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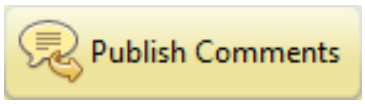

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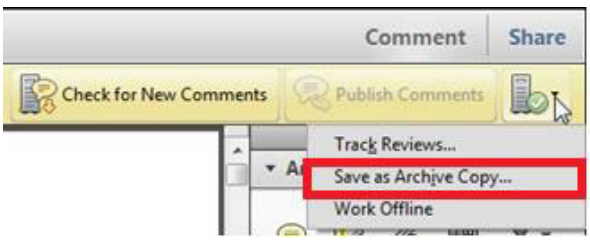

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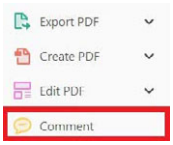

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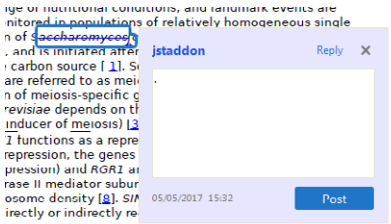

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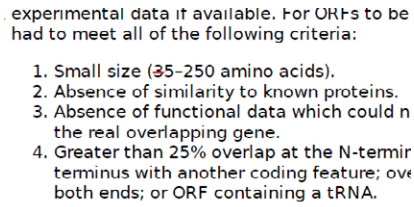

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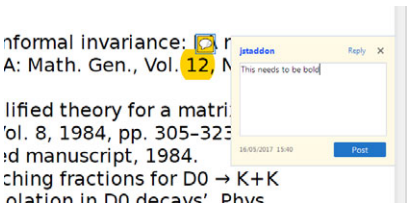

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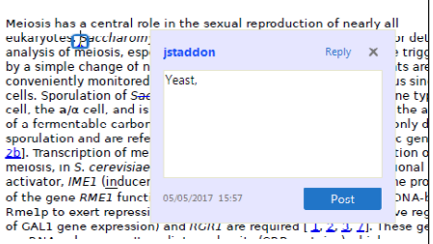

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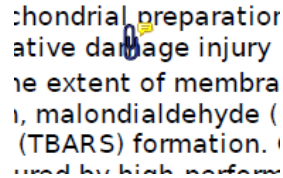

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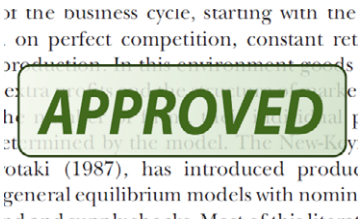

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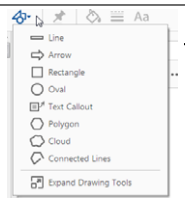

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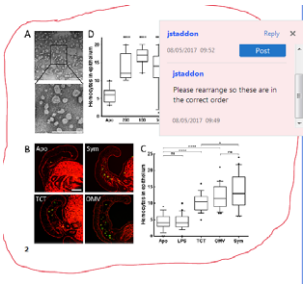

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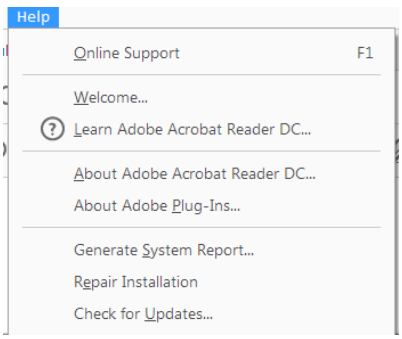

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Polyphenolic composition and antioxidant capacity of legume-based swards are affected by light intensity in a Mediterranean agroforestry system

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Abstract

BACKGROUND: In Mediterranean grazed woodlands, microclimate changes induced by trees influence the growth and development of the understory, although very little is known about its polyphenolic composition in relation to light intensity. We investigated the bioactive compounds and antioxidant capacity of different legume-based swards and variations as a result of full sunlight and partial shade. The research was carried out in a cork oak agrosilvopastoral system in Sardinia.

RESULTS: The highest values of (1,1-diphenyl-2-picrylhydrazyl) (DPPH) reached 7 mmol Trolox equivalent antioxidant capacity 100 g^{-1} dry weight (DW), total phenolics 67.1 g gallic acid equivalent kg^{-1} DW and total flavonoids 7.5 g catechin equivalent kg^{-1} DW. Compared to full sunlight, partial shade reduced DPPH values by 29% and 42%, and the total phenolic content by 23% and 53% in 100% legume mixture and semi-natural pasture, respectively. Twelve phenolic compounds were detected: chlorogenic acid in 80% legume mixture (partial shade) and verbascoside in pure sward of bladder clover (full sunlight) were the most abundant.

CONCLUSION: Light intensity significantly affected antioxidant capacity, composition and levels of phenolic compounds. The results of the present study provide new insights into the effects of light intensity on plant secondary metabolites from legume-based swards, highlighting the important functions provided by agroforestry systems.

Keywords: understory; *Trifolium spumosum*; bioactive compounds; HPLC; sunlight; partial shade

INTRODUCTION

In Europe, traditional agroforestry systems with a high natural and cultural value have been re-evaluated because of their important effects on ecosystem services and biodiversity.[1] Agroforestry systems include Mediterranean grazed woodlands, which are dominated by oak species, such as in Iberian dehesas and montados and Sardinian agrosilvopastoral farms.[2]

Plant assemblages vary from below-tree canopy areas to open areas[3] and, in some Mediterranean wood pastures, fodder crops are also grown to enhance the herbage on offer.[4] Forage mixtures mainly based on legume species or also including grasses have been widely established to improve pasture productivity and quality.[5–8] Other than supporting livestock farming, cork production and recreational activities, Mediterranean grazed woodlands provide a wide range of ecosystem services, such as carbon sequestration, water conservation, control of nutrient leaching, soil erosion and wildfires.[9–12]

Wood plants also modify the microclimate by reducing evapotranspiration and moderating extremes in soil temperatures and daily photosynthetically active radiation. Microclimate changes induced by woody plants influence the growth, development and maturity of the understory vegetation and, consequently, affect the quantity and quality of forage.[13] Herbage production usually decreases as light intensity decreases.[14] By contrast, Anderson and Moore[15] found a higher production of the understory subjected to moderate light intensity in an annual pasture vegetation growing under *Pinus radiata* D. Don. Kyriazopoulos *et al.*[16] reported similar results for natural herbaceous vegetation growing under *Prunus avium* L. The challenge for managers is thus to select the most appropriate forage species because this has a significant impact on the success of the entire silvopastoral system. Kyriazopoulos *et al.*[16] confirmed that grass–legume mixtures are more productive and of a higher nutritive value than pure grass stands under both full sun and moderate shade conditions.

