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Genetic and phytochemical difference between some Indian and Italian plants of *Withania somnifera* (L.) Dunal

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The geographical distribution of *Withania somnifera* (L.) Dunal is quite wide. However, in Italy, this species is very rare and grows spontaneously only in Sicily and in Sardinia. The PCR–RAPD technique has been utilized in this work to determine the genetic relationship among Sicilian, Sardinian and Indian samples and the HPLC analysis of whitaferin A was used as a marker to evaluate the phytochemical differences. The genetic difference between Indian and Sicilian plants of *W. somnifera* turned out to be smaller than that between Indian and Sardinian plants of this species. The phytochemical analysis as well showed that the Sardinian specimen strongly differed from the Indian and Sicilian ones in its contents of withaferin A. Our results seem to confirm the hypothesis that the Italian populations of this species may not be indigenous but naturalised. Due to the high withaferin A content of the Sardinian samples, these plants could be used as a source for pharmaceutical purposes.

Keywords: W. somnifera; Āyurveda; Withaferin A; Random Amplified Polymorphic DNA; HPLC; Antioxidant activity

1. Introduction

Withania somnifera (L.) Dunal is a small-medium undershrub belonging to the Solanaceae family. It is one of the most valued herbs in Ayurvedic medicine and for that reason it is used and largely cultivated in India. In Europe, this plant was first mentioned in 1683 by Hendrik Adriaan von Rheede tot Draakestein in his *Hortus Malabaricus*; he called it "Pevetti" and mentioned an ointment prepared with its leaves. Recently, in Italy, Pignatti, in 1982, called it "False alchechengi" because of the presence of a considerably expanded calyx around the fruit that is similar to that of *Physalis alkekengi* L. [1]. In Western regions, the use of this plant was limited and now completely forgotten. Conversely, in Āyurveda, the Indian traditional medicine, the roots of *W. somnifera* are the main constituent of many preparations [2]. According to the main ayurvedic classical texts, this plant is described as tonic, anabolic, useful in

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malnutrition, dysfunctions of the reproductive system, mental disorder (is used as tonic of the nervous system), as an aphrodisiac, a sedative, Medhya Rasāyana (''what promotes learning and a good memory''), and in case of pain, arthritis and inflammation and also geriatric problems. Pharmacological studies (adaptogenic, antiarthritic, anti-inflammatory, analgesic, antipyretic, antioxidant and immunostimulatory activity) confirmed many traditional uses of *W. somnifera*, as reviewed by Scartezzini and Speroni [2]. The most important phytochemical compounds in *W. somnifera* are withanolides and the most important among those ones is withaferin A, a highly oxygenated steroidal lactone. Withaferin A is mainly found in the leaves of the plant and it has recently been reported to have a potent anti-angiogenic activity *in vivo* at very low doses [3].

On the basis of the data found in the literature and in the specimens of the Herbarium of Florence, Troìa [4] has made a map showing the geographical spotted distribution of the species around the Mediterranean Sea. The interest on this species has been motivated on one hand by the very rare presence in Italy as a spontaneous plant and, on the other hand, by its content in phytochemical compounds with important pharmacological activities, in particular withaferin A. In the present study, we have analysed plants grown in different areas of Italy (in particular Sicily and Sardinia) through a genetic and phytochemical approach, and we have compared them with some Indian samples. Our aim was to identify any genetic and phytochemical diversities among plants grown in different areas, in order to eventually assign, as a preliminary approach, different chemotypic clustering.

To this purpose, the Random Amplified Polymorphic DNA (RAPD) technique has been utilised to determine genetic relationship, and the HPLC identification of withaferin A was used as a marker to evaluate the phytochemical differences.

