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Profiling of the Bioactive Compounds in Flowers, Leaves and Roots of *Vinca sardoa*

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Vinca sardoa (Stern) Pignatti (Apocynaceae) is largely distributed in Sardinia where it is considered a typical endemism. Since the plant is used in traditional folk medicine, the aim of the present work was to identify and quantify the polyphenolic metabolites, due to the well-known importance of polyphenolic compounds, as well as to evaluate antioxidant activity in different parts of the plant. The compounds were identified and determined in the methanol extracts of leaves, flowers, and roots by developing different LC-MS/MS methods. The obtained data show that leaves possess the highest amount of polyphenols, in particular quinic acid (3401 mg/100 g), chlorogenic acid (1082 mg/100 g), caffeoylquinic acid isomer 1 (190 mg/100 g), and robinin (633 mg/100 g). Likewise, antioxidant tests showed that leaves possess the main radical scavenging activities in both ABTS (49.19 ± 3.41 µg/mL, 30.88 ± 3.04 µg/mL at time zero and after 50 min, respectively) and DPPH assays (223.97 ± 30.81 µg/mL, 109.52 ± 12.89 µg/mL at time zero and after 30 min, respectively).Taking into account that leaves differed most from flowers and roots in the content of caffeoylquinic acid and chlorogenic acid, of which antioxidant properties are widely recognized, it is reasonable to assume that these two compounds are involved in the differences described. The relationship between the high polyphenolic content and interesting antioxidant activities, justifies its use in ethnobotany and may be suggest a use of this specie, after removal of the alkaloid fraction, in the pharmaceutical, phytotherapy, and cosmetic industries.

Keywords: Antioxidant activity, Sardinian endemic plants, Polyphenols, LC-MS.

Vinca sardoa (Stearn) Pignatti, (Apocynaceae), a perennial herbaceous plant, is largely distributed in Sardinia where is it considered a typical endemism [1-3]. In fact, Sardinian vegetation is the result of long processes of anthropization, and currently the vascular flora of Sardinia can be estimated in 2400 units. Among all Sardinian species and subspecies, approximately 5% is constituted from the endemic elements [4-5]. In Italy, there are four Vinca species: *Vinca minor* L., *V. major* L., *V. difformis* Pourret, and *V. sardoa* (Stearn) Pignatti. The first two ones are reported (leaf) in the European Pharmacopoeia IV edition (2002), but among them, the more important officinal specie is *V. minor*, which is also called periwinkle. It is used in the pharmaceutical industry for the extraction of the alkaloid from a hypogeum apparatus. The periwinkle is employed in popular medicine as resolving, digestive, astringent-antidiarrheal, vasodilatory-hypotensive, anti-inflammatory haemostatic, antianemic, and tonic in the convalescence. In Sardinia, *V. sardoa*, which is also called Sardinian periwinkle, is used in ethnobotany such as *V. minor* [6]. Only a few studies on *V. sardoa* are available in the literature concerning botanical characteristics [7] and alkaloids content in both the hypogeum apparatus and aerial part [8-12]. To the best of our knowledge, no studies are present in the literature regarding the occurrence of polyphenolic compounds and their content in this specie. The interest of plant polyphenols, a wide group of secondary metabolites that are also a common component in our diet, derives from the evidence of their potent antioxidant activity and their wide range of pharmacologic properties including anti-inflammatory, antiallergic, and antibacterial activities [13]. Thus, the aim of this research is to study the occurrence of polyphenol compounds and their content in different parts of the plant such as flowers, leaves, and roots and their related antioxidant activities, since the plant is used in traditional folk medicine.

Table 1: Determination of phenols by Folin-Ciocalteau's method.

Methanolic extracts (µL)	GAE *V. Sardoa* leaves (µg/mg dried plant part)	GAE *V. Sardoa* flowers (µg/mg dried plant part)	GAE *V. Sardoa* roots (µg/mg dried plant part)
100	66.7± 6.0	52.5 ± 6.6	52.7 ± 8.4
50	39.7 ± 3.7	31.1 ± 1.7	31.2 ± 4.5
25	21.5 ± 1.9	16.3 ± 2.0	16.9 ± 1.9
10	10.4 ± 0.5	9.2 ± 0.8	7.9 ± 0.8
5	7.2 ± 1.0	5.0 ± 0.6	4.2 ± 0.8
1	3.2 ± 1.7	3.5 ± 0.5	2.4 ± 1.5

Data were expressed as means ± SD of 3 independent experiments. Each results showed a positive correlation ($P < 0.001$) with DPPH and ABTS results.

