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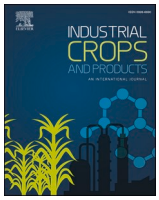

Advances in Sardinian *Withania somnifera* (L.) Dunal crops through phytochemical and biological approaches

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A R T I C L E I N F O

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A B S T R A C T

Withania somnifera (L.) Dunal is widely used in the Indian traditional system of medicine to promote general health, wellness, and longevity. Its pharmacological properties are attributed to a group of molecules called withanolides, among which Withaferin A holds great interest for its anti-carcinogenic action. For this reason, numerous studies in recent years have focused on different metabolic or genetic engineering solutions to increase its yield. Here, we present the Sardinian chemotype of *Withania somnifera* as a potential crop for the extraction of Withaferin A. *W. somnifera* was cultivated from Sardinian wild germplasm collected in the northeast of the island. After 18 months, the leaves and the roots were collected and their methanolic extract was analyzed by HPLC. 0.3 mg/g DW of Withanolide A (WA), 1.0 mg/g DW of Withanolide B (WB) and 17.7 mg/g DW of Withaferin A (WF) were detected in the leaf sample, while lower values were detected in the roots (0.1 mg/g WF, 0.3 WA mg/g, 0.1 mg/g WB, 0.2 mg/g WO). This research not only confirms the high Withaferin A content found in the wild population leaves, but shows how they are reproducible in cultivated specimens, highlighting Sardinian *W. somnifera* leaves as a potential source of high-content Withaferin A products. Finally, we focused on the leaves extract by characterizing the phenolic and flavonoid content, as well as the *in-vitro* antioxidant capacity by DPPH and ABTS assays, revealing a significant amount of phenolic compounds and a related free radical scavenging activity. The leaves extract was further characterized for its anti-aging properties for potential cosmetic application, by the inhibition of tyrosinase, elastase, and collagenase enzymes.

1. Introduction

Due to their innumerable properties and characteristics, herbal medicinal products have gained popularity worldwide. Herbal drugs have a wide range of biological properties, for which they are used in ancient folk traditions and in the pharmaceutical industry (Alves-Silva et al., 2023; Briskin, 2000; Sen and Samanta, 2014). In this context, *Withania somnifera* (L.) Dunal is the leading adaptogenic plant of Ayurveda medicine, which has been used for over 3000 years for its action of promotion of physical as well as mental health (Dar et al., 2015). *W somnifera* is a perennial plant belonging to the Solanaceae family, commonly known as Ashwagandha, Indian ginseng, or winter cherry (Dafni and Yaniv, 1994). The genus *Withania* is named after the English paleobotanist Henry Witham; the epithet "somnifera" is derived from the Latin words "*somnus*" and "*fero*" referring to the plant's relaxing and sleep-promoting properties (Akbar, 2020; Dar et al., 2015). Furthermore, it possesses a plethora of biological properties, ranging from anti-stress activity to actions against cardiovascular, neurodegenerative, cancer, or pathogen-related diseases (Ojha and Arya, 2009; Sehgal et al., 2012; Sharifi-Rad et al., 2021; Stan et al., 2008; Verma et al., 2021).

The well-known phytochemical profile of *W. somnifera* is characterized by the presence of withanolides, steroidal lactone compounds with

Abbreviations: DW, dry weight; GAE, Gallic Acid Equivalent; TFA, Trifluoroacetic Acid; TFC, Total Flavonoid Content; TPC, Total Phenolic Content; QE, Quercetin Equivalent; WA, Withanolide A; WB, Withanolide B; WF, Withaferin A; WO, Withanone.

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C28 ergostano skeleton (Elsakka et al., 1990). Among the above, Withaferin A was the first identified member of the withanolides group (Lavie et al., 1965), which is well distributed in leaves. So far, 35 withanolides, 12 alkaloids, and different sitoindosides have been identified (Kumar et al., 2021).

The pharmacological and medicinal properties of *W. somnifera* have been widely employed for physical and psychological benefits. Among the most remarkable are the anti-cancerous, antidepressant, anti-anxiety, anti-inflammatory, and anti-arthritic properties (Archana and Namasivayam, 1998; Costa et al., 2023; Gupta and Singh, 2014; Gurav et al., 2023; Salve et al., 2019; Stan et al., 2008). Again, its protective role was assessed in neurological disorders like Parkinson's and Alzheimer's, in cardiovascular diseases or also against the infection of SARS-CoV-2, when this compound act as antipyretic and anti-inflammatory (Saggam et al., 2021; Singh et al., 2022). In addition, recent evidence was reported on the ability of *W. somnifera* extract to counteract some behavioural and neurochemical effects of addictive drugs (Bassareo et al., 2019; Kasture et al., 2009; Peana et al., 2014).

Among the withanolides, Withaferin A stands out for its anticarcinogenic action, which was shown to be effective against several types of cancer (Kumar et al., 2023; Sivasankarapillai et al., 2020; Zhang et al., 2023). Therefore, in recent years there has been an increasing body of research on the biosynthetic pattern of withanolides, with a view to increase Withaferin A content, through genetic, molecular, or metabolomic approaches (Choyal et al., 2022; Dhar et al., 2006; Sharma et al., 2023).