2. Results

2.1. *Genetic data*

A preliminary screening was carried out with the aim of identifying the optimal conditions for PCR amplification. Sequences of the primers, annealing temperatures, electrophoretic conditions and size range of the amplification products are reported in table 1. For each of the six primers tested, optimal temperatures ranged from 47 to 58°C. These values, which turned out to be higher than those usually reported in standard protocols for RAPD markers (36–37°C), allowed to obtain unambiguous and more defined electrophoretic bands with a high reproducibility.

The six primers gave a total of 43 reproducible RAPD loci that have been used in the subsequent statistical analysis for their ability to discriminate the samples examined. An example of the RAPD profile generated by one primer used in this study is presented in figure 1. The matrix of fragment/primer combinations for the samples is available from the authors upon request.

2.2. *PCA and cluster analysis of genetic data*

RAPD data were subjected to cluster analysis and Principal Component Analysis (PCA), as described in the Experimental section. The matrix constructed with RAPD

Table 1. List of primers used and their sequences.

Primer	Sequence	Annealing temperature (°C)	Electrophoretic run (Vh)	Size (bp) min–max
1	5′-GGTGCGGGAA-3′	53	68	174–318
2	5′-GTTTCGCTCC-3′	51	64	182–437
3	5′-GTAGACCCGT-3′	49	66	183–298
4	5′-AAGAGCCCGT-3′	56	67	160–356
5	5′-AACGCGCAAC-3′	47	65	163–365
6	5′-CCCGTCAGCA-3′	58	66	200–629

Note: Annealing temperature, length of electrophoretic run and band size range settled for each primer are indicated.

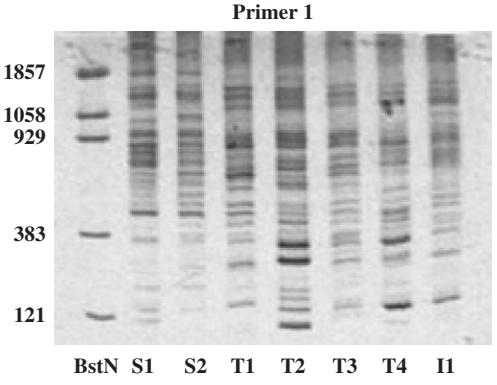

Figure 1. RAPD profiles in *W. somnifera* individuals using primer 1. Lane 1: DNA size marker in base pairs (pBR322 DNA-BstN I Digest); lanes 2–7: samples from Sardinia (S.1, S.2), from Sicily (T.1, T.2, T.3, T.4) and from India (I.1).

data was used to perform the cluster analysis by Nei and Li's and Jaccard's similarity coefficients and a dendrogram was generated to verify the genetic distance among all the samples (figure 2).

In the matrix of genetic distances calculated by Nei and Li's index, the values ranged from 0.28571 to 0.95652, while those calculated by Jaccard's index, the values were between 0.16667 and 0.91667.

As can be seen, the Sardinian samples appeared to be separated from both the Indian and Sicilian samples (figure 2). These later joined together in the same cluster (figure 2).

With regard to PCA analysis, the first principal component accounted for 26.49% of the variation, the second accounted for 23.00% and the third for 13.49%. In the relation between the first and second principal component $(1+2)$, as well as in that between the first and third principal components $(1+3)$, the Sardinian group sample appeared to be clearly separated from both the Indian and Sicilian groups (data not shown). This result was consistent with the cluster analysis.

It is clear from the dendrogram that the Sardinian samples branched out from the remaining Sicilian and Indian samples at a low similarity coefficient of approximately 0.4. The tie of Sardinian samples show a high similarity coefficient value close to 1 and this clearly indicated that these individuals were derived from the same mother plant. The Sicilian samples were arranged in two major clusters and the similarity coefficient ranged from 0.74 to 0.94.