A preliminary screening of total polyphenols content, was performed by Folin-Ciocalteau's method, and expressed as µg of GAE/mg of dried plant part (Table 1).

The best results were obtained in leaves, followed by flowers and roots, which is in line with the chromatographic data. Furthermore, a highly significant positive correlation was found between the total phenols of extracts of leaves, flowers, and roots obtained by Folin-Ciocalteau's method; also, DPPH and ABTS results, respectively, explained the role of phenols in antioxidant activity, supporting several literature data [14]. The total antioxidant activity of the methanolic extracts from flowers, leaves, and roots from *V. sardoa* were determined by DPPH and ABTS assays. Our results, expressed as IC_{50} values (µg/mL), revealed a moderate antioxidant activity if compared with Trolox, a homologue of vitamin E, used as reference (Table 2) and confirmed the data of Grujic et al. [15] obtained on *Vinca minor*. However, if we considered differences between groups, the scavenging of the DPPH radical was significantly higher in leaves and flowers than roots at time zero ($P < 0.001$) when the fast reaction began. After 30 min of reaction, the scavenging of the DPPH radical was similar in roots and flowers but differed significantly from leaves (Table 2).

Table 2: Scavenging of 50% of DPPH and ABTS radical by Trolox and V. sardoamethanolic extracts at different time points

	DPPH IC_{50}		ABTS IC_{50}	
	0 min	**30 min**	**0 min**	**50 min**
Trolox(μg /mL)	13.5	6.2	3.3	3.3
***V. Sardoa*lea ves** (μg/mL)	224.0 ± 30.8a	109.5 ± 12. 9a	49.2 ± 3.4a	30.9 ± 3.0a
***V. Sardoa* flowers** (μg/mL)	372.0 ± 22.3b	202.3 ± 11.3b	87.2 ± 6.0b	53.4 ± 2.9c
***V. Sardoa* roots** (μg/mL)	559.0 ± 61.0c	221.0 ± 17.4b	84.7 ± 5.9b	44.9 ± 0.7b

Data were expressed as means ± SD and lower case letters (a-c) indicate differences at α = 0. 001 between different parts of V. Sardoa