Regarding its distribution, *W. somnifera* is currently widespread around the world. It can be found in arid and warm climates like India, Israel, Africa, and America (Hepper, 1991), and in the Mediterranean basin (Scartezzini et al., 2007; Uddin et al., 2012), particularly in Spain, Portugal, and Italy, where the plant is perfectly adapted to the climate and grows spontaneously in Sicily and Sardinia (Rosa et al., 2022; Troia, 1995). The distribution in the Mediterranean area (Uddin et al., 2012) appears to be fragmented and ecological and biogeographical evidence suggest that it is now naturalised. In Sicily, it is found in several locations, such as Siracusa and Trapani (Pasta and Troia, 1994), while Sardinia specimens are found only in the Northeast of the Island.

Only one study has focused on the Sardinian *W. somnifera* specimens, evidencing a high concentration of Withaferin A (Scartezzini et al., 2007). Such approach, however, mainly compared the Sardinian with the Sicilian and the Indian chemotypes. Therefore, the study conducted by Scartezzini et al. (2007), represents an important starting point for the Sardinian chemotype analysis. However, there are several limitations from an analytical point of view, and in choosing to analyse only *W. somnifera* leaves, and only one metabolite without dealing with a full characterization of the withanolides. Therefore, considering the difference exhibited by the Sardinian specimens, this study analyzes the phytochemical profile of the cultivated *Withania somnifera* from wild germplasm, leaves and roots. The main objectives are first to confirm the high Withaferin A content also in the cultivated samples, in order to highlight *W. somnifera* as a potential crop, without affecting the wild population. For the first time, the authors develop a simple HPLC method for the standard quantification of Withaferin A, Withanone, and Withanolide A and B from cultivated Sardinian *W. somnifera* leaves and roots. We then focused on a further characterisation of the leaves extract, considering both the high content of Withaferin A found, and since they allow a sustainable, non-destructive harvesting of the entire plant. We performed a characterization of the relevant components of the extract through phenolic and flavonoid total quantification. Then, we assessed its antioxidant capacity by *in-vitro* assays and evaluated its cosmetic potential by performing tyrosinase, collagenase, and elastase inhibition.

2. Material and methods

2.1. Plant material and crop conditions

Wild germplasm of *W. somnifera* was collected from the only recognized population, in the Dorgali area, northeast of the Sardinian island (Scartezzini et al., 2007). A plant specimen was used for botanical accessions Herbarium CAG 990/V1 (University of Cagliari). After the collection, seeds were sown in November 2020 in a growth chamber (Angelantoni Scientifica, ABOT 29 CF) in standard conditions (12/12 light/dark photoperiod, 21± C° temperature). The seeds were monitored daily, showing 95% germination after 28–32 days (Supplementary data Fig. S1). Then, the seedlings were transferred in Planta Medica Greenhouse. In March 2021, *W. somnifera* plants were planted in open fields, in 2 m x 6 m beds, distance between replications 1.5 m, distance between each plant 0.45 m. The climate is typically Mediterranean, with hot summers and mild, relatively rainy winters (Supplementary data, Table S1) (Canu et al., 2015). Precipitation ranges from 74.42 mm in April 2021 to 231,67 mm in November 2021, while the average annual measured temperature ranges from 27.8 °C in August 2021 to 8.7 °C in January 2022 (Supplementary data Fig. S2). The soil type was a well-drained clay loam light-brown soil with a pH of 7–7.5 (in H_2O) (Sardinia Geodatabase, (SarSardegna Geoportale, 2023) (Supplementary Data, Table S1).

Considering the adaptation of *W. somnifera* to the Sardinian climatic conditions, water was only provided in case of severe drought and especially during the periods of planting, flowering and fruit ripening. No pesticide treatments or fertilization were used.

After 18 months of growth (May 2022) leaves were collected from 20 different individuals and placed in an air-forced ventilation oven at 40 °C (FD 115, BINDER) for 48/72 h and vacuum-stored. The dried leaves were then ground (0.1–0.5 mm) with an electric grinder. Root samples were collected from different plants and processed in the same way as leaves. Then, leaves and root samples were subjected to the extraction process.

2.2. Extract preparation

2 g of leaves and roots samples of *W. somnifera* were subjected to three exhaustive extractions with a total volume of 20 ml of 70:30 w/w methanol:water, and sonicated for 15 min at 22 ±1 °C. The samples were filtered through Whatman No. 1 filter paper and the solvents recovered using a rotary vacuum evaporator at 40 °C. To completely remove the water, and allow better preservation of the extract, the samples were frozen and freeze-dried (LIO 5PDGT lyophylizer, Cinquepascal S.r.l., Trezzano, with nXDS6i pump, Edwards Limited, Czech Republic). The obtained extracts were then redissolved in 2 ml of methanol (HPLC-grade), filtered by 0.45 μm filters, and then analyzed. The extraction was performed in duplicate.