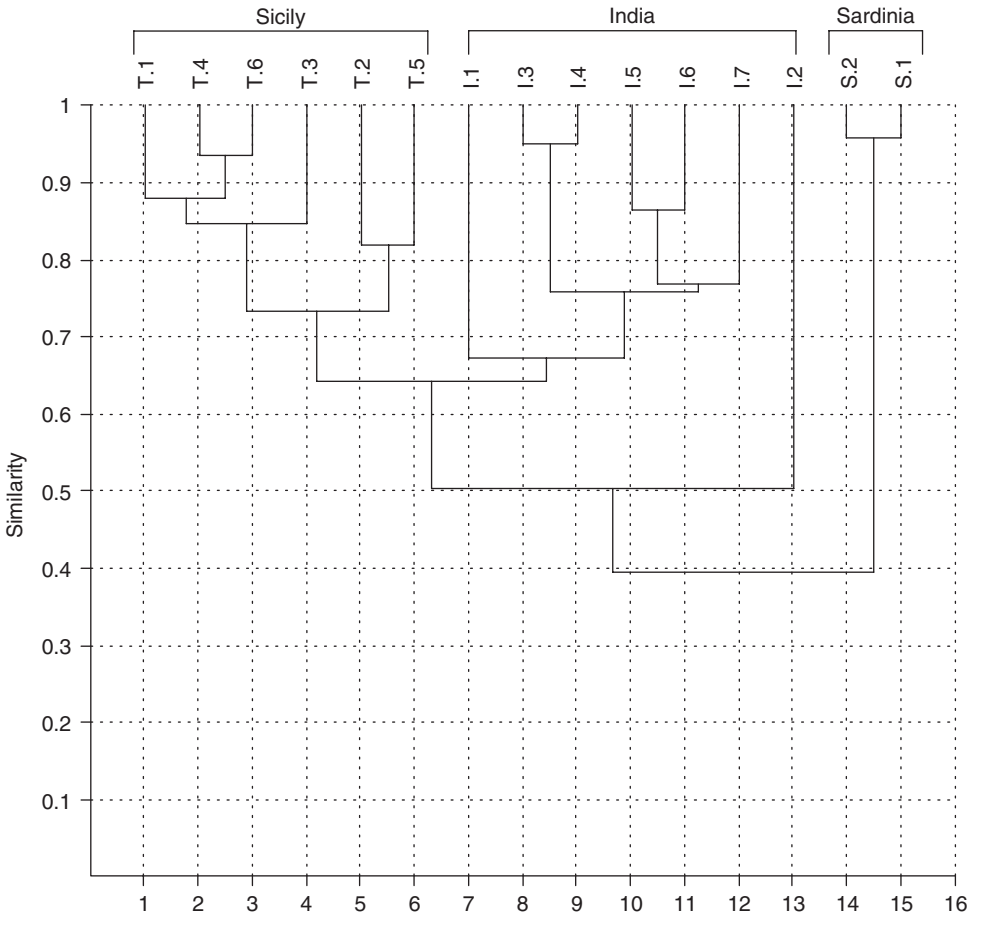

Figure 2. Dendrogram showing relationships among *W. somnifera* plants based on RAPD data.

Within the Sicilian samples, two subclusters (T.4 and T.6) with high similarity coefficient, two subclusters (T.2 and T.5) with similarity coefficient of approximately 0.8 and tied individuals (T.1 and T.3) were observed.

The tie of Indian samples showed a high level of genetic variation, the similarity coefficient ranged from 0.5 to close to 1. Interestingly, two samples, I.2 and I.1, diverged from the rest of the Indian individuals at a similarity coefficient 0.5 and 0.7, respectively. A comparison of the similarity coefficient generated through another method (Jaccard) did not reveal meaningful differences in the dendrogram (data not shown).