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However, Mediterranean grazed woodlands should be regarded not only as a primary forage supply but also as a valuable and rich source of plant secondary metabolites, as reported in ethno-pharmacology, ethnobotanical, and ethno-veterinary studies.[17–20]

Among plant secondary metabolites, phenolics are a class of commonly-found bioactive compounds, which includes several groups of different substances. Phenolic acids, flavonoids and tannins are the most important compounds as a result of their biological activities, and especially their antioxidant properties[21–23] and related implications in animal nutrition and welfare.[24] Levels of plant antioxidants vary as a result of temperature, light intensity, harvesting season and genetic factors.[25] Studies on vegetable crops have reported that the antioxidant activity and phenolic content of spinach and sweet potato leaves were greatly affected by artificial shade and sunlight intensity.[26,27] The highest content of total polyphenols and antioxidant activity of green edible amaranth leaves were found in plants grown in full sunlight.[28] Mole *et al.*[29] reported an increase in polyphenols with increased light intensity in leaves of *Acacia pennata* (L.) Wild, *Cynometra leonensis* L., *Diopyros thomasii* Hutch. & Dalz. and *Trema guineensis* Schum. & Thonn., which should be explained in terms of plant physiology and intermediate metabolism rather than resource allocation or a direct response to herbivory. The distribution and abundance of many phenolics can be explained as the plant response to preventing or minimizing photodamage, and not as a trade-off in resource allocation in limited resource environments, or as a response to herbivory.[30] High light intensity has been related to the higher antioxidant capacity and total polyphenol concentrations in berries (*Berberis microphylla* G. Forst) and *Thymus vulgaris* L.[31] Finally, flavonoids serve multiple functions in photoprotection as ultraviolet (UV)-screening against antioxidant functions and as antioxidants in photoprotection.[32]

Despite the important implications and potential benefits from the exploitation of plant secondary metabolites, very little is known regarding the polyphenolic composition of understory in relation to the contrasting exposure to full sunlight or shade. We hypothesize that legume plant secondary metabolites might be affected by different light conditions. The main aims of the present study were (i) to determine the level of bioactive compounds and antioxidant capacity of different legume-based swards and (ii) to investigate their qualitative and quantitative variations caused by the contrasting exposure to full sunlight and shade that typically occurs in a Mediterranean silvopastoral system.

MATERIALS AND METHODS

Locations, experimental design and legume-based swards

The research was carried out between 2015 and 2016 in a private farm (Buddusò municipality, 40°37′99″N, 9°15′33″ E, elevation 700 m a.s.l.) located in north eastern Sardinia (Italy). The climate is Mediterranean with hot dry summers. Long-term rainfall is 840 mm and the average annual temperature is 12.7 °C. From September 2015 to August 2016, the annual rainfall reached 680 mm and was 20% lower than the climatic mean; temperatures differed slightly from the long-term values.

The area is characterized by extensive agro-silvopastoral systems, typical of northern Sardinia and similar semi-arid areas of the Mediterranean basin. Land is used above all for traditional sheep/cattle farming with pasture as the primary feeding source. Natural pastures may occasionally be fertilized, and/or ploughed for the establishment of annual forage crops traditionally represented by barley, oats, oats-vetch mixtures and annual *Trifolium* spp.

The soil, classified as Typic, Dystric and Lithic Leptsol,[33] has an acid pH (5.4) and sandy texture, with contents of nitrogen (0.2%), phosphorous (5.7 ppm), organic matter (3.7%) and organic carbon (2.3%).

Open areas with full sunlight exposition (FS) and areas under tree canopy with partial shade conditions (PS), under a cork oak (*Quercus suber* L.) density of 450 trees ha^{-1}, were carefully identified. Light levels of photosynthetically active radiation were measured using a SunScan canopy analysis system (Delta-T Devices, Cambridge, UK). For both FS and PS, the following legume-based swards were compared:

(1) CNR ISPAAM mixture (L80GMIX), with 80% legume composition by *Trifolium subterraneum* L. (40%) and *Medicago polymorpha* L. (40%) and 20% *Lolium rigidum* Gaudin;
(2) Fertiprado commercial legume mixture (L100MIX), with 100% annual legume composition, 60% of which comprised *Trifolium subterraneum* L. The remaining legume species were *Ornithopus sativus* Brot. (20%), *Trifolium incarnatum* L. (6%) *Trifolium michelianum* Savi (4.5%) *Trifolium resupinatum* L. (3%) *Trifolium vesiculosum* (3%) *Trifolium isthmocarpum* Brot. (1.5%) and *Trifolium glanduliferum* Boiss. (1%).
(3) Unsown semi-natural pasture (L60SNPA), with 60% legume composition and a predominance of native unsown *Trifolium subterraneum* L. Other legumes were *Trifolium* spp. *Ornithopus compressus* L. Non-legume species were mainly represented by *Lolium* and *Avena* spp., *Asphodelus macrocarpus* Parl., *Hyoseris radiata* L., *Carlina corymbosa* L., *Sonchus oleraceus* L., *Plantago lanceolata* L., *Raphanus raphanistrum* L., *Rumex* spp, *Daucus carota* L., *Echium plantagineum* L. and *Thapsia garganica* L.
(4) Bladder clover, *Trifolium spumosum* L., pure sward (100BCLO), elite Sardinian accession.

Sown legume-based swards were established in September 2015, after soil ploughing and seedbed preparation. Before sowing, all plots were fertilized with 100 kg ha^{-1} of P_2O_5. Plot sizes were 5 × 3 m and plots were arranged in a completely randomized design with three replications.

Plant materials and sample preparation

Samples were harvested from each plot. In late spring, 240 days after sowing, shoot forage samples were cut from each plot at ground level, approximately at 5 cm, and immediately frozen in liquid nitrogen. Shoot subsamples were then freeze dried with Heto Lyolab 3000 (Heto-Holten A/S, Allerød, Denmark), ground to a fine powder and stored at −20 °C until analysis. Ground shoot samples (50 mg) were treated with a 2.5 mL methanol/water (8:2 v/v) mixture and shaken for 60 min. The samples were then centrifuged for 10 min at 1683 × *g* and the supernatant was stored at −20 °C until analysis. All the samples were analyzed in triplicate.

Total phenolic content

Total phenolics (TotP), non-tannic phenolics (NTP) and tannic phenolics (TP) of extracts were determined using the Folin–Ciocalteau reagent, in accordance with procedures previously described by Piluzza and Bullitta.[34] Results were expressed as g of gallic acid equivalent (GAE) kg^{-1} dry matter of plant material (g GAE kg^{-1} DM) by means of a calibration curve of gallic acid (5–30 mg L^{-1}, $r^2 = 0.999$).

The butanol assay[34] was used for quantification of the extractable condensed tannin content from samples, expressed as g delphinidin equivalent per kg^{-1} dry matter (g DE kg^{-1} DM) by means of a calibration curve of delphinidin (10–50 mg L^{-1}, $r^2 = 0.988$).

