Journal of Materials and Environmental Sciences
ISSN : 2028-2508
CODEN : JMESCN

J. Mater. Environ. Sci., 2018, Volume 9, Issue 6, Page 1662-1667

https://doi.org/10.26872/jmes.2018.9.6.182

http://www.jmaterenvironsci.com

Phenolic composition of Hedysarum flexuosum (Sulla) in Northwestern Morocco

A. Errassi[1], M. Ayadi[2], M. Chabbi[1], A. Noutfia[2], A. Jaber[2]

1. Laboratory of Materials and Valorization of Resources, Faculty of Science and Technology, University Abdelmalek Essaadi, Tangier, Morocco

2. Animal production research unit, National Institute of Agricultural Research, 78 Av. Sidi Mohamed ben Abdellah, Tangier, Morocco

Received 20 Jun 2017,
Revised 05 Dec 2017,
Accepted 13 Dec 2017

Keywords

- ✓ Northwest of Morocco;
- ✓ *Hedysarum flexuosum;*
- ✓ Phenols;
- ✓ Condensed tannins.

Pr. Mohamed Chabbi
mohamedchabbi2000@yahoo.fr
+212666068973

Dr. Ayadi Mohammed
ayadimohamed004@gmail.com
+212657944794

Abstract

Ten ecotypes of Sulla (*Hedysarum flexuosum*) from northwestern Morocco have been installed in the experimental station of INRA. At the vegetative stage, the budding and at the flowering stage, samplings were done to estimate the phenolic composition of this plant. The result shows that *Hedysarum flexuosum* is characterized by moderate levels of extractable phenolic compounds which vary significantly with the stage of maturation and also with the locality of the ecotype. Indeed, from the vegetative stage to the flowering, the total phenol content (TP) decreases from 2.60% to 0.92 %DM. The decrease is also from 1.31% to 0.32% for total tannins (TT) and from 0.79% to 0.17% for hydrolyzable tannins (HT). On the other hand, the condensed tannins (CT) content increased at the budding stage (0.82% vs 0.54 %DM) and thereafter decreased significantly at the flowering (0.14 %DM). Depending upon the ecotypes, the contents of TP and TT are ranging from 2.29% to 1.66% and from 1.09% to 0.63 %DM respectively. The ecotypes are also characterized by lower concentrations of CT with recorded values varying from 0.66% to 0.32%. In general, these concentrations are below the minimum limit (2 %DM of CT) that might affect either the forage nutritional value or the animal's health. However, a complementary study of non-extractable condensed tannins might reveal a fraction that could influence the palatability and the digestibility of proteins and fibers of Sulla.

1. Introduction

The Sulla is a forage legume characterized by a high protein content, but also by its palatability for ruminants. However, this plant is known for its high levels of condensed tannins (5.6 %DM [1] and 3.5 %DM [2]). Depending on their concentration and structure, and depending on the physiology of the animal and the diet composition, condensed tannins may have a beneficial effect (antiparasitic properties; a "by-pass ruminal" phenomenon which allows the escape of fatty acids and amino acids from ruminal degradation and therefore their absorption at the duodenal level; traps free radicals and as a result promotes antioxidant activities), or undesirable effect (decreased intake which is due to their astringent properties; decreased digestibility through the inactivation of digestive enzymes; the formation of sparingly soluble and non-degradable complexes with food macromolecules such as proteins and fibers; the inhibition of the ruminal microbiota) [3-6].

The study aimed to characterize *Hedysarum flexuosum* in terms of secondary metabolite composition (total phenols, total tannins, condensed and hydrolyzable tannins), depending upon the ecotype and the stage of growth.

2. Materials and methods

2.1. Plant material

Seeds of *Hedysarum flexuosum* ecotypes from pasture lands in northwestern Morocco (table 1) were cultivated in the Boukhalef experimental station of the National Institute of Agronomic Research, Tangier, Morocco. At each stage (vegetative stage, budding and flowering), fresh harvest samples were dried at 40 °C and thereafter grinded into 1 mm and stored at 4 °C.