2.3. HPLC analysis

HPLC analysis was performed using an HPLC chromatography system (1260, Agilent Technologies) equipped with a quaternary pump, a diode array detector operating at 225 nm, and an auto injector. Separation was achieved using a water reversed phase column (Zorbax Eclipse XDB-C18 RaPID Res 250 ×4.6 mm I.D.). The mobile phase consisted of Water (Solvent A) and Acetonitrile (Solvent B), both containing 0.01% of Trifluoroacetic Acid (TFA) (Table 1). The chromatographic run was performed at room temperature, at 1.0 ml/min of flow rate, and 5 μl of sample were injected. The gradient programme used for HPLC analysis is reported in Table 1.

Withaferin A, Withanolide A, Withanolide B, and Withanone (99.99% purity, Sigma Aldrich Milan, Italy) were used as standards and used for the preparation of the stock solutions (Supplementary data Fig. S3, Table S2). From the stock solutions, dilutions at different

Table 1
Gradient programme used for HPLC analysis.

Time (min)[a]	Solvent A (%)[b]	Solvent B (%)[b]
0 – 4.5	40	60
4.5 – 9	60	40
9 – 18	30	70
18–20	40	60

[a] Time is referred to the starting time of the HPLC gradient.
[b] Solvent A and B contained 0.01% of TFA.

concentrations were prepared by addition of MeOH, to final concentrations of 370, 270, 180, 90, 45, 30, and 15 μg/ml for Withaferin A and Withanolide A, and 202.50, 135, 67.50, 33.75, 22.50 and 11.25 μg/ml for Withanolide B and Withanone.

All the standard dilutions were then stored at 4 °C and were stable for at least 30 days. Calibration curves (Fig. 1) were obtained by comparing the concentration with respect to the response of the peak area, according to the equation: Area=m × concentration, in which *y* axis corresponds to the peaks area in mAU/s and *x* axis to the concentration expressed in μg/ml.

2.4. Total phenolic and flavonoid content

The Total Phenolic Content (TPC) of the leaves extract was measured by a colorimetric method reported in the literature (Everette et al., 2010) with fine/thin modifications, using the Folin-Ciocalteu reagent. Briefly, 20 μl of the sample extract was transferred to a glass test tube, and 1.58 ml of deionized water and 100 μl of Folin-Ciocalteu's reagent were added. The solution was stirred for a few seconds and incubated for 5 min. After the incubation, 300 μl of Na_2CO_3 solution (20% w/v) were added, mixed, and incubated for 2 h. The absorbance of the solution was measured at 765 nm by UV–VIS spectrophotometer (HONDA UV-31 SCAN). The calibration curve was set using gallic acid standard solutions with a concentration of 50, 100, 150, 250, and 500 μg/ml. Therefore, the content of extract phenolic compounds is expressed as mg of gallic acid equivalent (mg GAE)/ g dry sample.

The Total Flavonoid Content (TFC) in *W. somnifera* leaves was determined using the aluminium chloride method (Tuah et al., 2017). Flavonoids were measured by mixing in a test tube: 200 μl of the diluted methanolic extract with 40 μl of 10% (w/v) $AlCl_3$, 1.12 ml of water, 600 μl of MeOH, 40 μl of 1 M CH_3COOK. The samples were incubated for 30 min in the dark, absorbance was read at 415 nm. The Flavonoid content of dry leaves was estimated quantitatively and expressed in mg of quercetin equivalent (mg QE)/ g dry sample.

The TPC and TFC tests were performed in triplicates, and the absorbance was read in triplicates for each repetition. All the solutions were incubated and stored at 4 °C while the tests were carried out under semi-darkness conditions in a cold room.

2.5. Antioxidant capacity

Total antioxidant capacity was determined through DPPH assay and ABTS assay. The DPPH scavenging activity was determined according to Brand-Williams et al. (1995) with a slight modification. Briefly, 0.1 mM methanol solution of DPPH (1,1-diphenyl-2-picrylhydrazyl) was prepared and diluted to a final absorbance of 0.7 AU. Then, 3 ml of DPPH solution was added to 80 μl aliquots of various concentrations of *W. somnifera* leaves extract in methanol.

Eight different concentrations of the *W. somnifera* leaves extract were tested, from 8 to 1 mg/ml, respectively. The mixture was vortexed vigorously and incubated for 30 min at room temperature in dark conditions. After the incubation, the mixture was shaken, and 0.5 ml was poured into a cuvette for the spectrophotometer measurements. The absorbance of the solutions was then measured at 517 nm and compared with a DPPH solution without the sample. The free-radical scavenging percentage was calculated from the results and expressed as IC_{50}, the amount of antioxidant required to reduce the initial DPPH concentration of 50%. To further verify sample interference, each concentration to be tested was measured in the spectrophotometer (80 μl of sample and 3 ml of methanol). The control used was a TROLOX solution prepared in the same way as the samples.