Total flavonoid content

Total flavonoids (TotF) were quantified by colorimetric assay with the $AlCl_3$ method, in accordance with procedures reported previously.[22] TotF in samples were quantified by a catechin calibration curve (2.5–20 μg mL^{-1}, $r^2 = 0.999$). The results were expressed as g of catechin equivalent (CE) kg^{-1} dry matter (g CE kg^{-1} DM).

Determination of antioxidant capacity

Antioxidant capacity was determined by means of the 2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) and by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assays[35] with some modifications.[22] Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as the reference standard. For each assay, 0.1 mL of diluted sample was used, a calibrate standard curve with Trolox (2–12 μmol L^{-1}; $r^2 = 0.997$ for the DPPH assay and $r^2 = 0.998$ for the ABTS assay) was made. The results were expressed in terms of Trolox equivalent antioxidant capacity (TEAC), as mmol Trolox equivalents 100 g^{-1} dry weight of plant material (mmol TEAC 100 g^{-1} DW).

Reverse phase-high-performance liquid chromatography (HPLC) analysis of phenolic compounds

The phenolic compounds were analysed on an Agilent 1260 series HPLC instrument (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary pump (G1311B), degasser, column thermostat (G1316A), auto-sampler (G1329B) and diode array detector (G1315 B).

Chromatographic separation was carried out according to Karimi *et al.*[36] with some modifications, mainly with respect to the use of the column and gradient elution.

The column was a Zorbax Eclipse plus C_{18} (250 × 4.6 mm, 5 μm; Agilent Technologies). The flow rate was 0.8 mL min^{-1} and the column temperature was set to 30 °C. The injection volume was 10 μL, and the detection wavelengths were set to 280 and 350 nm. Elution was carried out with a binary mobile phase of solvent A (water and 0.1% trifluoroacetic acid) and solvent B (acetonitrile). The gradient elution was modified as follows: 0–5 min from 5% to 15% B, held for 5 min; 10–20 min from 15% to 25% B, held for 5 min; 25–30 min from 25% to 35% B, 30–35 min from 35% to 45% B, 35–40 min from 45% to 97% B, held for 5 min; and 45–60 min from 97% to 5% B. The post-running time was 5 min. Phenolic compounds were monitored at 280 and 350 nm. Data were processed using the OpenLAB CDS ChemStation edition 2012 (Agilent Technologies). Identification and peak assignment of polyphenolic compounds was based on a comparison of their retention times and spectra with analytically pure standard compounds, as well as by adding the standard solution to the sample. The concentrations of 12 standards [neochlorogenic acid, chlorogenic acid, rutin, verbascoside, 3,5-di-*O*-E-caffeoylquinic acid (3,5-DCQ), naringenin, isorientin, *p*-coumaric acid, luteolin 7-*O*-β-D-glucoside, luteolin, quercetin, gallic acid] were calculated in accordance with the external standard method curve (four known concentrations for each standard in duplicate, $r^2 = 0.99$) and expressed in g kg^{-1} DW.

Statistical analysis

Data were analysed using Statgraphics Centurion XVI.[37] Statistical significance was performed by two-way analysis of variance to test for differences between different legume-based swards and light intensity of full sunlight and partial shade. Fisher's test and Tukey's honestly significant test were used for post-hoc tests of significant differences between means as indicated. The regression analyses between polyphenols and antioxidant capacity were calculated using Excel 2016 (Microsoft Corp., Redmond, WA, USA). $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Light interception by cork trees was 85%, 77% and 70% in January, April and May, respectively as a result of the different solar azimuth angle of the seasons. Therefore, only 15%, 23% and 30% of the effective light radiation reached the understory of different legume-based swards.

The antioxidant capacity, total phenolic and total flavonoid contents of the different legume-based swards were significantly affected by the contrasting conditions of light intensity, as well as by the type of legume-based sward (Figs 1–7).

L100MIX and L60SNPA had the highest antioxidant capacity values and total phenolic and total flavonoid contents under FS. The peak values of DPPH were 6.6 and 7.0 mmol TEAC 100 g^{-1} DW, (Fig. 1), total phenolics were 67.1 and 50.1 g GAE kg^{-1} DW (Fig. 3), and total flavonoids were 6.4 and 7.5 g CE kg^{-1} DW, respectively (Fig. 6). Compared to full sunlight, PS reduced DPPH values by 29% and 42%, and the total phenolic content by 23% and 53%, in L100MIX and L60SNPA, respectively, and PS also reduced the total flavonoid content by 51% in L60SNPA.

L100MIX showed a condensed tannin (CT) content of 2.9 g DE kg^{-1} in the FS, which was twice as high as in PS. By contrast, PS significantly increased the CT content in 100BCLO by 13% (Fig. 7). Unfortunately, the CT concentrations found were too low to affect protein solubility and degradation in the rumen because the suggested minimum plant CT concentration needed to make forage bloat-safe is 5 g kg^{-1} DM or greater.[24,38] However, the results obtained suggest that the effects of light intensity should be investigated on legume species containing higher and/or optimal CT levels. The synthesis of flavonoids and phenolic acids depends on ecological and physiological factors. Light has been shown to be the key environmental factor influencing phenolic acids and flavonoids synthesis in most plants.[39]

A study on the effects of shade on the synthesis and accumulation of polyphenolic compounds in ginger (*Zingiber officinale* Roscoe) varieties indicated that phenolic acids and flavonoids are completely light dependent and that their biosynthetic rate is related to light intensity.[39] Conversely, 100BCLO showed that total flavonoids were unaffected by light intensity, whereas condensed tannins were higher in PS (Figs 6 and 7), indicating a legume species response.

Significant correlations were found between the antioxidant capacity by means of the ABTS and DPPH methods and the phenolic content (Table 1). ABTS and total phenolics showed a correlation of $r^2 = 0.8061$ (full sunlight) and $r^2 = 0.8558$ (partial shade), whereas statistically significant correlations were not found between antioxidant capacity and condensed tannins. Significant correlations were also found between antioxidant capacity and TotP and TotF in both FS and PS. Our findings agree with many studies regarding the relationship between antioxidant activity and total phenolic compounds.[23,35]

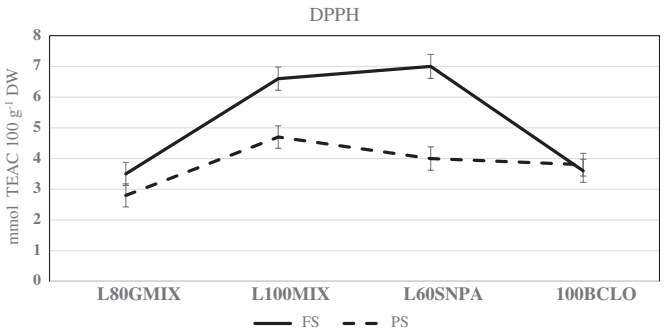

Figure 1. Antioxidant capacity (DPPH method) in shoot of legume-based swards growing in full sunlight (FS) and partial shade (PS). Vertical bars represent confidence interval. L80GMIX, CNR ISPAAM mixture; L100MIX, Fertiprado commercial mixture; L60SNPA, Unsown semi-natural pasture; 100BCLO, *Trifolium spumosum* L., pure sward.