Table 1: The ecological origin of the selected seeds

Ecotype	Locality or site	GPS coordinates	Characterization
1	Chrakka	35° 40' 55'' 5° 53' 948''	Late ecotype (at the end of blooming) on steep slope.
2	Chrakka	35° 40' 58'' 5° 53' 954''	Early ecotype (pod apparition).
3	Boughdour	35° 39' 626'' 5° 32' 857''	Ecotype with raised port, long stems, on flat soil, in full blooming.
4	Larbaa dalia	35° 40' 538'' 5° 48' 648''	Ecotype in full blooming, flat soil, raised port.
5	Axis Tetouan Larache	35° 34' 468'' 5° 39' 354''	Sloping soil, eroded, early ecotype, thinner stems.
6	Axis Tetouan Larache	35° 34' 820" 5° 40' 757"	Port slightly erect, very low slope, thicker stems, medium earliness.
7	Blocade 9 april	35° 31' 181" 5° 44' 538"	Slightly crawling port, thin stems, more pronounced flower color.
8	Highway Asilah	35° 22' 822" 6° 04' 287"	Rampant ecotype on a steep slope.
9	Highway Tahaddart	35° 30' 409" 5° 59' 260"	Less pronounced slope, rampant ecotype, premature ended blooming.
10	Highway Tahaddart	35° 36' 558" 5° 57' 690"	Raised port, premature ended blooming, eroded soil, stems of medium sizes.

2.2. Extraction

The total phenols were extracted by agitation of the mixture (test sample of 200 mg and 10 mL 70% aqueous acetone) in an ultrasonic bath for 20 minutes at room temperature followed by centrifugation at 4 °C for 10 min at 3000 times gravity (×g) [7]. The supernatant is recovered using a pipette Pasteur and a second extraction is done on the remaining precipitate.

2.3. Determination of Total Phenol concentration

Determination of total phenol in plant extract was done following procedure described by Makkar et al. (1993) [8]. The quantification of phenols and total tannins is carried out using spectrophotometer at 725 nm. The total phenol concentration was determined using the Folin-Ciocalteu reagent (1N, from sigma-aldrich) and an aqueous solution of sodium carbonate (Na_2CO_3, 20%). The absorbance is measured after incubation of the solutions in darkness at room temperature for 40 min. The obtained results were reported on a reference curve with tannic acid as standard and the TP content is expressed as tannic acid equivalent (TA) per 100 g of dry matter.

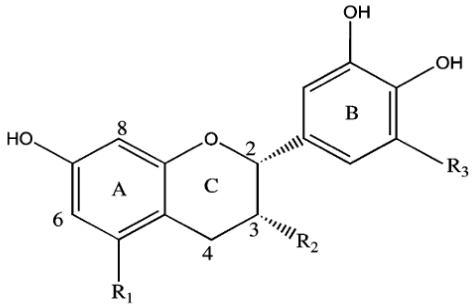
2.4. Estimation of Total Tannins

The plant extract is treated with poly-vinylpolypyrrolidone (PVPP, ~ 110 µm particle size) which has a high affinity with the tannins thus allowing their elimination by centrifugation. The total tannin concentration is measured by the difference between the total phenol content before and after treatment with PVPP [14].

2.5. Determination of free Condensed Tannins (Proanthocyanidines)

This determination was made according to the method described by Makkar (2003) [9]. After an oxidative depolymerization of the condensed tannins (figure 1) of the extract in an alcohol-acid solution (Butanol-HCl, 5/95, v/v) in the presence of a ferric reagent (2% ferric ammonium sulfate in 2N HCl), and after homogenization, the solutions are incubated in a boiling water bath at around 97 °C in darkness for one hour, leading finally to the production of anthocyanins of red color. Then, after cooling at ambient temperature, the absorbances of these solutions are measured at 550 nm. Free condensed tannins as leucocyanidin equivalent per 100 g of dry matter is calculated by the formula: $CT = A_{550\ nm} \times 78.26 \times DF / DM$.

