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Trend of Antioxidant Activity and Total Phenolic Content in Wild Edible Plants as Part of the Environmental Quality Assessment of Some Areas in the Central Italy

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Abstract: Polyphenols are secondary metabolites of interest due to their potential application in various fields. This study is supposed to analyse the content of total polyphenols, total tannins, condensed tannins and antioxidant activity of ten wild plant species of nutritive interest to better understand their potential applications. Furthermore, the effect of heavy metals on the production of the investigated secondary metabolites was analysed. The different phenolic compounds were determined in methanol extracts obtained from edible plants collected during three sampling periods (June, September and November–December) in four areas of the Central Italy. Analyses were carried out by applying standard methodologies. In particular, total polyphenols were determined by the Folin–Ciocalteu method, total tannins by the polyvinylpolypyrrolidone (PVPP) reagent and condensed tannins by the 4-(dimethylamino)cinnamaldehyde (DMCA). Antioxidant activity was determined by assessing the scavenging capacity of 2,2-diphenyl-1-picrylhydrazyl (DPPH). The results showed a satisfactory content of phenolic compounds and antioxidant activity for all species analysed. Furthermore, the existence of a negative correlation between the presence of heavy metals and phenolic compounds was found. Results proved the potential use of these plants for balanced feeding of ruminants.

Keywords: edible plants; antioxidant activity; polyphenols; tannins; heavy metals

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1. Introduction

Secondary metabolites of plants are an interesting topic for the scientific community as their uses and application in both human and animal health care is widespread [1,2]. Phenolic compounds are a class of secondary metabolites, used by the plants for carrying out physiological and fundamental activities, such as cell division processes, hormone regulation, nutrient mineralisation, reproduction and photosynthetic activity. Furthermore, phenolic compounds determine the plant's response to stresses when it is in sub-optimal conditions [3]. For example, active oxygen species (ROS) produced by plants during photosynthesis can damage them in specific conditions. More specifically, the amount of ROS produced is influenced by exposure to sunlight and when this deviates from normal conditions and is more impactful, the plant has to produce more ROS, damaging itself [4]. Therefore, plants are endowed with an efficient antioxidant system (i.e., free radical scavenging enzymes) and good antioxidant molecules (e.g., phenolic compounds) to contrast the negative effect of ROS [5]. During stress conditions, abiotic stress can determine an increase in the production of phenolic compounds. For example, abiotic stress given by heavy metals contamination causes activation of the phenylpropanoid biosynthetic pathway, leading to an increase in phenolic compound production [3]. More specifically,

heavy metals can inhibit the functioning of cytoplasmic enzymes and damage cells through oxidative stress when their concentrations exceed optimal values [6]. Heavy metals are able to deactivate enzymes involved in the detoxification of ROS: in particular, increased sensitivity to metal stress is presented by catalase and superoxide dismutase, whereas peroxidase can be deactivated by metal stress [7].

The attention to plant's phenolic compounds is due to their significant role in the health care of animals, with particular regards to ruminants. Indeed, phenolic compounds have a diverse action and effect, influenced by the type of compound, its concentration and astringency. In addition, factors such as physiological and sexual state, exposure to pathogens, and typical animal and environmental conditions can also modify the effect of phenolic compounds. For example, proanthocyanidins reduce the occurrence of bloat in ruminants due to the consumption of lucerne and white clover [8].

The current paper focuses attention on the analysis of phenolic compound concentrations and antioxidant activities of some of the most widespread species of wild edible plants (i.e., *Trifolium pratense*, *Scorzonera laciniata*, *Bromus erectus*, *Centaurea ambigua*, *Festuca circummediterranea*, *Medicago lupulina*, *Lotus corniculatus*, *Thymus longicaulis*, *Dorycnium pentaphyllum, Dactylis glomerata* and *Hippocrepis comosa*) in Central Italy as they are commonly used for feeding ruminants during grazing. The total phenolic content of some of these plants is documented in the scientific literature. More specifically, the remarkable content of phenolic acids and isoflavones in the extract of *T. pratense* has been reported [9]. *Scorzonera* species and *T. longicaulis* present flavonoids [10,11] whilst flavonols and isoflavonoids have been detected in *L. corniculatus* extract [12]. *D. pentaphyllum* and *D. glomerata* present the phenolic compounds quercetin and flavonoids, respectively [13,14]. Conversely, for *B. erectus, C. ambigua, F. circummediterranea, M. lupulina* and *H. comosa* no papers are available about their phenolic compound composition and antioxidant activity.

In the present work, antioxidant activity, total polyphenols, tannins and condensed tannins contents (i.e., proanthocyanidins) were determined after sampling of various species of wild edible plants from four different areas of Central Italy during three different times of the year. This was necessary to assess the impact of weather conditions on phenolic compound content and antioxidant activity. Furthermore, the effect of heavy metals contamination on total polyphenols content and antioxidant activity has been analysed. To the best of our knowledge, this is the first paper where the phenolic compound composition and antioxidant activity of ten wild edible plant species were simultaneously analysed.