Table 1. Correlations (r^2) established between total phenolics (ToTP), non-tannic phenolics (NTP), tannic phenolics (TP), condensed tannins (CT), total flavonoids (ToTF) and antioxidant capacity (ABTS, DPPH) from shoot of legume-based swards growing in full sunlight (FS) and with partial shade (PS)

	ABTS		DPPH		Ptot	
	FS	PS	FS	PS	FS	PS
DPPH	0.9325***	0.6230*				
ToTP	0.8061***	0.8558***	0.7057**	0.5680*		
NTP	0.6682**	0.9672***	0.4679*	0.6000*	0.8341***	0.8471***
TP	0.7229**	0.6106*	0.7491***	0.4317*	0.8927***	0.9102***
CT	0.0841 ns	0.1620 ns	0.0191 ns	0.0940 ns	0.4381*	0.0113 ns
ToTF	0.8924***	0.7733**	0.8888***	0.4014*	0.6108*	0.5700*

***$P \leq 0.0001$. **$P \leq 0.001$. *$P \leq 0.05$.

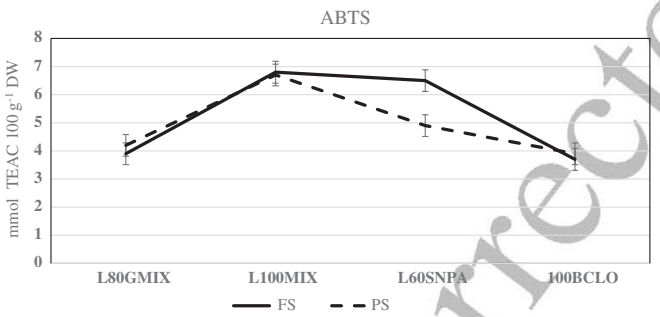

Figure 2. Antioxidant capacity (ABTS method) in shoot of legume-based swards growing in full sunlight (FS) and partial shade (PS). Vertical bars represent confidence interval. L80GMIX, CNR ISPAAM mixture; L100MIX, Fertiprado commercial mixture; L60SNPA, Unsown semi-natural pasture; 100BCLO, *Trifolium spumosum* L., pure sward.

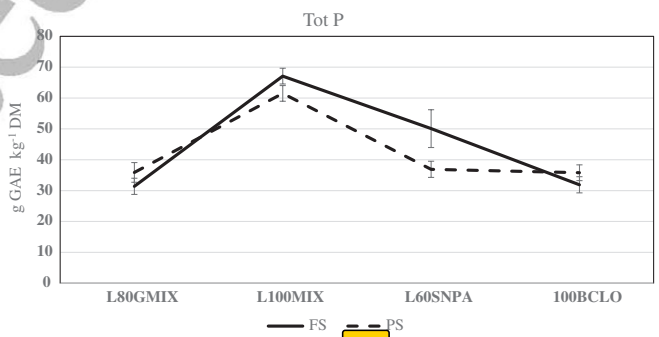

Figure 3. Total phenolic contents (T[illegible] g GAE kg^{-1} DM) in shoot of legume-based swards growing in full sunlight (FS) and with partial shade (PS). Vertical bars represent confidence interval. L80GMIX, CNR ISPAAM mixture; L100MIX, Fertiprado commercial mixture; L60SNPA, Unsown semi-natural pasture; 100BCLO, *Trifolium spumosum* L., pure sward.

Among the 30 individual phenolic compounds that were screened, 12 phenolic compounds were detected in the different legume-based swards subjected to a contrasting light intensity. These were neochlorogenic acid, chlorogenic acid, rutin, verbascoside, 3,5-DCQ, naringenin, isorientin, *p*-cumaric acid, luteolin-7-*O*-glucoside, luteolin, quercetin and gallic acid. Seven of these compounds are reported in Table 2 because they are present and common in almost all the various legume-based swards. Of the compounds not reported in Table 2, *p*-coumaric acid (0.11 g kg^{-1} in FS and 0.06 g kg^{-1} in PS) and luteolin-7-*O*-glucoside (4.25 g kg^{-1} in FS and 1.15 g kg^{-1} in PS) were detected only in L100MIX and only traces of L80GMIX in FS. In addition, quercetin was detected in L60SNPA (0.07 g kg^{-1} in FS and 0.035 g kg^{-1} in PS, respectively) and in 100BCLO only in FS (0.062 g kg^{-1}). Luteolin was found only in L60SNPA (0.11 and 0.03 g kg^{-1} in FS and PS, respectively) and below the detection limit in L80GMIX in FS. L60SNPA was the only legume-based sward containing gallic acid (0.163 g kg^{-1} in PS but below the detection limit in FS).

In an HPLC analysis of the polyphenolic composition in a permanent mountain pasture, similar to our results, Fraisse *et al.*[40] identified the following phenolic acids: neochlorogenic acid, chlorogenic acid, verbascoside and 3,5-DCQ, as well as flavonoids such as luteolin-7-*O*-glucoside and isorientin. In the same study, 1,5-DCQ, schaftoside and apigenin were also detected; however, these phenolics were not detected in the present study.

Table 2. HPLC analysis of polyphenolic compounds (g kg^{-1} DM) from shoot of legume-based swards growing in full sunlight (FS) and partial shade (PS)

	Neochlorogenic acid			Chlorogenic acid			Isorientin			Rutin			Verbascoside			3,5-DCQ			Naringenin		
Retention time	9.49			11.39			17.4			20.52			21.18			24.50			37.4		
	FS	PS		FS	PS		FS	PS		FS	PS		FS	PS		FS	PS		FS	PS	
L80GMIX	0.09 a	0.35 b	***	2.36 a	2.71 a	NS	ND	ND		0.3 bc	0.19 b	*	0.3 c	Tr	***	0.1 c	ND	NS	0.32 a	0.18 b	***
L100MIX	0.08 a	Tr		2.03 ab	0.44 c	***	0.17 ab	0.23 b	NS	0.85 a	0.21 ab	***	ND	ND		0.31 b	0.23 ab	*	0.17 b	0.44 a	***
L60SNPA	0.05 a	0.05 c	NS	1.44 b	1.64 b	NS	0.71 a	4.78 a	***	0.33 b	ND		1.33 b	ND		0.47 a	0.58 a		Tr	Tr	
100BCLO	0.05 a	0.46 a	***	0.23 c	0.45 c	NS	ND	ND		0.19 c	0.28 a	*	2.84 a	2.06	***	ND	0.13 a		ND	ND	

L80GMIX, CNR ISPAAM mixture; L100MIX, Fertiprado commercial mixture; L60SNPA, Unsown semi-natural pasture; 100BCLO, *Trifolium spumosum* L., pure sward.
In the columns of light exposure, means followed by the same lowercase letter are not significantly different at $P \leq 0.05$.
In the [illegible] LS indicates a test for light intensity effect on each legume-based sward.
ND, not detected; NS, not significant; Tr trace quantities.
$^{***}P \leq 0.0001$. $^{**}P \leq 0.01$. $^{*}P \leq 0.05$.