ABTS radical cation scavenging activity was measured according to Li et al. (2008) with some modifications. The $ABTS^{\bullet+}$ solution was prepared by the reaction of the same quantities of a 7 mM aqueous ABTS solution with 2.45 mM potassium persulfate ($K_2S_2O_8$) solution. After

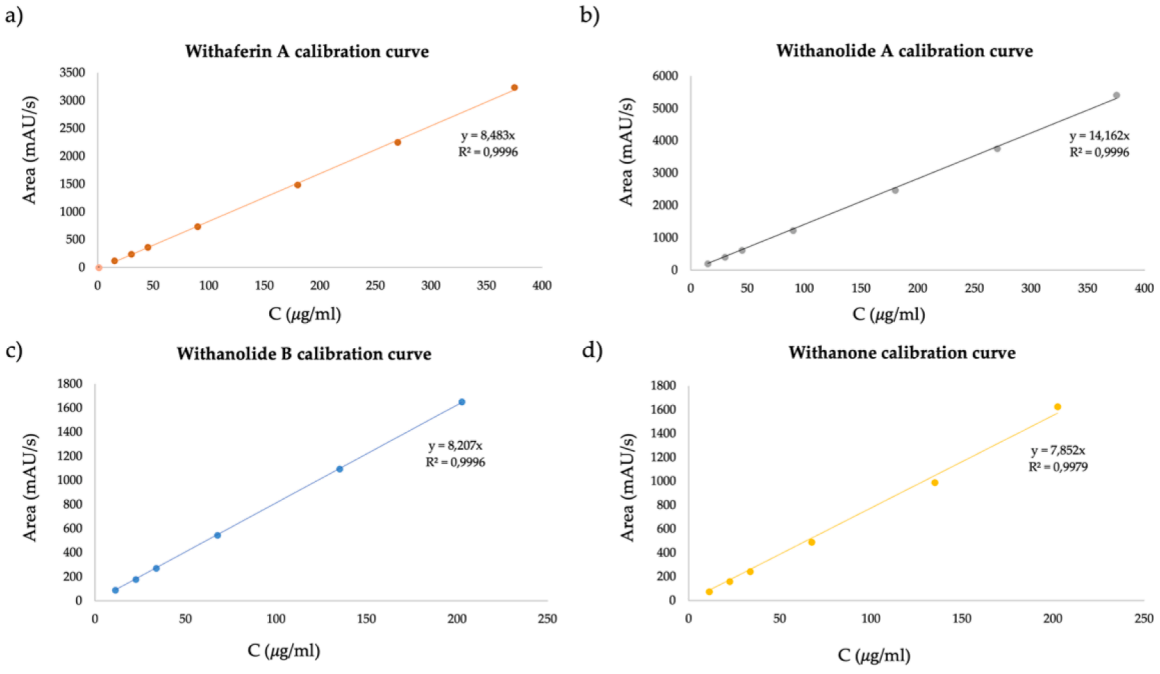

Fig. 1. Calibration curves of the standard compounds. The dots represent the measured values, the line the obtained Regression curve. a) Withaferin A calibration curve, b) Withanolide A calibration curve, c) Withanolide B calibration curve, d) Withanone calibration curve.

16 h of incubation in the dark, the solution was diluted to reach a final absorbance of 0.7 AU. Subsequently, 3.920 ml of $ABTS^{\bullet+}$ working solution and 80 µl of each sample dilution were mixed and incubated in the dark for 6 min. The mixture was shaken, and 0.5 ml was poured into a cuvette for the spectrophotometer measurements. The absorbance of the solutions was then measured at 734 nm against an $ABTS^{\bullet+}$ solution without the sample. The standard, used as a control, was a TROLOX solution. For each tested concentration the sample interference was measured.

2.6. Enzymatic inhibition

The results of all the assays described below were expressed as a percentage of the blank control. The percentage of inhibition of enzyme activity inhibition was calculated as (%) by the following equation:

$$\frac{A-B}{A} \times 100 \quad (1)$$

where A represents the difference in the absorbance of the control sample between an incubation time of 0.5 and 1.0 min, and B represents the test sample's absorbance difference in the same time range. Data from activity experiments were recorded using an Ultrospec 2100 spectrophotometer (Biochrom Ltd., Cambridge, UK).

The leaves extract of *W. somnifera* and the standard of Withaferin A, used as standard in HPLC analysis, have been used for all inhibitory activities.

2.6.1. Tyrosinase inhibition assay

The tyrosinase inhibitory activity of the samples was analysed using 3,4-dihydroxy-L-phenylanine (L-DOPA), as previously described (Di Petrillo et al., 2018) with slight modifications. The reaction solution containing phosphate buffer (50 mM, pH 6.5) and mushroom tyrosinase (72 U/ml), with or without test samples (0.5 mg/ml), was pre-incubated for 10 min at room temperature and mixed with L-DOPA (0.5 mM). After adding the substrate, the activity was monitored at 492 nm for dopachrome formation in the reaction mixture.