2. Materials and Methods

2.1. Study Sites

In the present paper, ten different species of wild edible plants (i.e., *Trifolium pratense*, *Scorzonera laciniata*, *Bromus erectus*, *Centaurea ambigua*, *Festuca circummediterranea*, *Medicago lupulina*, *Lotus corniculatus*, *Thymus longicaulis*, *Dorycnium pentaphyllum*, *Dactylis glomerata* and *Hippocrepis comosa*) were analysed. The investigated samples were collected from four different sites in Capracotta municipality (Molise region, Central Italy): (1) Monteforte (geographic coordinates 41.797489, 14.291578), (2) Verrino (41.816970, 14.293321), (3) Guardata (41.847971, 14.277226) and (4) Guado Cannavina (41.849737, 14.337791). Through careful exploration of investigated territories, the most present species have been identified and then collected at each site. Sampling was repeated over three different periods of the year: June (Sampling 1), September (Sampling 2) and November/December (Sampling 3) for a total of 36 plant samples. After collection, the plant samples were transported to the Chemistry laboratory of University of Molise for preliminary treatment before proceeding with chemical analysis.

2.2. Pre-Treatment and Extraction

Prior to chemical analysis for the determination of antioxidant activity and total polyphenol and tannin content, the leaf samples were dried in an oven to constant weight, at 40 °C for 72 h to remove the water. Samples were thus minced to obtain a homogeneous

powder. For each plant species, 0.150 ± 0.005 g of minced samples were extracted in 3.0 mL of methanol (Sigma-Aldrich, Milan, Italy, 80% v/v). It is worth pointing out that the presence of possible interferents in the methanol extract was not investigated since the subsequent analyses for the determination of the phenolic compounds were specific for such compounds. Hence, samples were sonicated using an ultrasonic bath (Sonica, Ultrasonic cleaner, Milan, Italy) for 45 min, incubated at room temperature (25 °C) in a dark environment for 15 min and subsequently centrifuged for 15 min at 5000 rpm. The centrifugation was conducted to separate the extraction solvent from minced leaf. The supernatant was then recovered for subsequent analysis.

Extraction of phenolic compounds was carried out only on leaf samples, since, even if a first accumulation occurs mainly in the roots, canes and stem, the definitive distribution of these substances is in the leaf tissues [15].

2.3. Determination of Total Polyphenol Content (TPC)

From each of the 36 leaf samples, previously treated as described in Section 2.2., an extract in methanol (3 mL) was obtained and then used for the determination of the total polyphenol content, following the Folin–Ciocalteu method [16], slightly modified on the basis of experimental requirements. Firstly, a calibration line was constructed for gallic acid (MP Biomedicals, LLC, San Diego, CA, USA), a phenolic carboxylic acid used as a reference standard. Briefly, 0.5 mL of Folin–Ciocalteu reagent (Titolchimica, Rovigo, Italy), previously diluted 1:10 with deionised water, was added to 1.0 mL of five gallic acid solutions at known concentrations (5, 10, 20, 40 and 50 μg mL^{-1}). To each solution, 3.0 mL of Na_2CO_3 (7%) (Acros, Geel, Belgium) and an appropriate volume of deionised water were added to obtain a final volume of 5 mL. All solutions were incubated in the dark for 60 min at room temperature. The solutions were then centrifuged at 4000 rpm for 3 min and the supernatant was recovered and transferred into cuvettes for absorbance measurement by means of a VIS spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan) at a wavelength of 765 nm. The same procedure was applied to each extract to determine the total polyphenol content. The obtained results were expressed as mg GAE (Gallic Acid Equivalent) per g of dry matter (DM), which is a general reference for expressing the content of phenolic compounds. All the experiments were carried out in triplicate.

2.4. Determination of Total Tannin Content

The total tannin content of the investigated plant extracts was estimated by subtracting the so-called "non-tannin" polyphenols to the total polyphenols, after addition of polyvinylpolypyrrolidone (PVPP), a polymer capable of adsorbing tannins [17]. In particular, their determination was carried out following Sultana et al. 2012 [18], slightly modified. Specifically, 0.5 g of PVPP and 1 mL of deionised water were added to 1 mL of methanol extract. Samples were stirred using a vortex (mod. RX3, Velp Scientifica, UsmateVelate, Italy), incubated at 4 °C for 10 min and then centrifuged at 5000 rpm for 5 min. The supernatant was recovered to determine the total polyphenol content without tannins with the previously described assay. Therefore, the total tannin content was determined by calculating the difference between the total polyphenol content of each plant extract before and after adding PVPP. The results were expressed as tannic acid equivalent per gram of dried plant sample (mg TAE g^{-1} DM). All the experiments were carried out in triplicate.

2.5. Determination of Condensed Tannins (Proanthocyanidins)

For the determination of condensed tannins, the method proposed by Heil and co-authors 2002 [19] (slightly modified) was followed [17]. Briefly, 100 μL of methanol extract were thoroughly mixed with 1 mL of 4-(dimethylamino)cinnamaldehyde DMAC (Sigma-Aldrich) (0.1% of DMAC in methanol- HCl 9:1 v/v) within a cuvette. After 5 min of incubation at room temperature, the adsorbance of each sample was measured at 640 nm by using a UV-Vis spectrophotometer (UV-1601, Shimadzu). Concentration of condensed tannins present in the extract was determined using the calibration curve of catechin, which

is used as reference standard for the analysis of condensed tannin compounds. All the experiments were carried out in triplicate.