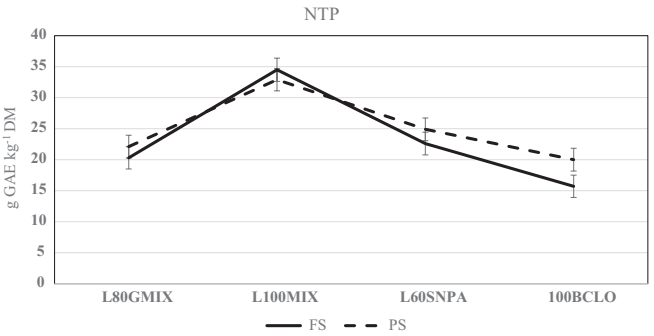

Figure 4. Non-tannic phenolic contents (NTP, g GAE kg^{-1} DM) in shoot of legume-based swards growing in full sunlight (FS) and partial shade (PS). Vertical bars represent confidence interval. L80GMIX, CNR ISPAAM mixture; L100MIX, Fertiprado commercial mixture; L60SNPA, Unsown semi-natural pasture; 100BCLO, *Trifolium spumosum* L., pure sward.

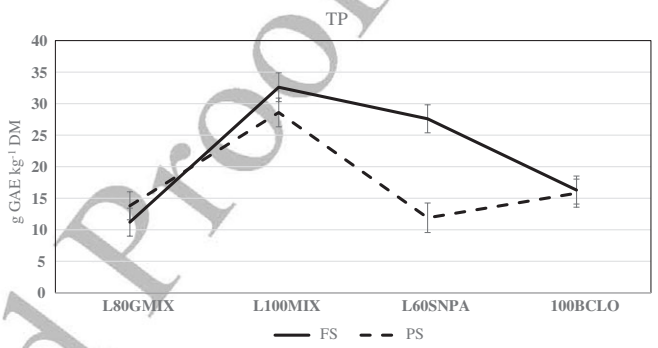

Figure 5. Tannic phenolic contents (TP, g GAE kg^{-1} DM) in shoot of legume-based swards growing in full sunlight (FS) and with partial shade (PS). Vertical bars represent confidence interval. L80GMIX, CNR ISPAAM mixture; L100MIX, Fertiprado commercial mixture; L60SNPA, Unsown semi-natural pasture; 100BCLO, *Trifolium spumosum* L., pure sward.

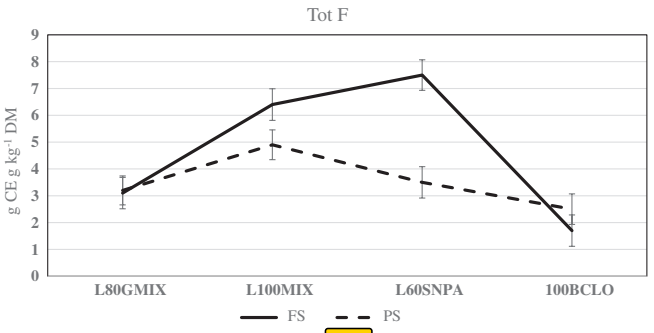

Figure 6. Flavonoid contents (To[illegible] g CE kg^{-1} DM) in shoot of legume-based swards growing in full sunlight (FS) and with partial shade (PS). Vertical bars represent confidence interval. L80GMIX, CNR ISPAAM mixture; L100MIX, Fertiprado commercial mixture; L60SNPA, Unsown semi-natural pasture; 100BCLO, *Trifolium spumosum* L., pure sward.

Regardless of the light intensity conditions, neochlorogenic and chlorogenic acids were always detected (Table 2). Rutin was not detected in L60SNPA grown in PS; 3,5-DCQ was not found in L80GMIX in PS and in 100BCLO grown in FS. Under PS, L80GMIX showed a higher content of neochlorogenic acid (0.35 g kg^{-1}) and a higher content of rutin (0.3 g kg^{-1}) in FS.

In FS, L100MIX revealed a higher content of neochlorogenic acid (0.08 g kg^{-1}), chlorogenic acid (2.03 g kg^{-1}), rutin (0.85 g kg^{-1})

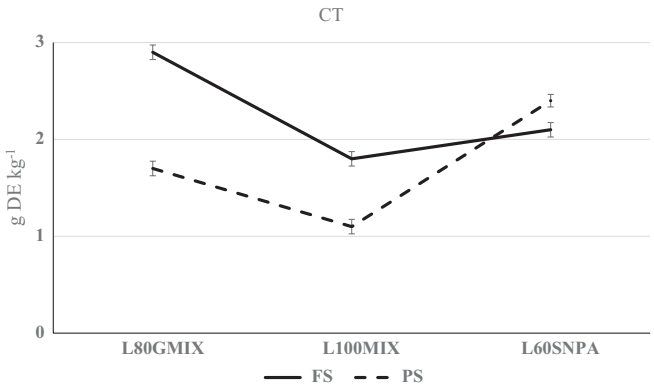

Figure 7. Condensed tannins (CT, g DE kg^{-1} DM) in shoot of legume-based swards growing in full sunlight (FS) and with partial shade (PS). Vertical bars represent confidence interval. L80GMIX, CNR ISPAAM mixture; L100MIX, Fertiprado commercial mixture; L60SNPA, Unsown semi-natural pasture; 100BCLO, *Trifolium spumosum* L., pure sward.

and 3,5-DCQ (0.31 g kg^{-1}) than PS; naringenin was higher in PS (0.44 g kg^{-1}).

Fraisse *et al.*[40] reported values of neochlorogenic acid at three stages of pasture growth (0.17, 0.65 and 0.26 g kg^{-1}) and chlorogenic acid (1.80, 4.90 and 2.36 g kg^{-1}), which were similar to our results. Verbascoside was not detected in L100MIX and the contents in the other legume-based swards were in accordance with those of Fraisse *et al.*[40] Chlorogenic acid in PS L80GMIX and verbascoside in 100BCLO in FS were found to be the most abundant phenolic acids. Among the flavonoids, luteolin-7-*O*-glucoside was the most abundant in L100MIX in FS, and isorientin was the most abundant in L60SNPA in PS. As a result of their valuable antioxidant activity, several studies have highlighted the potential role of these compounds in preventing various diseases associated with oxidative stress, such as cancer and cardiovascular and neurodegenerative diseases.[41,42] Chlorogenic acid is an ester of caffeic acid with quinic acid that occurs in many plants and fruits. A recent study reported that a sheep diet supplemented with coffee pulp (up 16%) did not affect their productive parameters, although it increased the antioxidant capacity of the diet and the production of volatile fatty acids in the rumen, at the same time as reducing the oxidative stress.[43] The coffee pulp used in this experiment contained predominantly chlorogenic acid as an antioxidant. As a result of the predominance of chlorogenic acid found in legume-based sward, it is reasonable to assume that chlorogenic acid might significantly contribute to the antioxidative properties of legume-based sward extracts.