2.6.2. Collagenase inhibition assay

Collagenase from *Clostridium histolyticum* (1 U/ml) was prepared in 50 mM Tricine, 10 mM calcium chloride, 400 mM sodium chloride buffer at pH 7.5; the enzyme was incubated with the extract (0.1 mg/ml) for 15 min, and, once the N-(3-[2-Furyl]-acryloyl)-Leu-Gly-Pro-Ala (FALGPA) substrate (1.6 mM) was added, the absorbance was registered at 340 nm (Era et al., 2021).

2.6.3. Elastase inhibition assay

The inhibition elastase was determined by measuring the production of *p*-nitroaniline during the reaction of the enzyme with the N-Succ-(Ala) 3-nitroanilide (SANA) substrate. A reaction mixture containing porcine pancreatic elastase (3.3 g/ml) in 0.1 M Tris- HCl buffer (pH 8.0) was incubated with or without the test sample (0.1 mg/ml) for 20 min. After adding the SANA 1.6 mM, the enzyme activity was monitored at 410 nm.

3. Results

3.1. Plant material

The seeds were collected from the Sardinian wild germplasm. After 28–32 days, they showed a 95% germination rate (Supplementary data Fig. S1). The plants were easily cultivated in the open field; Fig. 2 shows a plant example specimen.

3.2. Extract preparation and HPLC analysis

The sample preparation was optimized by an exhaustive extraction in methanol, to allow an immediate and easy comparison with the majority of *W. somnifera* studies. The methanolic leaves and roots extracts were then characterized by HPLC. The HPLC method developed was effective, allowing us to evaluate the concentration of the main known metabolites in *W. somnifera* leaves and roots: Withaferin A, Withanolide A, Withanolide B, and Withanone (Fig. 3).

The analysis of the leaves extract evidenced the presence of Withanolide A and B in the Sardinian specimens, in average with those found in other studies, and respectively of 0.3 and 1.0 mg/g DW. The high concentration of Withaferin A reported for the wild Sardinian *W. somnifera* chemotype (Scartezzini et al., 2007) was also confirmed here in the cultivated specimens, where we quantify 17.77 mg/g of Withaferin A in dry samples. We also reported that the concentration of Withanone was under the detection limit. The results are reported in Table 2. After characterization, the same extract was tested to assess its relative antioxidant capacity and phenolic and flavonoid content

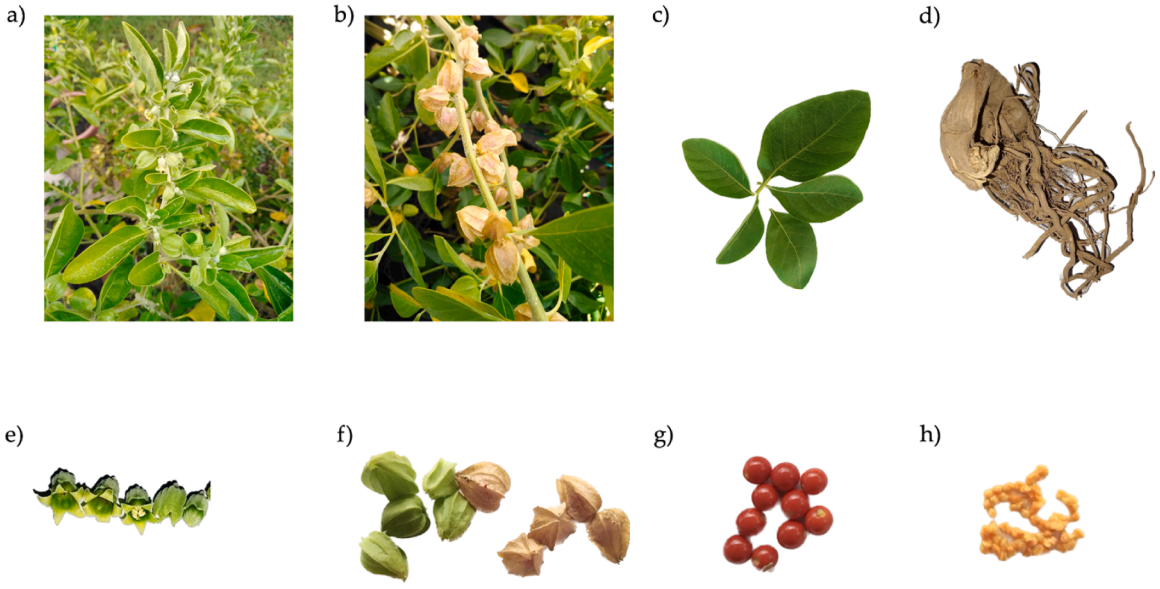

Fig. 2. Different parts of cultivated *W. somnifera* specimen. a) vegetative stage (15 months); b) fruitification stage; c) leaves; d) dry roots (18 months); e) flowers; f) unmatured (green), and matured fruits (brown) with accrescent calyx; g) berries; h) seeds.