2.6. Determination of Antioxidant Activity

Antioxidant activity of the investigated wild edible plants was determined by assessing the scavenging capacity of 2,2-diphenyl-1-picrylhydrazyl (DPPH), following the method proposed by Molyneux [20], slightly modified. For each sample, 1 mL of DPPH solution (0.015 g of DPPH in 100 mL of methanol) was added to 1 mL of each extract. Then, 3 mL of methanol were added up to a volume of 5 mL. Samples were incubated in the dark at room temperature for 30 min. The absorbance (A) of each sample was measured using a UV-Vis spectrophotometer (Shimadzu UV-1601) at a wavelength of 517 nm. Measurements were also performed on control samples consisting of 1 mL of DPPH solution and 4 mL of methanol. Antioxidant activity values—obtained by applying the formula $[(A_{control} - A_{sample})/A_{control}] \times 100$—were correlated with the concentration of leaf extracts. In this way, it was possible to calculate the so-called IC_{50}, which is an index of the antioxidant activity [21]. All the experiments were carried out in triplicate.

2.7. Determination of Heavy Metals

Heavy metals (namely, Cu, Cd, Ni, Pb and Zn) were extracted from leaf samples by means of aqua regia mineralisation, following Sastre et al. [22], slightly modified. Briefly, 0.50 ± 0.05 g of plant sample was processed in the digester tube with 3 mL of hydrogen peroxide and then mineralised with 9 mL of hydrochloric acid (Baker Instra-Analyzed, Fisher Scientific, Waltham, MA, USA) and 3 mL of nitric acid (68%) for 40 min at 180 °C. The mineralised samples were filtered by means of Whatman filters n. 42 (pore size: 2.5 µm) before proceeding with the analysis by using an atomic absorption spectrophotometer (SpectrAA 220 FS, Varian, Santa Clara, CA, USA), equipped with a hollow cathode lamp, specific for each metal. Wavelengths used for the detection of heavy metals are as follows: 324.8, 228.8, 232, 217 and 213.9 nm for Cu, Cd, Ni, Pb and Zn, respectively.

Heavy metal concentrations in leaf samples were determined by using calibration curves. During the analysis, procedural blanks were performed to confirm the goodness of the results. More specifically, procedural blanks were conducted following the same procedure described above, without the leaf samples, to confirm the absence of heavy metals contamination of the glassware and reagents. For each metal investigated, procedural blanks were performed.

3. Results

3.1. Total Polyphenol Content

The results regarding total polyphenol content obtained from the analysis of the extracts during three different sampling times in four diverse areas are reported in Table S1, available in Supplementary Materials. As shown in Table S1, the total polyphenol content varied significantly between the investigated edible species. The extract of *F. circummediterranea*, which was collected during the third sampling at the first site (Monteforte) showed the lowest value (0.76 ± 0.05 mg GAE g^{-1} DM), whilst the highest one was found for *T. longicaulis* (17.03 ± 0.30 mg GAE g^{-1} DM) collected at the second site (Verrino) during the second sampling time.

3.2. Total Tannin Content

Results obtained from the determination of the total tannin content are reported in Table S2 available in Supplementary Materials. Particularly, the tannin content varied a lot in relation to the investigation period. *F. circummediterranea* extract contained 0.55 ± 0.05 mg TAE g^{-1} DM of tannins whilst *T. longicaulis* presented 15.50 ± 0.05 mg TAE g^{-1} DM of tannins, which represented the highest concentration.

3.3. Condensed Tannins (Proanthocyanidins)

The analysis of condensed tannins showed quite significant variability, as reported in Table S3, available in Supplementary Materials. The lowest value was shown by *B. erectus* (0.13 ± 0.02 μg mL^{-1}) collected during the second sampling time in the Monteforte area whilst the highest one was found in *D. pentaphyllum* (22.8 ± 0.1 μg mL^{-1}) collected during the second sampling period in the Verrino area. Significant concentrations of condensed tannins were detected in *T. pratense* (10.51 ± 0.08 μg mL^{-1}) and *L. corniculatus* (6.03 ± 0.05 μg mL^{-1}), collected during the third and second sampling period in the Monteforte and Verrino areas, respectively.

3.4. Antioxidant Activity

Results obtained from the determination of the antioxidant activity of the investigated wild edible plants highlighted that the highest antioxidant capability was detected in *T. pratense* extract, which showed the lowest value of IC_{50} (0.31 ± 0.01 mg mL^{-1}). On the contrary, *M. lupulina* showed the highest value of IC_{50} (9.83 ± 1.13 mg mL^{-1}), which corresponds to the lowest antioxidant activity among the analysed plant species. Results are reported in Table S4, available in Supplementary Materials.

3.5. Heavy Metals

From the determination of heavy metals in the investigated wild edible plants resulted that there was no contamination by cadmium (Cd), nickel (Ni) or lead (Pb). Indeed, the obtained values were lower than the limit of detection (LOD). On the other hand, copper (Cu) and zinc (Zn) were detected in all the examined plant samples, although in varying concentrations. For example, the lowest value for Cu was 1.2 μg g^{-1} detected in *F. circummediterranea* whilst the highest one was of 20.4 μg g^{-1} in D. glomerata. For Zn, values ranged from 1.1 μg g^{-1} in *T. pratense* to 26.5 μg g^{-1} in *H. comosa*. More detailed results are reported in Table S5 available in Supplementary Materials.