Based on the results of the present study, the legume pure sward 100BCLO grown in full sunlight is rich in verbascoside and therefore could be a natural source of this compound. Verbascoside is a caffeoyl phenylethanoid glycoside, mainly found in the families of the Lamiales order, with antimicrobial, anti-inflammatory and antioxidant properties.[44,45] However, the presence of verbascoside has not been reported in other HPLC studies on other clover species, namely *T. resupinatum* L., *Trifolium pratense* L. and *Trifolium repens* L.[46–48] The effects of the administration of verbascoside on the plasma oxidative status and specific blood and milk production parameters have been evaluated in Lacaune ewes during the peripartum period.[49] It was reported that the use of verbascoside provided benefits in terms of several blood parameters, oxidative status and milk production, particularly in the immediate postpartum period. Casamassima *et al.*[50] and Vizzarri *et al.*[51] reported the supplementation of rabbit feeding with plant extracts, also based on verbascoside, with positive effects on blood parameters, plasma oxidative markers, productive performance and meat quality, as well as possible beneficial effects on animal health. It is worth highlighting that bladder clover (100BCLO) is a very promising aerial seeding annual legume, which represents a productive alternative to annual *Medicago* spp. in fine-textured Mediterranean soils.[52] To our knowledge, this is the first reported chemical characterization of *T. spumosum* shoot.

The oral administration of bay leaf (*Laurus nobilis* L.) and its isolated flavonoids, such as kaempferol, quercetin and luteolin, were shown to be useful in reducing hyperlipidemia of local Iraqi female rabbits.[53] Isorientin was only detected in L100MIX and L60SNPA with a higher content in PS, 0.23 and 4.78 g kg^{-1}, respectively. Fraisse *et al.*[40] reported values in isorientin of 1.05, 0.84, 0.56 g kg^{-1}.

In the leaves of *Medicago sativa* L., Karimi *et al.*[36] found phenolic acids and flavonoids, including gallic acid, naringenin and quercetin, which was a similar finding to the results of the present study, except for rutin, which was absent in *M. sativa* leaves.[36] By contrast, Karimi *et al.*[36] found apigenin, pyrogallol, caffeic acid, syringic acid, kaempferol and myricetin, which were not detected in the present study.

The striking feature is that each phenolic compound showed differences as a result of the light intensity and type of legume-based swards, leading to variable concentrations and composition of polyphenols contained in the forage of legume-based swards on offer to ruminants.

Some studies have reported a higher polyphenol content in full sunlight;[28,31] however, our results also revealed a higher content of isorientin and 3,5-DCQ in L60SNPA, as well as of naringenin in L100MIX, with a significant ($P \leq 0.01$) effect of PS on the synthesis of phenolic acids and flavonoids (Table 2). Ghasemzadeh and Ghasemzadeh[39] reported that the flavonoid accumulation of quercetin, apigenin, luteolin and myricetin in ginger varieties was affected considerably by shade, with the leaves having a higher flavonoid content under a 60% shade level compared to 0% shade. It was also reported that caffeic acid was only detected from ginger grown under a 0% shade, whereas tannic acid only accumulated in ginger leaves grown under a 60% shade level. The increase in phenolic acids, such as intermediates in lignin biosynthesis, indicates typical anatomical changes.[54] An important issue is whether the enhanced production of secondary metabolites under different light intensities is a result of the increased carbon production through photosynthesis or to the stress induced by different light intensities, which stimulates secondary metabolite production.[39]

Another important factor is the enzyme activity involved in the biosynthesis of phenolic compounds. A high content in some phenolic compounds could inhibit flavonoid synthesis, as a result of inhibiting the enzyme activity of phenylalanine ammonia lyase.[55] This enzyme is involved in the biosynthesis of phenolic acids, which show activity induced by high light intensity and UV.[56] The key enzyme in the flavonoid pathway is chalcone synthase, which is extremely sensitive to UV and blue light.[57,58]

By contrast to previous assumptions, the present study demonstrated that the reduction in light intensity by partial shade enhances the synthesis of phenolic acids and the flavonoid compounds of different legume-based swards. One study, aimed at evaluating the effects of light on growth and the accumulation of secondary metabolites of the legume medicinal plant *Glycyrrhiza*

uralensis Fisch., reported that a low light intensity significantly increased the concentration of glycyrrhizic acid and the flavonoid liquiritin.[59] An appropriate light control obtained within agroforestry systems might therefore increase the secondary metabolite content of that plant.

Finally, recent studies have reported that secondary metabolites also play a major role in ecosystem processes, such as plant succession or litter decomposition, by governing the interplay between plant matter and soil organisms. The ecological role of phenolic acids, flavonoids and tannins has been reviewed recently by Chomel *et al.*[60]

CONCLUSIONS

Our research provides new insights into the effects of light intensity on plant secondary metabolites from legume-based swards grown under contrasting conditions of partial shade in Mediterranean grazed woodlands and full sunlight conditions.

Both the contribution of light intensity and the legume species affected the concentration and composition of polyphenol compounds, as well as the antioxidant capacity of the legume-based swards under study.

The phenolic acid verbascoside and the flavonoid luteolin-7-*O*-glucoside were the most abundant compounds in full sunlight. Chlorogenic acid and the flavonoid compound isorientin were predominant under partial shade. Because antioxidant capacity and the content of plant secondary metabolites determined in the legume-based swards could potentially affect the nutritional properties of forage, their variations caused by contrasting light intensities thus represent a particular benefit of agroforestry systems, which could be exploited as an additional service at farm levels.

Future multidisplinary investigations are required to clarify the specific role of the most important phenolic compounds identified in animal diets, as well as to test their beneficial effects as supplementary treatments.