2.3. *PCA and cluster analysis of phytochemical data*

Methanolic extracts of leaves from all the samples were utilised for a HPLC fingerprint, and for the quantitative determination of withaferin A. This compound was found only in the Indian and Sardinian samples, while in the Sicilian samples, the content of

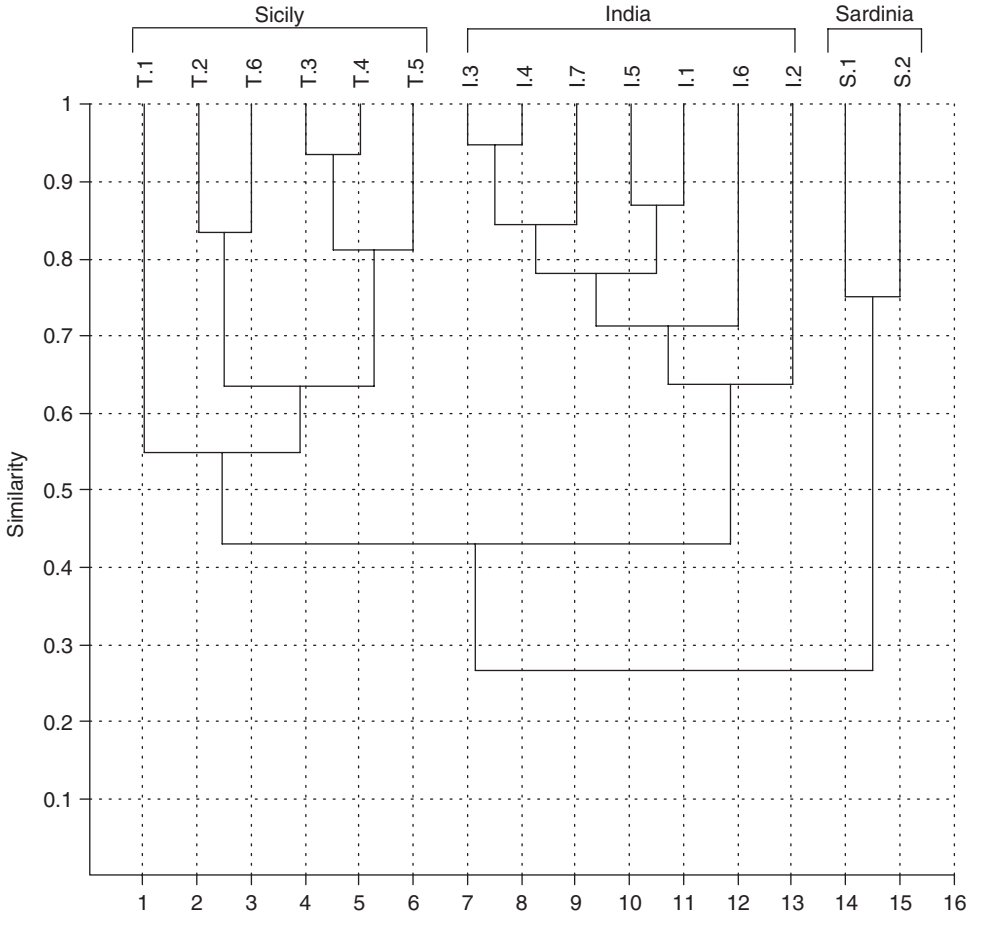

Figure 3. Dendrogram showing relationships among *W. somnifera* plants based on HPLC data.

withaferin A was below the detection limit. Sardinian samples showed a withaferin A pool 7–25 times higher than the Indian samples. The content of this steroidal lactone in the Sardinian samples was very close to that reported for the Mediterranean chemotypes [5].

Cluster analysis and PCA were applied to HPLC fingerprint data. A matrix with the phytochemical data was performed in order to apply the Cluster analysis by Nei and Li's and Jaccard's similarity coefficient and a dendrogram was constructed to evidence the relations among all the samples examined (figure 3). Samples from different origin formed separate clusters, depending on their origin. Sardinian samples formed one cluster clearly separated from those of the Indian and Sicilian samples (figure 3). Nevertheless, clusters formed by the Indian and Sicilian samples were closer to each other compared to the Sardinian one.