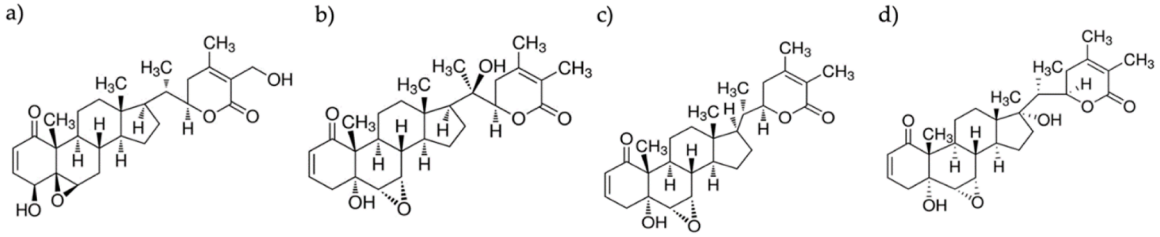

Fig. 3. Structure of the main *Withania somnifera*'s compounds, which concentrations in the methanolic leaves and roots extracts were assessed through the HPLC protocol. a) Withaferin A; b) Withanolide A; c) Withanolide B; d) Withanone.

Table 2
Concentration of Withanolides in *Withania somnifera's* leaves and roots methanolic extract.

Compound	Leaves sample		Roots sample	
	Conc. (mg/g DW)[a]	Percentage % (w/w)[b]	Conc. (mg/g DW)[a]	Percentage % (w/w)[b]
Withaferin A	17.7 ± 0.011	1.77	0.1 ± 0.002	0.01
Withanolide A	0.3 ± 0.001	0.03	0.3 ± 0.003	0.03
Withanolide B	1.0 ± 0.002	0.1	0.1 ± 0.001	0.01
Withanone	Not detected	-	0.2 + 0.002	0.02
Total Withanolides quantified	**18.9**	**1.89**	**0.7**	**0.07**

[a] Concentrations are expressed in mg/g dry leaves and roots, based on the calibration curves of each compound reported in the Methods section. The reported values are expressed by the means of three replicate standard deviation (SD).
[b] Percentage (w/w) are the mean value expressed in grams of compound in 100 g.

(Homayun et al., 2016). The obtained chromatogram of the leaves extract, and its main peaks are reported in Fig. 4.

The results of HPLC analysis of the root extract showed significantly lower amounts of total withanolides with respect to the leaves (Fig. 5). This is due to the Withaferin A and Withanolide B content, in both cases higher in the leaves (Table 2). In contrast, an opposite trend is shown by Withanone content, which was below the detection limits in the leaves, while was detected at a concentration of 0.2 mg/g DW in the roots (Table 2).

Compared with the literature, the withanolide content of the roots appears lower (Sabir et al., 2013; Khajuria et al., 2004; Malik et al., 2007).

3.3. Total phenolic and flavonoid content

The leaves extract was subsequently tested for its Total Phenolic Content (TPC) and Total Flavonoid Content (TFC). TPC and TFC were calculated on the basis of the calibration curve of the standards, respectively Gallic Acid and Quercetin, and then expressed in equivalent

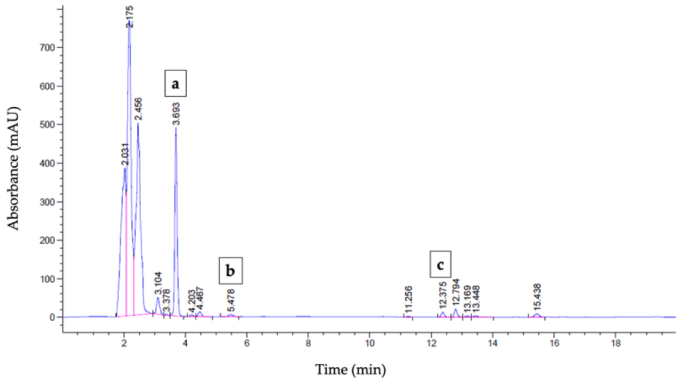

Fig. 4. HPLC chromatogram of *Withania somnifera*'s leaves methanolic extract. The number above each peak indicates the retention time, while the letter indicates the compound: a) Withaferin A peak; b) Withanolide A peak; c) Withanolide B peak. No Withanone was detected.

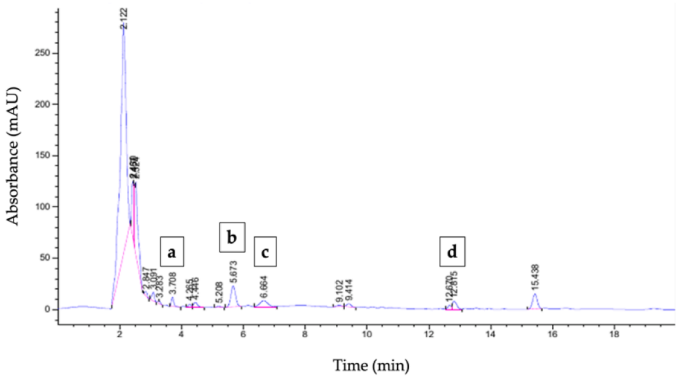

Fig. 5. HPLC chromatogram of *Withania somnifera's* roots methanolic extract. The number above each peak indicates the retention time, while the letter indicates the compound: a) Withaferin A peak; b) Withanolide A peak; c) Withanone peak; d) Withanolide B peak.

mg of standards / g of dry material. The obtained results are reported in Table 3.

