inflammations properties and gastroduodenal disorder treatment in Spain. They have considered attention because of their physiological function, including cardio protective action and hepatoprotective activity which was mainly attributed to the antioxidant potential that might occur by reduction of lipid peroxidation and cellular damage. Therefore,it is urgent need of new and potent antioxidant agents all over the world.

Research frontiers

The present research work depicted investigation of antioxidant activity, total phenolic and flavonoid content of ethanol (E) and ethyl acetate (A) extracts of *I. viscosa* aerial parts, which were assessed by estimating different three *in vitro* methods: DPPH, phosphomolybdenum and reducing power assays, from three regions in Morocco (Imouzzer, Sefrou and Taounate).

Related reports

The phenolic content estimated was probably responsible for the free radical-scavenging activity of *I. viscosa*. As reported, phenolic compounds were attributed to the overall antioxidant activities. The folklore medicine has evidence of effectiveness of herbs in treating various hepatoprotective activity which was mainly attributed to the antioxidant potential and might occur by reduction of lipid peroxidation and cellular damage.

Innovations and breakthroughs

I. viscosa, locally called "Bageraman", is a medicinal plant used in various ayurvedic formulations to treat diabetes, inflammation, tuberculosis, anemia and as cataplasm for rheumatic pain. It has been used for its antiseptic, skin inflammations properties and gastroduodenal disorder.

In the present study, the authors have demonstrated the antioxidant activity of *I. viscosa* ethanol and ethyl acetate extracts.

Applications

From the literature survey, it has been found that many contributions concerning the chemistry of numerous species of *Inula* have been reported. The antioxidative effectiveness in natural sources has been reported to be mostly due to phenolic compounds.

This scientific study showed that the flavonoid content did not vary relative to the location of *I. viscosa*. The plant *I. viscosa* is a good source of phenols and natural antioxidants that might have health benefits.

Peer review

This was a valuable research work in which authors have demonstrated the the greatest antioxidant properties of *I. viscosa* extracts in all systems of assays.

The activity was assessed based on the phenolics, which have been considered powerful antioxidants *in vitro* and proved to be more potent antioxidant assays that justified and partially supported the popular usage of the tested plants

The highest antioxidant activity was found in the plant from Sefrou and its great biomass in this region suggested that *I. viscosa* is a good source of natural antioxidants compounds that might has benefits for health.

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