These results can be explained taking into account that the reaction in methanol was very fast and changed rapidly over time until 30 min when the antioxidant activity of the samples reached their peak. The percentage of inhibition of the ABTS radicals' activity, which the other method used to measure the antioxidant, is shown in Table 2: it confirms that leaves had a higher antioxidant activity and differed significantly from flowers and roots. On the other hand, flowers and roots are similar at time zero, while after 50 min, the best antioxidant activity was achieved by roots. Although the antioxidant capacities measured by the two assays were in line, the $ABTS^{\bullet+}$ method gave the best results. The superiority of the $ABTS^{\bullet+}$ assay over DPPH is explained because $ABTS^{\bullet+}$ is usable over a wider range of pH, and it is more rapid than that of the DPPH assay. Another important difference is that $ABTS^{\bullet+}$ can be solubilized in both aqueous and in organic media, so that the antioxidant activity of both hydrophilic and lipophilic in the samples can be measured. In contrast, DPPH can only be dissolved in alcoholic media [16-17], thus limiting its applicability. With the purpose to screen polyphenolic compounds occurring in flowers, leaves, and roots of *V. sardoa*, a solution of each reference standard (see experimental, "reagent and chemical" section) (1 μg/mL) was directly introduced into the mass spectrometer one after another at a flow rate of 10 μL/min using a syringe pump (Harvard Apparatus, Australia). For each precursor ion, the product ion (ESI-MS/MS) was also selected and studied to choose the best transitions in the MRM mode. The most sensitive transitions were selected, and a LC-MS/MS MRM screening method was developed. Successively, *V. sardoa* samples were analysed by using the LC-MS/MS MRM method. Thus, 21 polyphenolic compounds were identified in different parts of *V. sardoa*such as *p*-coumaric acid, caffeic acid, quinic acid, ferulic acid, clorogenic acid, phloridzin, catechin, kaempferol, sinapic acid, syringic acid quercetin-rhamnoside, isoquercitrin, rutin, robinin, isorhamnetinrutinoside, eriocitrin, myricitrin, quercetin galactoside, myricetingalactoside, among two isomers of caffeoylqionic acid (Table 3). The MRM method was adequately modified selecting only the most sensitive transitions of occurring phenolic compounds in *V. sardoa* samples. Thus, the developed analytical method was applied to determine 21 compounds in flowers, leaves, and roots. Quantitative analysis was performed by the external standard method. Calibration curves were obtained by plotting the area of ES against the known concentration of compounds; the curves were linear in the range of 0.01-10 μg/mL. The chromatographic profile contained all the peaks corresponding to the compounds under investigation, with appreciable intensity for quantitative purpose. Retention times, transitions, and quantitative data for analyzed compounds are reported in Table 3. By comparing the total contents of 21 compounds among flowers, leaves, and roots, the results were found to be as follows: leaves > flowers > roots. In particular, the contents of quinic acid (3401.13 μg/100 g), clorogenic acid (1081.99 mg/100 g), robinin (632.86 mg/100 g), and caffeoylquinic acid isomers (190.13 mg/100 g and 44.64 mg/100 g) were significantly higher in leaves ($P < 0.01$) than in the flowers (3239.74 mg/100 g, 520.93 mg/100 g, 536.78 mg/100 g, 6.56 mg/100 g, and 14.46 mg/100 g, respectively) and roots (226.57 mg/100 g, 6.77 mg/100 g, 0.94 mg/100 g, 6.77 mg/100 g, and 0.86 mg/100 g, respectively), as shown in Table 3. However, some compounds were found only in the determinate sample. Quercetin rhamnoside, myrycitrin, quercetin galactoside, and myricetin galactoside seem to be characteristic of flowers, whilst syringic acid was revealed only in roots. All the identified compounds were reported for the first time in *V. sardoa*. To the best of our knowledge, the following compounds including quinic acid, phloridzin, quercetin-rhamnoside, isoquercitrin, isorhamnetin rutinoside, eriocitrin, myricitrin, quercetin galactoside, myricetin galactoside, catechin, and the two isomers of chlorogenic acid were herein reported in the *Vinca* genus for the first time. Antioxidant activity was measured by DPPH and ABTS assays. Our results revealed that leaves had higher antioxidant activity, as described by Saral et al. [18], obtained on *Vinca major* where leaves showed a highest DPPH scavenging and higher amounts of phenolic acid and flavonoids if compared with flowers and roots. Taking into account that leaves differed most from flowers and roots in their content of caffeoyl quinic acid and chlorogenic acid, of which antioxidant properties are widely recognized [19], it is reasonable to assume that these two compounds are involved in the differences described here. Many studies of antioxidant abilities on volatiles proved that compounds with phenolic moieties like caffeoylquinic acid and chlorogenic acid show high antioxidant ability [20-27]. Moreover, although caffeoyl quinic acid and chlorogenic acid were well represented only in leaves, the amounts of both quinic acid and robinin were similar in leaves and flowers and were found in small quantities in roots; this explains the positive correlation between scavenging capacity and total phenols in the manner described above (leaves > flowers > roots). Also, we can assume that despite quercetin rhamnoside, myrycitrin, quercetin galactoside, and myricetin galactoside were found only in flowers, their contribution to the antioxidant activity were low. This is the first quali-quantitative study on *Vinca sardoa*. 21 compounds were identified and determined in the methanolic extracts of leaves, flowers, and roots by using LC-MS/MS methods. The obtained data show that leaves possess the highest amount in polyphenols, in particular quinic acid, clorogenic acid, caffeoylquinic acid isomer 1, and robinin. The high amounts of these phenolic compounds could be linked with interesting antioxidant activities. The significant relationship between total polyphenols and antioxidant activity indicated that total phenolic contents can be used as an indicator for assessing the antioxidant activities of *Vinca sardoa*. Finally, our results show the value of the *Vinca sardoa* as a source of antioxidant polyphenolic compounds and justifies its use in ethnobotany.