4. Discussion

The positive effect of some plant compounds on humans and animals seems to be increasingly highlighted by epidemiological evidence. Prevention, in humans and animals, of diseases resulting from oxidative stress could be based on the use of plants with antioxidant activity [11]. It has been reported that phenolic compounds act as antioxidant, anti-inflammatory and anti-carcinogenic agents [23], besides being able to enhance the growth performance of an animal fed with them [24]. The current study aimed to analyse the antioxidant activity and the content of phenolic compounds in some wild edible plants grown under natural environmental conditions in order to evaluate their possible use in animal nutrition.

It has been well-documented that plants produce phenolic compounds as a response to environmental stresses. For example, high light, low temperatures, pathogen infection and nutrient deficiency lead to an increase in the production of free radicals and oxidative species in plants [25]. Laboratory experiments showed that mild light stress increased the production of phenolic compounds and antioxidant activity [26]. Generally, plant metabolism is affected by solar radiation and, according to the species, secondary metabolites (i.e., phenolic acids and flavonoids) can accumulate in their tissues [27].

The mechanisms by which plants physiologically response to UV radiation are not fully understood yet. Different theories have been proposed, but they are not proven by scientific evidence. The most accredited theory supposes that plants detect UV radiation by means of their ability to generate ROS. In this case, the increase in the production of phenolic compounds is related to an increase in transcription as a response to the oxidative stress [28]. As for the antioxidant activity, the scientific literature reported that the relationship between antioxidant activity and solar radiation (UV-A and UV-B radiation) is not "unique", but is influenced by the availability of plant water. More specifically, scientific evidence would suggest that antioxidant activity increased only after an exposure

to UV-A and UV-B radiation under dried conditions [29]. Temperature is another parameter that could influence both antioxidant activity and phenolic compounds content [30], but it can significantly vary according to the species [29]. Generally, cold-tolerant plants seem to present a higher concentration of phenolic compounds and a stronger antioxidant activity [30]. More precisely, synthesis of phenolic compounds in leaves is related to the activity of specific enzymes. For example, phenylalanine ammonia lyase (PAL) is an important enzyme for the synthesis and production of phenolic compounds, including tannins. Scientific literature reported that low temperatures determine an increase in the PAL activity, leading to an increase in the production of phenolic compounds [31]. Our findings seem to confirm this behaviour only in the case of condensed tannins (Figure S4). Indeed, the production of condensed tannins increased during winter and decreased in spring. So, it is possible to suppose that this behaviour can change according to the species.

As for antioxidant activities, results of investigated plants are reported in Figure 1. More specifically, the inhibition concentration (IC_{50}) values of plants during the sampling times are showed. A low value of IC_{50} indicates a strong antioxidant activity, meaning that low concentrations of antioxidant substances in the extract can inhibit 50% of the radical reaction [32].

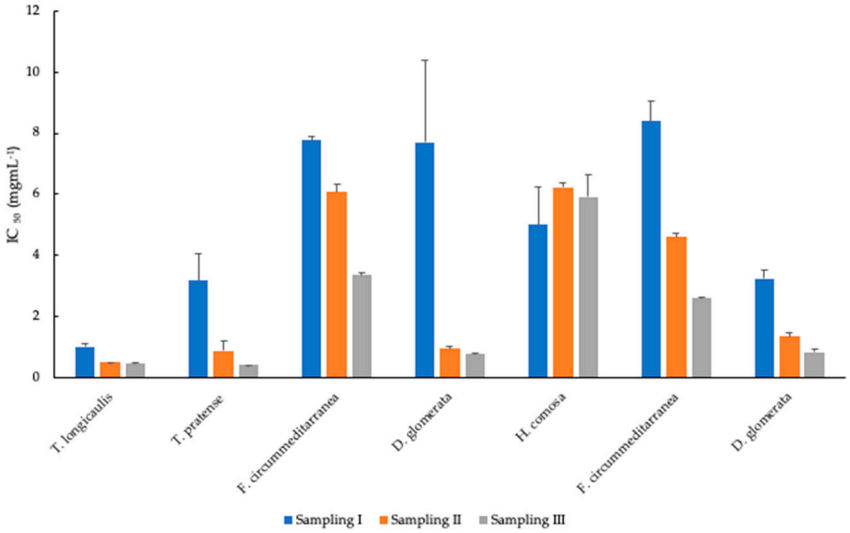

Figure 1. The dependence of IC_{50} values of investigated plants, which is inversely related to the antioxidant activities. For this evaluation, only plants available at all three sampling times were considered. *F. circummediterranea* and *D. glomerata* were reported twice because they were collected from two different areas (Guardata and Guado Cannavina, respectively). A low value of IC_{50} indicates a strong antioxidant activity since low concentrations of extract are able to inhibit 50% of the radical reaction [32].