With regard to PCA analysis of fingerprint data, the first principal component accounted for 34.75% of the variation, the second accounted for 19.38% and the third for 11.75% (data not shown). Considering the relation between the first and second principal component (1 + 2) and that between the first and third principal

component (1 + 3), the Sardinian group samples were confirmed to be clearly separated from the Indian and Sicilian groups (data not shown). This is in agreement with the results from the cluster analysis. Moreover, similar results were obtained by considering genetic and phytochemical data.

It is clear from the dendrogram that the Sardinian samples branched out from the remaining Sicilian and Indian samples at a low similarity coefficient of approximately 0.3. The tie of Sardinian samples showed a similarity coefficient value of 0.75. The Sicilian samples were arranged in two major clusters and the similarity coefficient ranged from 0.55 to 0.94. Interestingly, one sample (T.1) diverged from the rest of the Sicilian individuals at a 0.55 similarity coefficient. Within Sicilian samples, two subclusters (T.3 and T.4) with high similarity coefficient, two subclusters (T.2 and T.6) with similarity coefficient of approximately 0.82 and tied individuals (T.1 and T.5) were observed. The tie of Indian samples showed a high level of genetic variation, the similarity coefficient ranged from 0.63 to close to 1. Interestingly, one sample (I.2) diverged from the rest of the Indian individuals at a 0.63 similarity coefficient and also samples I.6 and I.7 diverged from the rest of the individuals at a similarity coefficient of 0.7 and 0.85, respectively.

Comparison of the similarity coefficient generated through Jaccard method did not reveal meaningful differences in the dendrogram (data not shown).

2.4. *Antioxidant activity*

The antioxidant activity of all extracts was evaluated by the DPPH (1,1-diphenyl-2-picrylhydrazyl) test. In table 2, the antioxidant activity of methanolic leaf extracts from all samples of *W. somnifera* is reported. The average IC_{50} values of Indian, Sardinian and Sicilian samples was 3.28 , 2.04 and 2.67 $mg\,mL^{-1}$, respectively. Thus, Sardinian samples turned out to have the highest antioxidant activity. Considering that the plant has been shown to possess antioxidant activity [2–6], we tested this activity to

Table 2. Antioxidant activity assayed by the DPPH test of different methanolic extract of *W. somnifera* (data are expressed as IC_{50} $mg\,mL^{-1}$).

W. somnifera samples	IC_{50} ($mg\,mL^{-1}$)	Mean
I.1	2.67 ± 0.12	$3.28^{a} \pm 0.10$
I.2	3.05 ± 0.08	
I.3	3.78 ± 0.13	
I.4	3.66 ± 0.09	
I.5	3.29 ± 0.03	
I.6	3.40 ± 0.11	
I.7	3.14 ± 0.12	
S.1	1.82 ± 0.17	$2.04^{b} \pm 0.16$
S.2	2.25 ± 0.14	
T.1	3.26 ± 0.13	$2.67^{c} \pm 0.11$
T.2	2.50 ± 0.11	
T.3	2.55 ± 0.09	
T.4	2.60 ± 0.10	
T.5	2.56 ± 0.14	
T.6	2.52 ± 0.11	

Note: Different letters (a, b, c) indicate significant differences ($p < 0.0013$).

evaluate a possible relationship among the *W. somnifera* populations. The statistical analysis (ANOVA) showed that the difference among mean groups of sample was significantly different with $p < 0.0013$.