Experimental

Reagents and chemicals: Solvents used for extraction, HPLC-MS grade methanol, acetonitrile and formic acid were purchased from Sigma-Aldrich Chemical Company (St Louis, MO). HPLC grade water (18 mΩ) was prepared by using a Millipore (Bedford, MA, USA) Milli-Q purification system. Standards reference compounds such as *p*-coumaric acid, caffeic acid, quinic acid, ferulic acid, chlorogenic acid, phloridzin, catechin, kaempferol, sinapic acid, syringic acid were purchased from Sigma Aldrich; standards of quercetin-rhamnoside, isoquercitrin, rutin, robinin, isorhamnetin rutinoside, eriocitrin, myricitrin, quercetin galactoside, myricetin galactoside were purchased from Extrasynthese (Genay, France).

Table 3: Retention times, transitions, and quantitative data for analyzed compounds.

Compound	RT	Selected Transition	Leaves	Flowers	Roots
***p*-coumaric acid**	10.6	(163/119)	1.6 ± 0.1b**	2.3 ± 0.4c**	0.4 ± 0.0a**
Caffeic acid	9.2	(179/135)	8.8 ± 0.6a*	1.2 ± 0.1b*	ND
Quinic acid	1.3	(191/85)	3401.1 ± 38.9c**	3239.7 ± 98.8b**	226.6± 2.9a**
Ferulic acid	11.3	(193/134)	2.9 ± 0.4c**	2.2 ± 0.1b**	1.0 ± 0.0a**
Clorogenic acid	9.4	(353/191)	1082.0 ± 56.6b°	520.9 ± 3.7ab°	6.8 ± 0.3a°
Phloridzin	13.1	(435/273)	0.1 ± 0.0c**	0.1± 0.0b**	0.1 ± 0.0a**
Quercetin-rhamnoside	12. 7	(447/300)	ND	1.0 ± 0.0	ND
Isoquercitrin	11.6	(463/299)	0.4 ± 0.0ab°	1.0 ± 0.1b°	0.0 ± 0.0a°
Rutin	11.3	(609/300)	7.8 ± 0.7b**	15.9 ± 0.4c**	0.3 ± 0.0a**
Robinin	10.7	(739/593)	632.9 ± 10.8c**	536.8 ± 12.0b**	0.9 ± 0.0a**
Isorhamnetinrutinoside	12.2	(623/315)	0.0 ± 0.0a*	0.2 ± 0.0b*	ND
Eriocitrin	10.7	(595/287)	0.1 ± 0.0	0.1 ± 0.0	ND
Myricitrin	11.4	(463/316)	ND	0.3 ± 0.0	ND
Quercetin galactoside	11. 7	(463/300)	ND	0.0 ± 0.0	ND
Myricetingalactoside	10. 6	(479/316)	ND	0.3 ± 0.0	ND
Catechin	9.1	(289/245)	1.1 ± 0.1b*	0.3 ± 0.0a*	ND
Kaempferol	16.2	(285/186.9)	0.6 ± 0.1	0.5 ± 0.0	ND
Caffeoylquinic acid isomer 1	8.4	(353/191)	190.1 ± 2.2b°	6.6 ± 0.3a°	6.8 ± 0.3ab°
Caffeoylquinic acid isomer 2	9.8	(353/191)	44.6 ± 0.6b°	14.5 ± 1.3ab°	0.9 ± 0.0a°
Sinapic acid	11.4	(223/164)	0.9 ± 0.0b*	ND	0.8 ± 0.0a*
Syringic acid	9.8	(197/121)	ND	ND	1.8± 0.1

Data were expressed as mean ± SD of 3 samples. The statistical analysis were conducted with (*) t test or (**) One way Analysis of Variance (ANOVA) when data followed a normal distribution or (°)Kruskal-Wallis One Way Analysis of Variance on Ranks where data were not normal distributed. Lower case letters (a-c) indicate differences at α = 0.05 between different parts of *V. Sardoa*. ND = not determined

Sample collection: The plant material was collected during spring 2015 in the Archipelago of la Maddalena exactly on the main island. The identification of the species was carried out by Dr. Mario Chessa and a voucher specimen was deposited in the herbarium SASSA (Sassari) with identification number 820. The whole plant was divided in roots leaves and flowers, and dried at room temperature. After a treatment with ammonia 10%, the material was subjected to continuous extraction by Soxhlet apparatus: first with hexane to operate a defatting and remove the alkaloid fraction,.then with solvents in increasing polarity (chloroform and methanol: water 7:3 v/v) to separate different metabolites.Methanolic extracts then, were subjected to instrumental analysis.