Our findings (Figure 1) proved that, in general, the highest antioxidant activity was shown by plant samples collected during the third sampling period (November/December) whereas the lowest values were detected in plants collected during the first sampling in June. Low temperature is a stress condition for plants, which can determine an excessive excitation of the respiratory and photosynthetic electron transport systems. In this scenario, low temperature can determine an increase in the production of ROS that are able to damage plant cells systems. Hence, to control the ROS levels, it is vital for plants to adjust their antioxidant systems [33]. However, a different trend was observed for H. comosa extracts, with the lowest antioxidant activity observed in September and the highest one in June. This different trend could be related to the genetic component of the plant itself. H. comosa is characterised by a significant resistance to low temperatures. The adult plants of this species show no damage up to temperatures of −18 °C, while the younger plants up to −13 °C [34]. This can explain why its antioxidant activity of H. comosa tends to

reduce during Autumn–Winter. Among all analysed species, T. pratense presented the highest antioxidant power (expressed by IC_{50} value at 0.31 ± 0.01 mg mL^{-1}) during the months of November and December. The antioxidant activity (expressed as IC_{50}) of some extracts of T. pratense collected in Turkey determined by using the DPPH method was 0.32 mg mL^{-1} [35], which is comparable to our results. However, among external factors influencing the determination of the antioxidant activity of a plant extract, the solvent used is one of the most significant. Nantitanon and colleagues, in fact, proved that antioxidant activity changed depending on the type of solvent used for the extraction [36].

As for total polyphenols content in analysed plants, results are shown in Figure 2a, where a comparison with the antioxidant activity values has been done.

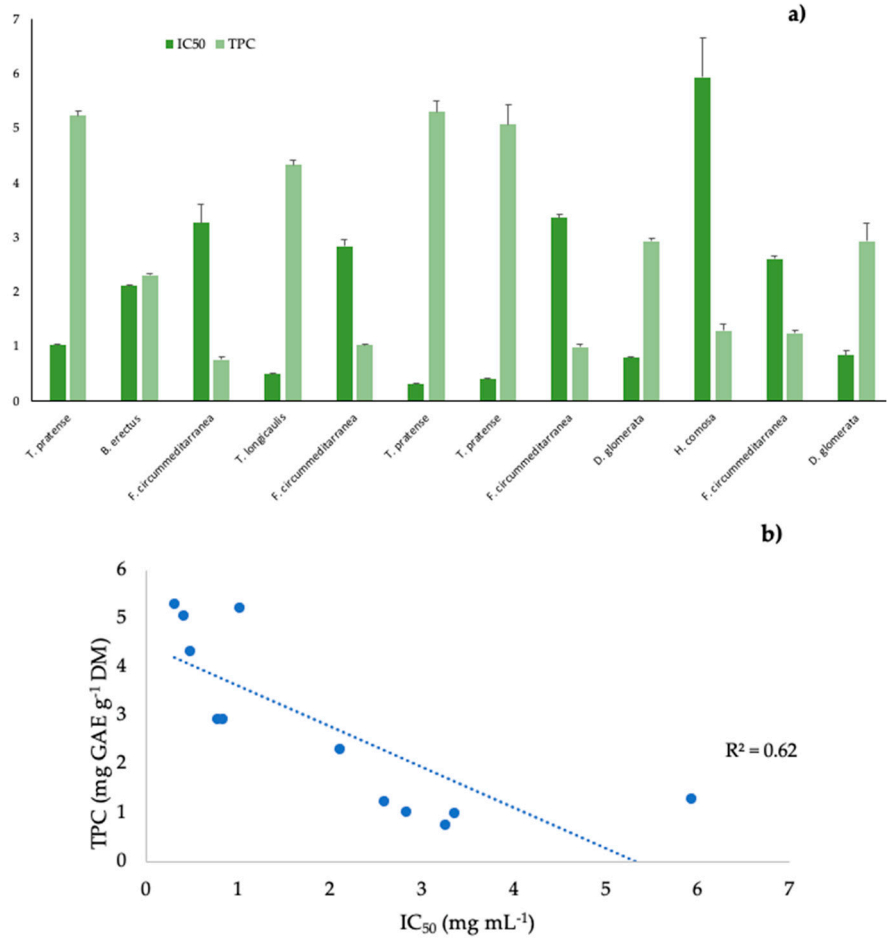

Figure 2. (**a**) Comparison between total polyphenol content (mg GAE g^{-1} DM) and antioxidant activity (IC_{50} mg mL^{-1}) of some of the investigated wild edible plants, collected during the third sampling (November–December). Plant species reported more than once were collected from different sites, specifically Monteforte, Verrino, Guardata and Guado Cannavina. The Y axis represents the antioxidant activity and total polyphenol content of the affected plants in the graph. (**b**) Correlation between antioxidant activities and total polyphenols content in plants.

The study of the potential correlation between total polyphenols content and the antioxidant activities has been carried out only on the third sampling, as it was the longest one in terms of time. As showed by Figure 2b, there is a generally moderate negative correlation (−0.79) between the two parameters investigated, meaning that there is a strong positive correlation between antioxidant activity and total polyphenol content. As stated before, low value of IC_{50} correspond to high value of antioxidant activity.