3. Discussion

The intraspecific variations and polymorphism are usual phenomena in Solanaceae and according to Atal and Schwarting [7], five distinct morphological forms of *W. somnifera* are present in India and also genetic variation within *W. somnifera* was revealed [8]. *W. somnifera* is the species of its genus with a wider geographical distribution. Troìa [4], on the basis of historical literature and specimens of the Herbarium of Florence, has individuated the geographical spotted distribution of the species around the Mediterranean Sea. In Italy, this species is very rare as a spontaneous plant, growing in Sicily and Sardinia [4,9–12]. Despite the wide distribution, the presence around the Mediterranean Sea shows a fragmented distribution, that, (together with other ecological and biogeographical considerations) suggests the hypothesis that in this area, *W. somnifera* is not indigenous but is naturalised. It is probable that this species has been introduced around the Mediterranean Sea like a medicinal plant and subsequently naturalised in some places. Some authors have reported that *W. somnifera* was introduced in Egypt, Minorca and Morocco [13–15]. Recently, *W. somnifera* has been naturalised in North Africa [16]. In Italy, this species is considered rare. In the past, there were many places where it was reported in Sicily, but more recently, it has become very rare and is almost not available in the natural form [4]. The last report in Sicily only regards one site [17] and from this site comes the sample studied in the present work. Actually, the Sardinian *W. somnifera* plants are found only in Dorgali in the Northeast of the island (M. Ballero, personal communication).

Morphological studies on the Sardinian specimens picked up [18] have shown a morphological similarity between this and morphotypes III and IV already described in literature according to Atal and Schwarting [19] and histological resemblance with models present in the north of India, near Delhi. The results of this study, obtained using RAPD markers, indicate that there are some differences between Indian, Sardinian and Sicily samples. In detail, a definite difference has been seen between Sardinian chemotype and the Sicilian one. This evidence probably, could be due to a different history of the arrival of the plant in those two islands, considering both the time and the country of origin. In fact, Sicily has been invaded by Arabian people, coming from North Africa, for a long time (IX–XI century AC), whereas Sardinia, a region less rich from an agricultural standpoint and more difficult to dominate, has not had such an invasion [4]. The genetic difference between the Indian and Sicilian populations was smaller than that between the Indian and Sardinian populations.

The phytochemical analysis showed that the Sardinian specimen differed from Indian and Sicilian ones.

Furthermore, the antioxidant activity among group of *W. somnifera* samples was significantly different with $p < 0.0013$ and also in this case, the Sardinian sample showed a higher antioxidant activity. The withaferin A content (expressed as percentage wt/wt dry) of Indian samples (I.1–I.7) varied from 0.014 ± 0.001 to 0.058 ± 0.004, whereas in the Sardinian samples (S.1 and S.2), it was between 0.4 ± 0.01 and 0.5 ± 0.02. The withaferin A could not be traced in the Sicilian samples (T.1 and T.6).

The high content of withaferin A of Sardinian specimen found in the Israel can be related with chemotype I described by Abraham *et al*. [20].

With regard to the antiangiogenic activity of withaferin A [3], the Sardinian samples could be used to start a cultivation of plant with higher withaferin A content to produce a raw material for the pharmaceutical industry.

4. Experimental section

4.1. *Plant material*

Fruits and leaves of seven wild samples of Indian *W. somnifera* (designated as I followed by sequence numbers) were collected in North India at Noida, in different sites, near New Delhi, and authenticated by Dr M. R. Uniyal (Maharishi Āyurveda Product Ltd., Noida, India) and by Dr P. Scartezzini. Fruits and leaves of two wild samples of *W. somnifera* from Sardinia were collected in Dorgali, North-East of the island (designated as S.1 and S.2), and both samples were authenticated by Prof. M. Ballero (Department of Botanical Science, University of Cagliari). Wild fruits of six samples of *W. somnifera* and leaves of six samples from Sicily (designated as T followed by sequence numbers, in which the "T" indicates "Trinacria", the old name of Sicily island) were collected at Pellegrino Mountain near Palermo in the North-West of the island and authenticated by Dr A. Troìa. All samples have been collected in the same period, when ripened fruits were present.