3.4. Antioxidant capacity

After the HPLC characterization and the evaluation of phenolic and flavonoid compounds, the leaves extract was then tested for its *in-vitro*

Table 3
Phenolic and Flavonoid contents of the methanolic extract of *Withania somnifera* leaves.

Total Phenolic and Flavonoid content (± SD)		
	TPC[a]	**TFC**[b]
***W. somnifera* leaves extract**	178.19 ±4.49	8.45 ± 0.04

[a] TPC is expressed in mg GAE/g dry material, based on the Gallic Acid calibration curve (y = 1.1579x+0.0148, with R^2 = 0,9957);
[b] TFC is expressed in mg QE/g dry material, on the basis on the Quercetin calibration curve (y = 6.7451x+0,0126, with R^2 = 0.9996).

antioxidant capacity with ABTS and DPPH assays. The Sardinian wild chemotype was reported to have major antioxidant activity with respect to the other chemotype (Scartezzini et al., 2007). The results showed a good scavenging activity and are expressed in IC_{50} (half maximal inhibitory concentration), in Table 4.

3.5. Enzymatic inhibition

To evaluate the potential anti-aging use of the cultivated Sardinian *W. somnifera* leaves extract, we tested its inhibitory activity against the principal enzymes contributing to the skin-aging process. The effect of *W. somnifera* leaves extract on tyrosinase, collagenase, and elastase inhibition is reported in Table 5. The results show that the extract has an inhibitory activity, even if it shows different efficiency, which is higher in elastase inhibition, and progressively lower with collagenase and tyrosinase inhibition, respectively. Notably, with respect to the other studies, here we evidenced the inhibition activity of the extract against all the three tested enzymes. The inhibitory activity of Withaferin A, the compound most present in the extract, was evaluated under the same experimental conditions. In the presence of the standard compound, the activity of the enzymes considered was unchanged to the control (data not shown).

4. Discussion

Withania somnifera (L.) Dunal, known by the Indian name Ashwagandha, is a perennial plant used since ancient times in the Ayurveda and Unani systems (Gurav et al., 2023; Uddin et al., 2012). Considering its wide territorial spread worldwide, several attempts have been made to reconstruct the phylogenetics of the different strains and, consequently, the different chemical characteristics (Dhar et al., 2006; Kalra and Kaushik, 2017; Nittala and Lavie, 1981). It is now well established from various studies that there are three different chemotypes (Kalra and Kaushik, 2017). According with this classification, the Sardinian *W. somnifera* belongs to the Chemotype III, with low Withaferin A content. However, Scartezzini et al. (2007) showed a high Withaferin A content in the Sardinian specimens. The latter is the only study that focuses on Sardinian chemotype profile, with the limitation of mainly highlighting the difference with Indian and Sicilian samples, without any further characterization (Scartezzini et al., 2007).

To fill the gap in this knowledge, here we analyze the chemical profile of Sardinian *W. somnifera* leaves and roots, focusing on withanolides characterization.

The developed HPLC protocol allowed us to characterize the Sardinian chemotype for their content in Withanolides. In the leaves, the Withanone's content was under the detection limit, while average concentration of Withanolide A and Withanolide B were detected, also considering that they are more abundant in the roots than in the leaves (Girme et al., 2020; Kumar et al., 2020; Nile et al., 2019; Girme et al., 2020; Kumar et al., 2020; Nile et al., 2019; Tomar et al., 2019).

Surprisingly, the Withaferin A content here found appears higher than those reported in the literature (Pandey et al., 2018). In fact, we obtained double or triple values when considering chemotypes known for high Withaferin A concentration. When considering low/medium concentration chemotypes, our values appear much higher (30–5 times higher) (Kaur et al., 2021; Pandey et al., 2018).

Table 4
Antioxidant capacity of *W. somnifera* leaves extract.

Free-radical scavenging - IC_{50} (μg/ml)		
	DPPH scavenging[a]	**ABTS scavenging**[b]
***W. somnifera* leaves extract**	127.67 ±5.68	48.86± 2.32

[a]DPPH and ABTS scavenging are expressed in μg/ml; the results are expressed by the means of three replicates ± standard deviation (SD).

Table 5
Inhibitory activity of *W. somnifera* leaves extract on the selected enzymes.

	Enzymatic inhibitory activity (Inhibition % ± SD)		
	Tyrosinase[a]	Collagenase[b]	Elastase[b]
***W. somnifera* leaves extract**	21.9 ± 0.2	16.3 ± 0.3	25.5 ± 3.9

[a] *W. somnifera* extract at 0.5 mg/ml
[b] *W. somnifera* extract at 0.1 mg/ml.