During the third sampling, the highest content of total polyphenols was revealed in the methanol extract of *T pratense* (Figure 2a). From the scientific literature, it is known

that *T. pratense* is considered as an important source of polyphenols. Kucukboyaci et al. reported a concentration of 153 mg GAE g^{-1} DM of total polyphenols in an ethanol extract of this species [35]. *T. longicaulis* showed a significant content of polyphenols. Ozturk's paper stated the important content of polyphenols in this species, revealing a value of 188 mg GAE g^{-1} DM in a methanol extract [37]. Furthermore, the concentration of phenolic compounds in the extract is dependent on the type of solvent. Some authors identified hot water as the best solvent to maximise their extraction [36], but more recent studies have suggested the use of methanol [37]. *H. comosa* also revealed significant concentration of polyphenols in its extract. Therefore, our findings not only seem to confirm the remarkable phenolic compound content of *T. longicaulis* and *T. pratense*, but also identify *H. comosa* as an important and exploitable source of them, underling the potential use of this plant species and its extracts in animal nutrition, food, cosmetic and pharmaceutical preparation.

In addition, *T. pratense* showed in all three cases low values of IC_{50} that correspond to high concentrations of total polyphenols. As a matter of fact, the lower the IC_{50}, the higher the total polyphenol content [24]. This trend can be observed also in the extract of *T. longicaulis* and less clearly in *D. glomerata*. These plants seem to confirm the existence of a relationship between the total polyphenol content and antioxidant activity. Furthermore, in the case of the extract of *F. circummediterranea* and *H. comosa*, to high values of IC_{50} correspond low concentrations of polyphenols. However, in the case of *B. erectus* extract, this relationship was not confirmed. As a matter of fact, in each plant species, there are several compounds that contribute to the overall antioxidant activity [37].

The analysis of total tannins showed a comparable trend with the total polyphenol content (Table S2). For example, *T. longicaulis* showed the highest concentration of total tannins and polyphenols and, similarly, in the case of *F. circummediterranea*, a low value of polyphenols corresponds to a low value of total tannins. *T. longicaulis*, in fact, presented a concentration of 15.50 mg TAE g^{-1} DM, which is slightly higher than concentrations reported for other species of *Thymus*. Indeed, in the methanol extract of *T. satureioides* the total tannin content detected was of 4.63 mg TAE g^{-1} DM, whereas the lowest content (2.62 mg TAE g^{-1} DM) was revealed in *T. broussonetii* [38]. Although little scientific evidence is available regarding possible interspecific variation in terms of total tannin content, comparison of our results with those of other papers would seem to confirm the strong impact of genetic material on the tannin content of the different *Thymus* species. Furthermore, significant concentrations of tannins were detected in *T. pratense* and *H. comosa*. While the considerable tannin content was fairly well known in the case of *Thymus* spp., no information was available in the literature for the species *T. pratense* and *H. comosa*. Our findings proved the richness of these species in tannin content. These compounds are able to prevent the initiation of the chain, the peroxide decomposition, the hydrogen abstraction and trapping of radicals [38].

Condensed tannin content was investigated in the current paper. The highest concentration was found in *D. pentaphyllum* (22.9 μg mL^{-1}), followed by *T. pratense* and *L. corniculatus*, with concentrations of 10.51 and 6.03 μg mL^{-1}, respectively. Condensed tannins, at low concentrations, have beneficial actions in ruminants: these compounds reduce the degradation of forage proteins in the rumen by binding to them. They also reduce the concentration of proteolytic bacteria in the rumen. Furthermore, an increase in milk production has been proven, along with a reduction in the risk of bloat and internal parasite load [39]. Hence, the presence of condensed tannins in significant concentrations in some of the analysed plant species can represent an opportunity for their use in animal feeding.

Overall, the obtained results showed that the concentration of condensed tannins in the analysed edible species did not have a regular trend over time (increase/decrease in concentration in relation to the harvest period). In this regard, it is useful to observe the data relating to one of the most representative species, *Festuca circummediterranea* (Table 1). Although the values related to the second and third collection periods at the Monteforte, Verrino and Guardata sites were very similar, there were important differences between the samples collected at Guardata and Guado Cannavina during the first sampling.

Moreover, the *Festuca* samples taken at the latter site seem to follow a different trend. From our findings, it is not possible to identify a correlation between the geographical area of sampling and the antioxidant activity of plants as well as for the other parameters investigated, whereas potential correlations were identified between sampling seasons and parameters investigated. More precisely, only plants which were present during all three sampling times were taking into account for the studying of correlations between seasons of sampling and antioxidant activities (Figure S1). It resulted that there is a strong positive correlation for *F. circummediterranea* collected from Guardata and Guado Cannavina and the mean temperature recorded during the sampling (0.98; 0.87), meaning that the antioxidant activity of considered species decreased during the summer (mean temperature recorded 22.5 °C), whereas it increased during the winter ((mean temperature recorded 6.0 °C). Moderate correlations were found in the case of *B. erectus* (0.49) and *T. longicaulis* (0.65), collected from Monteforte and Verrino, respectively, but also in these cases, the highest antioxidant activities have been found during the winter. As for correlation between total polyphenols content and sampling season, results are shown in Figure S2. More specifically, *T. longicaulis* and *F. circummediterranea* showed strong positive correlations with the temperature values recorded during the samplings (0.93; 0.91; 0.92). *B. erectus* showed a moderate correlation (0.72). Total tannin content was positively correlated with the season sampling (Figure S3): *B. erectus*, *T. longicaulis* and *F. circummediterranea* showed correlation values higher than 0.75 (0.75; 0.96; 0.94; 0.95). Instead, condensed tannin content showed a negative correlation with the sampling seasons (Figure S4). Particularly, *B. erectus* (−0.11) is strongly negatively correlated with sampling seasons as well as *T. longicaulis* and *F. circummediterranea* collected in Guado Cannavina (−0.95; −0.65). A weak negative correlation was observed in the case of *F. circummediterranea* (0.15) collected in Guardata.