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REFERENCES

1 Mosquera-Losada MR, Moreno G, Pardini A, McAdam JH, Papanastasis V, Burgess PJ *et al.*, Past, present and future of agroforestry systems in Europe, in *Agroforestry – The Future of Global Land Use*. Springer, Netherlands, pp. 285–312 (2012).
2 Moreno G, Aviron S, Berg S, Crous-Duran J, Franca A, García del Jalón S *et al.*, Agroforestry systems of high nature and cultural value in Europe: provision of commercial goods and other ecosystem services. *Agrofor Syst* (2017). https://doi.org/10.1007/s10457-017-0126-1.
3 Garbarino M and Bergmeier E, *Plant and Vegetation Diversity in European Wood Pastures. European Wood-pastures in Transition: A Social-ecological Approach*. Earthscan from Routledge, Abingdon, pp. 113–131 (2014).
4 Eichhorn MP, Paris P, Herzog F, Incoll LD, Liagre F, Mantzanas K *et al.*, Silvoarable systems in Europe - past, present and future prospects. *Agrofor Syst* **67**:29–50 (2006).
5 Rochon JJ, Doyle CJ, Greef JM, Hopkins A, Molle G, Sitzia M *et al.*, Grazing legumes in Europe: a review of their status, management, benefits, research needs and future prospects. *Grass Forage Sci* **59**:197–214 (2004).
6 Sulas L, The future role of forage legumes in the Mediterranean climatic areas, in *Grasslands: Developments Opportunities Perspectives*, ed. by Reynolds SG and Frame J. FAO, Rome, and Science Publishers, New Hampshire, pp. 29–54 (2005).
7 Crespo D, The role of legumes on the improvement of grazing resources and the conservation of the 'Montado-Dehesa system', in *Proceedings of the International Workshop Diversity of Fabacae fourragères et de leurs symbiotes*, ed. by Abdelguerfi A, pp. 298–308 (2006).
8 Re GA, Porqueddu C, Sanna F, Sulas L, Franca A, Piluzza G *et al.*, Effect of seed component ratios and cutting regime on the performances of annual ryegrass and burr medic mixtures. *Afr J Agric Res* **11**:2893–2902 (2016).
9 Teixeira RFM, Domingos T, Costa APSV, Oliveira R, Farropas L, Calouro F *et al.*, Soil organic matter dynamics in Portuguese natural and sown rainfed grasslands. *Ecol Model* **222**:993–1001 (2011).
10 Franca A, Sanna F, Nieddu S, Re GA, Pintus GV, Ventura A *et al.*, *Effects of Grazing on the Traits of a Potential Fire in a Sardinian Wooded Pasture, Options Méditerranéennes Série A: séminaires méditerranéens*, Vol. **102**. CIHEAM, Centre international 434 de hautes études agronomiques méditerranéennes, pp. 307–311 (2012).
11 Ruiz-Mirazo J and Robles AB, Impact of targeted sheep grazing on herbage and holm oak saplings in a silvopastoral wildfire prevention system in south-eastern 586 Spain. *Agrofor Syst* **86**:477–491 (2012).
12 Francaviglia R, Benedetti A, Doro L, Madrau S and Ledda L, Influence of land use on soil quality and stratification ratios under agro-silvo-pastoral Mediterranean management systems. *Agric Ecosyst Environ* **183**:86–92 (2014).
13 Kyriazopoulos AP, Abraham EM, Parissi ZM, Koukoura Z and Nastis AS, Forage production and nutritive value of *Dactylis glomerata* and *Trifolium subterraneum* mixtures under different shading treatments. *Grass Forage Sci* **68**:72–82 (2013).
14 Devkota NR and Kemp PD, Morphological aspects of pasture species in the shade in relation to various management practices under silvopastoral systems. *J Inst Agric Animal Sci* **19–20**:1–26 (1999).
15 Anderson GW and Moore RW, Productivity in the first seven years of a *Pinus radiata*-annual pasture agroforest in Western Australia. *Aust J Exp Agric* **27**:231–238 (1987).
16 Kyriazopoulos AP, Koukoura Z and Nastis AS, Effects of wild cherry (*Prunus avium*) and grazing on the herbaceous understorey and its crude protein content. *Grassl Sci Eur* **4**:187–190 (1999).
17 Benítez G, González-Tejero MR and Molero-Mesa J, Knowledge of ethnoveterinary medicine in the Province of Granada, Andalusia, Spain. *J Ethnopharmacol* **139**:429–439 (2012).
18 Di Novella R, Di Novella N, De Martino L, Mancini E and De Feo V, Traditional plant use in the national park of Cilento and Vallo Di Diano, Campania, Southern, Italy. *J Ethnopharmacol* **145**:328–342 (2013).
19 Piluzza G, Virdis S, Serralutzu F and Bullitta S, Uses of plants, animal and mineral substances in Mediterranean ethno-veterinary practices for the care of small ruminants. *J Ethnopharmacol* **168**:87–99 (2015).
20 Polat R, Cakilcioglu U, Kaltalioğlu K, Ulusan MD and Türkmen Z, An ethnobotanical study on medicinal plants in Espiye and its surrounding (Giresun-Turkey). *J Ethnopharmacol* **163**:1–11 (2015).
21 Conforti F, Sosa S, Marrelli M, Menichini F, Statti GA, Uzunov D *et al.*, In vivo anti-inflammatory and in vitro antioxidant activities of Mediterranean dietary plants. *J Ethnopharmacol* **116**:144–151 (2008).
22 Piluzza G and Bullitta S, Correlation between phenolic content and antioxidant properties in twenty-four plant species of traditional ethnoveterinary use in the Mediterranean area. *Pharm Biol* **49**:240–247 (2011).
23 Sulas L, Re GA, Bullitta S and Piluzza G, Chemical and productive properties of two Sardinian milk thistle (*Silybum marianum* (L.) Gaertn.) populations as sources of nutrients and antioxidants. *Gen Res Crop Evol* **63**:315–326 (2016).
24 Piluzza G, Sulas L and Bullitta S, Tannins in forage plants and their role in animal husbandry and environmental sustainability: a review. *Grass Forage Sci* **69**:32–48 (2013).
25 Tamura Y, Environmental changes and genetic variation of accumulation of bioactive compounds in plantain (*Plantago lanceolata* L.). *Bull Natl Agric Res Cent Tohoku Reg* **100**:75–92 (2002).
26 Howard LR, Pandjaitan N, Morelock T and Gil MI, Antioxidant capacity and phenolic content of spinach as affected by genetics and growing season. *J Agric Food Chem* **50**:5891–5896 (2002).

27 Islam MS, Yoshimoto M, Ishigure K, Okuno S and Yamakawa O, Effect of artificial shade and temperature on radical scavenging activity and polyphenolic composition in sweetpotato (*Ipomoea batatas* L.) leaves. *J Am Soc Hortic Sci* **128**:182–187 (2003).

28 Ali MB, Khandake L and Oba S, Changes in pigments, total polyphenol, antioxidant activity and color parameters of red and green edible amaranth leaves under different shade levels. *J Food Agric Environ* **8**:217–222 (2010).

29 Mole S, Ross JA and Waterman PG, Light-induced variation in phenolic levels in foliage of rain-forest plants. *J Chem Ecol* **14**:1–21 (1988).

30 Close DC and McArthur C, Rethinking the role of many plant phenolics–protection from photodamage not herbivores? *Oikos* **99**:166–172 (2002).

31 Arena ME, Postemsky PD and Curvetto NR, Changes in the phenolic compounds and antioxidant capacity of *Berberis microphylla* G. Forst. berries in relation to light intensity and fertilization. *Sci Hortic* **218**:63–71 (2017).