In the root extract all the tested withanolides were found. However, the extract showed a marketed reduction in the withanolides content, amounting to a total of 0.07% (g/100 g). The main difference is observed in Withaferin A content (from 1.77% to 0.01%), and Withanolide B content (from 0.1% to 0.01%). For this reason, further characterizations were carried out on the leaf samples, which have a higher concentration of withanolides.

There may be multiple explanations for the high Withaferin A content of Sardinian specimens of *W. somnifera*. Firstly, it may be due to the different genetic structure, as evidenced by Scartezzini et al. (2007). On the basis of genetic analyses comparing the Sicilian and Indian populations, they assumed that the Sardinian colonisation of *W. somnifera* probably has an older origin in respect to the Sicilian one, therefore this species is no longer to be considered autochthonous but naturalized (archephyta). On the other hand, Sardinia is part of the Mediterranean biodiversity hotspot, which covers 2% of the earth's surface, but accounts for 60% of endemisms (Cocco et al., 2022; Lopez-Alvarado and Farris, 2022; Sanna et al., 2020). Therefore, it is also possible that the increase in this metabolite is due to interaction with the environment and its biotic and abiotic factors. In fact, it should also be noted that some research shows that high concentrations of Withaferin A were induced by interaction with endophytic fungi (Pandey et al., 2018), which could indeed produce some metabolites of the withanolides class themselves (Sathiyabama and Parthasarathy, 2018).

Previous studies on *W. somnifera* showed an increase in the flavonoid and phenolic content in response to oxidative stress stimuli (Fernando et al., 2013; Polumackanycz et al., 2023; Tabassam et al., 2020). Here, the cultivated specimens appeared characterized by a significant amount of phenolic compounds.

Furthermore, taking into account the scavenging action of phenols and flavonoids metabolites, we tested the antioxidant activity of *W. somnifera* leaves extract through preliminary *in-vitro* assay (Kandil et al., 1994; Singh. et al., 2013). The assays showed good antioxidant activity and good reproducibility among the replicates. Considering the lower flavonoids quantity found in the extract, this action can be addressed to the well-known action of phenols.

For the first time, the inhibitory effect of *W. somnifera* leaves extract on three enzymes involved in skin aging is highlighted in this study. The extract proved to be a multitarget inhibitor, even if not with maximum efficiency on the three enzymes.

Our results showed that the inhibitory effect is not attributable to the Withaferin A present in the extract. Therefore, it is possible to attribute the obtained effect to the other component (e.g. withanolides, phenolic or flavonoid compounds) or consider the synergistic effects of all the compounds present in the extract.

Regarding the anti-aging properties, Chiocchio et al. (2018) studied the inhibitory effect of *W. somnifera* against elastase and tyrosinase, but on the root, evidencing no inhibitory activity (respectively 4% and 0% inhibition). Our results demonstrated that the leaves' extract modestly inhibited all three enzymes tested. The simultaneous effect on the three crucial enzymes in the treatment of skin aging could be enhanced by fractionation and separation of the extract.

5. Conclusion

The current study provides the first available characterization of the cultivated Sardinian *W. somnifera* (L.) Dunal. Compared to current literature, here we provide the first characterisation of both roots and leaves, analysing the content in different withanolides. Moreover, we demonstrated that the leaves from cultivated specimens maintain a high content of Withaferin A.

In addition to the high content of this anticarcinogenic metabolite, we focused on the leaves extract with a view of sustainable exploitation, as the collection of the plant material does not involve the eradication of the entire plant.

The preliminary characterization by *in-vitro* tests revealed *W. somnifera* as a good source of antioxidant molecules, and phenolic compounds. Leaves extract showed multi-target anti-aging activity, however, not attributable to Withaferin A. Because the bioactive compounds in the plant material are multi-component mixtures, further studies on *W. somnifera* are needed to identify and isolate its active compounds.

In conclusion, the present study allows the identification of Sardinian *Withania somnifera* as a crop species, relevant especially for its high Withaferin content, even in the cultivated specimens. However, to improve cultivation and characterization, the next steps will be the analysis of seasonality, and the test of eco-friendly solvents such as ethanol.

Domestication of new genetic resources rich in secondary metabolites of economic interest could also have repercussions in the social field by implementing the local economy and enhancing the Sardinian territory.

These steps may enable the large-scale production of high-quality plant material for pharmaceutical, cosmetic or nutraceutical purposes and over time promote the conservation *in-situ* of the wild population of Sardinian *Withania somnifera* through targeted reintroduction.

CRediT authorship contribution statement

Delia Maccioni: Conceptualization, Investigation, Writing – original draft preparation. **Danilo Falconieri**: Investigation, Data curation. **Elio Acquas**: Funding acquisition, Writing – review & editing. **Sanjay Bhaskar Kasture**: Resources, Writing – review & editing. **Benedetta Era**: Validation, Writing – review & editing, Visualization. **Antonella Fais:** Validation, Writing – review & editing, Visualization. **Emma Cocco:** Conceptualization, Investigation, Data curation, Writing – original draft preparation, Visualization. **Andrea Maxia**: Resources, Writing – review & editing, Supervision, Funding acquisition.

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.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2023.117887.

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