Table 1. Condensed tannin concentration in extracts of *F. circummediterranea* collected during the three sampling times in the four study areas. Results are expressed as μg mL^{-1}. Standard deviation values are reported. n.a. means that the sample was not available.

Site	Sampling I	Sampling II	Sampling III
Monteforte	n.a.	0.67 ± 0.08	1.74 ± 0.32
Verrino	n.a.	0.63 ± 0.06	1.97 ± 0.43
Guardata	2.49 ± 0.42	0.77 ± 0.04	1.67 ± 0.56
Guado Cannavina	0.67 ± 0.08	1.23 ± 0.03	1.21 ± 0.65

However, even if the data of all the analysed plant species sampled during the months of November–December (Sampling III) would seem to show a general moderate correlation between the antioxidant activity and the total polyphenol content (Figure 2b), considering only the plants present at all three in samplings (i.e., *B. erectus*, *F. circummediterranea*, *T. longicaulis*), there appears to be no such correlation (more information are available in the Supplementary Materials Figure S5). The correlations of the parameters investigated with the sampling periods (mean temperatures recorded) show that, for the species *B. erectus*, *F. circummediterranea* and *T. longicaulis*, low temperatures determine an increase in antioxidant activity but a low content of total polyphenols. On the contrary, this trend seems to not be confirmed for condensed tannin content. In their case, in fact, higher temperatures determine a reduction in the production of condensed tannins.

Lastly, the potential interaction between total polyphenol content and the presence of heavy metals has been analysed. Laboratory experiments proved that groups of *Zea mays* species treated with heavy metals increased in the production of polyphenols. Particularly, scientific evidence proved that Cd, Cu and Pb could determine an increase in the production of phenolics as a response to this abiotic stress [29]. For this purpose, the determination of heavy metals was carried out to better understand if there was a significant impact in polyphenol compound production in the examined wild edible plants.

Among heavy metals investigated, only Cu and Zn were detected. Their impact on the production of polyphenols by wild plants and their antioxidant activities were analysed.

To this purpose, *F. circummediterranea* and *T. pratense* were taken into account as they are the most representative species of the investigated territories, meaning that they were present at all sites investigated during all sampling times.

The data analysis showed that the correlation values were −0.45 for *F. circummediterranea* and −0.83 for *T. pratense*, indicating a slight negative correlation and a good negative correlation, respectively (Figure 3) between HMs concentrations and total polyphenols contents. Negative correlations were observed also for the antioxidant activities both for *F. circummediterranea* and *T. pratense* (−0.86; −0.89). Our findings seem to suggest a very weak negative correlation between total polyphenols content and HMs total concentration for *F. circummediterranea* and a clear negative one for *T. pratense*, meaning that HMs concentrations do not entail an increase in total polyphenols content production. More specifically, it is possible to suppose that in the case of *T. pratense*, an increasing HMs concentration determines a reduction in the production of polyphenols. The results indicate that higher concentrations of HMs can reduce the antioxidant activity of plants investigated. The effect of heavy metals on polyphenol content is poorly studied and little information is available in the scientific literature. However, some scientific evidence suggests that susceptibility to environmental stresses, such as heavy metals, can lead to a reduction in polyphenol content [40].

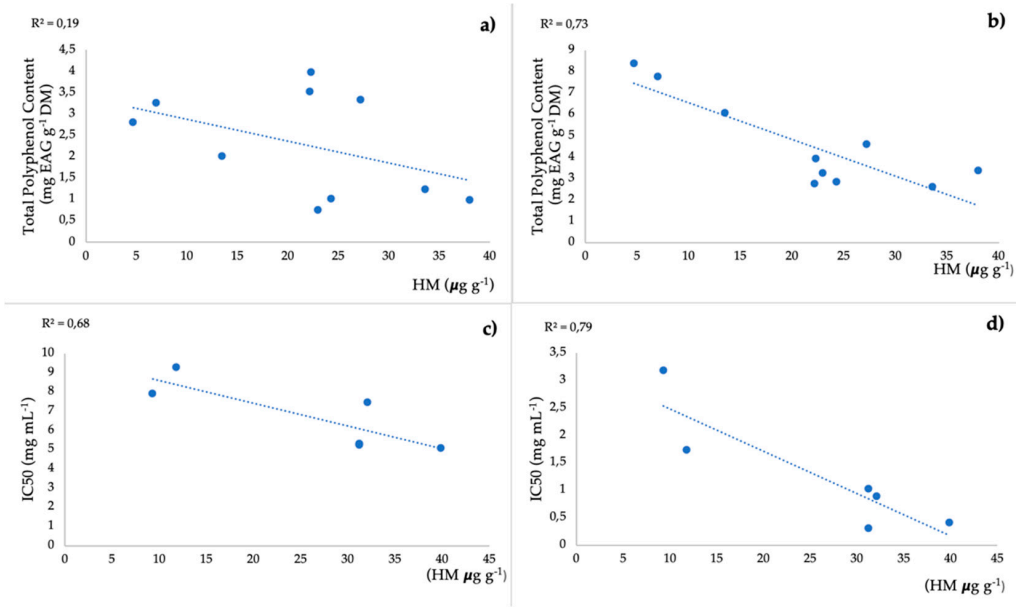

Figure 3. Correlation between total HMs concentrations and total polyphenol content in extracts of *F. circummediterranea* (**a**) and *T. pratense* (**b**), and total HMs concentrations and antioxidant activities of *F. circummediterranea* (**c**) and *T. pratense* (**d**).