4.2. *DNA extraction and amplification*

DNA was obtained from seeds of each individual plant, after lyophilisation. Extraction of DNA was carried out according to the procedure of Conte and Cristofolini [21]. Tubes (0.5 mL) of the "Ready-To-Go" RAPD Analysis Kit (GE Healthcare Life Sciences) were used for RAPD reactions. Six random decamer primers were supplied as part of the purchased kit (table 1). Ten nanograms of the required template DNA and the primer of choice were added to each reaction mixture along with water to make up to a total volume of 25 μL. Several annealing temperatures were tested in a series of preliminary amplifications. In order to ensure reproducibility of the results, each primer–sample combination was repeated at least twice. The amplification products were separated on polyacrylamide mini-gels (PhastGel Gradient 10–15, GE Healthcare Life Sciences) and visualised with silver stain according to a procedure adapted from Bassam *et al*. [22]. The molecular weight standard pBR322 DNA-BstN I Digest (New England BioLabs) was run in the outer lanes and used as a reference for sizing the fragments obtained. Different lengths of the electrophoretic runs and band size ranges were also settled for the six primers (table 1). A permanent scanned record was obtained for each mini-gel and used for later scoring and analysis.

4.3. *Data scoring and statistical analysis*

Analysis of the RAPD loci was performed using the Phoretix software (Phoretix, AB.EL Science Ware Srl); amplified fragments were scored as present or absent in order

to produce a binary matrix; those bands with an intensity lower than the established minimum threshold were not scored because such fragments were poorly reproducible.

Two genetic similarity matrices were calculated using the Nei and Li's and Jaccard's coefficients, respectively [23,24]. The resulting matrices were used to construct dendrograms using the unweighted pair group method with arithmetic averaging (UPGMA) procedure. Calculations were done using a software package palaeontological statistics (PAST) [25]. The same software was used to conduct PCA of the RAPD data, based on a variance/covariance matrix.

4.4. *HPLC identification of withaferin A*

A total 5 g of dried finely powdered leaf samples was extracted twice with methanol for 48 h. After filtration, the methanol solvent was evaporated and the residue suspended again in methanol and filtered through a Millex GV 0.22 μm filter before injection.

Analysis was performed using a Jasco PU-1580 HPLC instrument (Jasco Europe s.r.l., Modena, Italy) equipped with 20 μL loop and a Jasco UV-1575 variable wavelength detector set at 215 nm. A Waters C18 Spherisorb 5 μm ODS2 Column was used in isocratic condition with mobile phase consisting of a mixture (60 : 40 v/v) of acetonitrile and water with 0.1% phosphoric acid at a flow rate of 1 mL min^{-1}. Withaferin A was obtained from ChromaDex, Inc. and separation was performed according to the method suggested by the company (Daimler St.Ana, CA, USA).

Standard solutions of withaferin A, in the 5–50 μg mL^{-1} concentration range, were used to identify and quantify this analyte in plant extracts. Determinations were repeated at least three times per sample.

Data scoring was performed by constructing a matrix in which the presence or absence of the most significant chromatographic peaks was reported for each sample. Subsequently, statistical PCA and cluster analysis were applied as described above.

4.5. *Antioxidant activity*

The antioxidant activity of *W. somnifera* extracts was assessed by their scavenging effect on the DPPH radical [26,27]. Samples of *W. somnifera* methanolic extracts of dry leaves (0.100 mL) were added to a methanol solution of DPPH (1×10^{-4} M, 2.9 mL), shaken vigorously and kept in the dark for 30 min. The absorbance of samples was measured with a spectrophotometer (Perkin-Elmer 554) at 517 nm, with methanol used as blank. The antioxidant activity of each extract was determined according to the percentage of DPPH decolouration. The antioxidant activity of *W. somnifera* extracts was expressed as IC_{50}. IC_{50} value was defined as the concentration (mg mL^{-1}) of the extract required for bleaching the DPPH radicals by 50%. The DPPH radical, methanol and phosphoric acid were purchased from Sigma (Milan, Italy). For each of the data collected, relative standard deviation was calculated. Analyses of variance (ANOVA) was performed. All computations were made using the statistical software PRISM.

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