32 Agati G and Tattini M, Multiple functional roles of flavonoids in photoprotection. *New Phytol* **186**:786–793 (2010).

33 Soil Survey Staff, *Keys to Soil Taxonomy*, 12th edn. USDA–Natural Resources Conservation Service, Washington, DC (2014).

34 Piluzza G and Bullitta S, The dynamics of phenolic concentration in some pasture species and implications for animal husbandry. *J Sci Food Agric* **90**:1452–1459 (2010).

35 Surveswaran S, Cai YZ, Corke H and Sun M, Systematic evaluation of natural phenolic antioxidants from 133 Indian medicinal plants. *Food Chem* **102**:938–953 (2007).

36 Karimi E, Oskoueian E, Oskoueian A, Omidvar V, Hendra R and Nazeran H, Insight into the functional and medicinal properties of *Medicago sativa* (Alfalfa) leaves extract. *J Med Plants Res* **7**:290–297 (2013).

37 StatPoint Technologies Inc, *Statgraphics Centurion XVI User Manual*. StatPoint Technologies Inc., Warrenton, VA (2009).

38 Li YG, Tanner G and Larkin P, The DMCA HCl protocol and the threshold proanthocyanidin content for bloat safety in forage legumes. *J Sci Food Agric* **70**:89–101 (1996).

39 Ghasemzadeh A and Ghasemzadeh N, Effects of shading on synthesis and accumulation of polyphenolic compounds in ginger (*Zingiber officinale* Roscoe) varieties. *J Med Plants Res* **5**:2435–2441 (2011).

40 Fraisse D, Carnat A, Viala D, Pradel P, Besle JM, Coulon JB *et al.*, Polyphenolic composition of a permanent pasture: variations related to the period of harvesting. *J Sci Food Agric* **87**:2427–2435 (2007).

41 López-Lázaro M, Distribution and biological activities of the flavonoid luteolin. *Mini Rev Med Chem* **9**:31–59 (2009).

42 Rocha LD, Monteiro MC and Teodoro AJ, Anticancer properties of hydroxycinnamic acids – a review. *Cancer Clin Oncol* **1**(2012).

43 Salinas-Rios T, Ortega-Cerrilla ME, Sánchez-Torres-[illegible]da MT, Hernández-Bautista J, Díaz-Cruz A, Figueroa-Velasco JL *et al.*, Productive performance and oxidative status of sheep fed diets supplemented with coffee pulp. *Small Rum Res* **123**:17–21 (2015).

44 Avila JG, de Liverant JG, Martıćnez A, Martıćnez G, Muñoz JL, Arciniegas A *et al.*, Mode of action of Buddleja cordata verbascoside against *Staphylococcus aureus*. *J Ethnopharmacol* **66**:75–78 (1999).

45 Cardinali A, Pati S, Minervini F, D'Antuono I, Linsalata V and Lattanzio V, Verbascoside, isoverbascoside, and their derivatives recovered from olive mill wastewater as possible food antioxidants. *J Agric Food Chem* **60**:1822–1829 (2012).

46 Vlaisavljević S, Kaurinović B, Popović M and Vasiljević S, Profile of phenolic compounds in *Trifolium pratense* L. extracts at different growth stages and their biological activities. *Int J Food Prop*: (2016).

47 Hanganu D, Benedec D, Vlase L, Olah N, Damian G, Silaghi-Dumitrescu R *et al.*, Polyphenolic profile and antioxidant and antibacterial activities from two *Trifolium species*. *Farmacia* **65**:449–453 (2017).

48 Kassem ME, Marzouk MM, Mostafa AA, Khalil WK and Booles HF, Phenolic constituents of *Trifolium resupinatum* var. *minus*: protection against rosiglitazone induced osteoporosis in type 2 diabetic male rats. *J Appl Pharm Sci* **7**:174–183 (2017).

49 Casamassima D, Palazzo M, Martemucci G, Vizzarri F and Corino C, Effects of verbascoside on plasma oxidative status and blood and milk production parameters during the peripartum period in Lacaune ewes. *Small Rum Res* **105**:1–8 (2012).

50 Casamassima D, Palazzo M, Vizzarri F, Costagliola C, Corino C and Di Costanzo CA, Dietary effects of plant extracts, based on verbascoside, lycopene and horseradish on several blood variables and plasma oxidative status in growing rabbits. *Liv Sci* **206**:148–153 (2017).

51 Vizzarri F, Palazzo M, D'Alessandro AG and Casamassima D, Productive performance and meat quality traits in growing rabbit following the dietary supplementation of *Lippia citriodora*, *Raphanus sativus* and *Solanum lycopersicum* extracts. *Livest Sci* **200**:53–59 (2017).

52 Loi A, Nutt BJ, Howieson JG, Yates RJ and Norman HC, Preliminary assessment of bladder clover (*Trifolium spumosum* L.) as an annual legume for ley farming systems in southern Australia. *Crop Pasture Sci* **63**:582–591 (2012).

53 Al-Samarrai OR, Naji NA and Hameed RR, Effect of Bay leaf (*Laurus nobilis* L.) and its isolated (flavonoids and glycosides) on the lipids profile in the local Iraqi female rabbits. *Tik J Pure Sci* **22**:72–75 (2017).

54 Diaz J, Bernal A, Pomar F and Merino F, Induction of shikimate dehydrogenase and peroxidase in pepper (*Capsicum annum* L.) seedlings in response to copper stress and its relation to lignification. *J Plant Res* **161**:179–188 (2001).

55 Shui YC, Feng X and Yan W, Advances in the study of flavonoids in Ginkgo biloba leaves. *J Med Plants Res* **3**:1248–1252 (2009).

56 Kumari R, Singh S and Agrawal SB, Effects of supplemental ultraviolet-b radiation on growth and physiology of *Acorus calamus* L. (sweet flag). *Act Bio Cra Ser Bot* **51**:19–27 (2009).

57 Logemann E, Tavernaro A, Shulz W, Somssish IE and Hahlbrock K, UV light selectively co induces supply pathways from primary metabolism and flavonoid secondary product formation in parsley. *Proc Nat Acad Sci USA* **97**:1903–1907 (2000).

58 Loyall L, Uchida K, Braun S, Furuya M and Frohnmey H, Glutathione and a UV light-induced glutathione S-transferase are involved in signaling to chalcone synthase in cell cultures. *Plant Cell* **12**:1939–1950 (2000).

59 Hou JL, Li WD, Zheng QY, Wang WQ, Xiao B and Xing D, Effect of low light intensity on growth and accumulation of secondary metabolites in roots of *Glycyrrhiza uralensis* Fisch. *Biochem Syst Ecol* **38**:160–168 (2010).

60 Chomel M, Guittonny-Larchevêque M, Fernandez C, Gallet C, DesRochers A, Paré D *et al.*, Plant secondary metabolites: a key driver of litter decomposition and soil nutrient cycling. *J Ecol* **104**:1527–1541 (2016).