5. Conclusions

The aim of the present work was to analyse the antioxidant power and total polyphenol content of some wild plant species of nutritive interest. The extraction processes applied to the leaf samples allowed determination of the phenolic contents of leaves tested and are reported in the Supplementary Materials. The results showed a good content of phenolic compounds and an interesting antioxidant activity, highlighting the possibility of their appropriate use in animal feed. The influence of sampling seasons on the production of phenolic compounds and antioxidant activities was analysed.

The results show that, in general, the antioxidant activity is higher during the winter period. This aspect confirms what is reported in the scientific literature. Phenolic compound contents, on the other hand, are higher when temperatures are higher. During the third sampling, the analysed plant species showed a positive correlation between antioxidant activity and total polyphenol content. More precisely, the IC_{50} values were lower in

the winter period, indicating a higher antioxidant activity and corresponding to a high concentration of total polyphenols. However, the analysis of the species present during all three sampling periods showed the absence of such a correlation. It is therefore possible to assume that this correlation is species-dependent. Furthermore, the work was supposed to analyse a potential correlation between the presence of heavy metals and total polyphenolic compounds. Although a possible ability of heavy metals to increase the production of phenolic compounds is reported in the literature, our data indicate a negative correlation. This is the first study to focus on the analysis of phenolic compounds, antioxidant activity and the effect of heavy metals on twelve wild edible plant species. Further studies will be necessary for in-depth analyses about the relationship between antioxidant activities and polyphenols in these species.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/separations10020092/s1, Table S1: Total phenol content in wild edible plants harvested in the four study areas investigated during the three sampling periods. Results are expressed as mg GAE g^{-1} DM ± SD, where DM is dry matter and SD is standard deviation. n.a. means that the sample was not available; Table S2: Total tannin content in wild edible plants harvested in the four study areas investigated during the three sampling periods. Results are expressed as mg TAE g^{-1} DM± SD, where DM is dry matter and SD is standard deviation. n.a. means that the sample was not available; Table S3: Condensed tannin content in wild edible plants harvested in the four study areas investigated during the three sampling periods. Results are expressed as μg mL^{-1} of extract ± standard deviation (SD). n.a. means that the sample was not available; Table S4: Antioxidant activity of wild edible plants investigated in the four study areas investigated during the three sampling periods. Results are expressed as IC_{50} (mg mL^{-1}) with related standard deviation (DS). n.a. means that the sample was not available; Table S5: Heavy metals concentrations in wild edible plants analysed expressed as μg g^{-1} with standard deviation. Cd, Ni and Pb were below LODs values; Table S6: Heavy metals concentrations in wild edible plants analysed expressed as μg g^{-1} with standard deviation. Cd and Pb were below LODs values; Figure S1: Correlation between the antioxidant activity and the sampling period, considering the mean temperatures of each sampling time. The correlation has been evaluated only when plant species was present during all period of sampling. (a) *B. erectus* collected from Monteforte; (b) *T. longicaulis* collected from Verrino; (c) *F. circummediterranea* collected from Guardata; (d) *F. circummediterranea* collected from Guado Cannavina; Figure S2: Correlation between the total polyphenols content and the sampling period, considering the mean temperatures of each sampling time. The correlation has been evaluated only when plant species was present during all periods of sampling. (a) *B. erectus* collected from Monteforte; (b) *T. longicaulis* collected from Verrino; (c) *F. circummediterranea* collected from Guardata; (d) *F. circummediterranea* collected from Guado Cannavina; Figure S3: Correlation between the total tannin content and the sampling period, considering the mean temperatures of each sampling time. The correlation has been evaluated only when plant species was present during all period of sampling. (a) *B. erectus* collected from Monteforte; (b) *T. longicaulis* collected from Verrino; (c) *F. circummediterranea* collected from Guardata; (d) *F. circummediterranea* collected from Guado Cannavina; Figure S4: Correlation between the condensed tannin content and the sampling period, considering the mean temperatures of each sampling time. The correlation has been evaluated only when plant species was present during all periods of sampling. (a) *B. erectus* collected from Monteforte; (b) *T. longicaulis* collected from Verrino; (c) *F. circummediterranea* collected from Guardata; (d) *F. circummediterranea* collected from Guado Cannavina; Figure S5: Correlation between the antioxidant activity and total polyphenols content of (a) *B. erectus* collected from Monteforte; (b) *T. longicaulis* collected from Verrino; (c) *F. circummediterranea* collected from Guardata; (d) *F. circummediterranea* collected from Guado Cannavina during all sampling periods. Correlation value (−0.25; 0.32; 0.97; 0.59).

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