Determination of total phenols: Total phenols were estimated by a colorimetric assay based on procedures described by Lizcano et al. [28], with some modifications [29]. Gallic acid (1-200 µg) was used for constructing the standard curve. Results were expressed as µg of gallic acid equivalent (GAE) per mg of the dried plant part.

Antiradical activity by diphenyl-1-picarylhydrazyl (DPPH) assay: The radical scavenging activity assay was performed according the method proposed by Brand et al. [30], with some modifications [29]. A Trolox calibration curve in the range 0.25-7.5 µg/mL was used as positive reference. Experiments were carried out in triplicate, and results were expressed as mean ± SD.

Antioxidant Capacity Assay: The ABTS free radical-scavenging activity of each sample was determined according to the method described by Petretto et al. [31].

ESI-MS and ESI-MS/MS analyses: Full scan ESI-MS, MS/MS analyses of standards were performed on an AB Sciex 4000 Q-Trap (Foster City, CA, USA) spectrometer. Standard solutions (1µL in methanol: water 50:50) were infused at 10 µL/min for tuning, the optimized parameters: fragmentation reactions selected for each compound, dwell times, and Declustering Potential, Entrance Potential, Collision Energy, Collision Cell Exit Potential values were acquired in the negative ion MS and MS/MS modes.

HPLC–ESI-MS/MS analysis: The dried samples were dissolved methanol in solution 1 mg/mL and filtered through 0.20-µm syringe PVDF filters (Whatmann International Ltd., UK). A 4000 QTRAP with Turbo V and Turbo Ionspray interface from AB Sciex (Foster City, CA, USA) was used for UHPLC-ESI-MS/MS analysis. Multiple reaction monitoring (MRM) mode was used for quantification. Liquid chromatography was performed with a Flexar UHPLC AS system (Perkin-Elmer, USA) consisting of degasser, Flexar FX-10 pump, autosampler and PE 200 column owen. Five microliters of each samples were injected into a XSelect CSH C18 column (Waters, Milford, MA) (100 x 2.1 mm i.d., 2.5 µm d) kept at 47°C. Mobile phase was composed by a mixture of H_2O containing0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B) and elution was carried out according to the following flow and solvent gradient: 0-10 min, linear gradient 0%-23% solvent B and the flow changes from 300 µL/min to 350 µL/min; 10-13 min, isocratic gradient at 23% solvent B and the flow constant at 350 µL/min; 13-16 min, linear gradient 23%-100% B and flow retrieves to 300 µL/min. The source temperature was held at 500°C. The voltage applied was -4500. Data acquisition and processing were performed using Analyst software 1.6.2.

Preparation of standard solutions: A sample (1 mg) of each standard was weighted accurately into a 1 mL volumetric flask, dissolved in ethanol 70% (v/v) and the volume made up to the mark with ethanol. The resulting stock solution was diluted with methanol in order to obtain reference solutions containing 0.01, 0.05, 0.1, 1, 5, and 10 µg/mL of external standards. The calibration curves, for each compound, were made by linear regression by plotting the peak area of external standard against their known concentrations. The result represents the average of curves performed by three injections of each concentration. All quantitative data were elaborated with the aid of Analyst software (AB Sciex).

Method validation: LC–MS/MS method was validated according to the European Medicines Agency (EMEA) guidelines relating to the validation of analytical methods [32]. All procedures were performed according previous literature data [33-34].

Statistical analysis: All experiments were repeated three times. All statistical analyses were performed comparing methanolic extracts from flowers, leaves and roots from *V. sardoa* with unpaired Student's *t*-test, when the data followed a normal distribution, using SigmaStat v 3.5 software. The distribution of the sample was evaluated by the Kolmogorov-Smirnov and Shapiro tests. The strength of association between variables was analyzed with the Pearson product moment correlation coefficient when the data were normally distributed. A $P \leq 0.05$ was considered statistically significant.

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