With: $A_{550\ nm}$: absorbance measured at 550 nm, DF: dilution factor, DM: dry matter of the treated sample.



R_1	R_3	Class
OH	H	Proanthocyanidin
OH	OH	Prodelphinidin
H	H	Profisetinidin
H	OH	Prorobinetinidin

Figure 1: The basic repeating unit in condensed tannins. If R_1=R_2=OH, R_3=H, then the structure is that for (-)-epicatechin. The groups at R_1 and R_3 for other compounds are indicated beside the structure. R_2= O-galloyl in the catechin gallates [26].

Hydrolyzable tannins are deduced by the difference between the total tannins and the condensed tannins.

2.6. Statistical analysis

The obtained results were subjected to variance analysis using the General Linear Model (GLM) of SAS (version 9.0) [10], with ecotype, harvest and their interaction as principal factors. The linear model used was:

$$y_{sej} = \mu + T_s + T_e + T_s x T_e + \varepsilon_{sej}$$

Where, μ is the overall average, T_s is the stage of harvest, T_e is the ecotype, $T_s x T_e$ is their interaction and ε_{sej} is the random error. Mean differences were considered significant at $P<0.05$, they were determined using the Tukey's multiple range test.

3. Results

The obtained results show that the phenolic compound composition of *Hedysarum flexuosum* varies very significantly ($P < 0.001$) depending on the stage of harvest and the ecological seed's origin. The total phenol content varies from 1.66% to 2.29 %DM (table 2) with an average of 1.90 %DM. Depending on the stage of development, the TP content decreases considerably from the vegetative stage (2.60 %DM) to the flowering with value of 0.92 %DM (figure 2). The average total tannin content is 0.90% (9g equivalent tannic acid / Kg DM) with a variation ranging between 0.63% and 1.09 %DM. The TT content of *H. flexuosum* evolves in the same way as TP with the stage of growth (figure 2).

Table 2: Phenolic composition (%DM) of *Hedysarum flexuosum* ecotypes from the northwestern Morocco (± standard error)

Ecotype	TP	NTP	TT	CT	HT
E1	2.17 ± 0.28^{ab}	1.09 ± 0.13^{b}	1.09 ± 0.15^{a}	0.62 ± 0.17^{ab}	0.47 ± 0.14^{b}
E2	2.07 ± 0.36^{bc}	1.00 ± 0.13^{c}	1.07 ± 0.24^{a}	0.44 ± 0.10^{d}	0.63 ± 0.20^{a}
E3	1.75 ± 0.26^{de}	1.03 ± 0.11^{bc}	0.72 ± 0.16^{b}	0.32 ± 0.07^{e}	0.40 ± 0.14^{bcd}
E4	1.72 ± 0.187^{de}	1.09 ± 0.10^{b}	0.63 ± 0.10^{b}	0.52 ± 0.09^{c}	0.11 ± 0.02^{f}
E5	1.75 ± 0.21^{de}	0.78 ± 0.09^{e}	0.97 ± 0.23^{a}	0.33 ± 0.06^{e}	0.65 ± 0.24^{a}
E6	2.29 ± 0.28^{a}	1.24 ± 0.12^{a}	1.05 ± 0.20^{a}	0.61 ± 0.11^{ab}	0.44 ± 0.10^{bc}
E7	1.76 ± 0.27^{de}	1.00 ± 0.13^{c}	0.76 ± 0.14^{b}	0.44 ± 0.10^{d}	0.32 ± 0.08^{cde}
E8	1.66 ± 0.19^{e}	0.90 ± 0.09^{d}	0.76 ± 0.10^{b}	0.51 ± 0.11^{c}	0.25 ± 0.04^{e}
E9	1.84 ± 0.27^{d}	0.88 ± 0.10^{d}	0.97 ± 0.17^{a}	0.66 ± 0.11^{a}	0.31 ± 0.10^{de}
E10	2.01 ± 0.24^{c}	1.06 ± 0.11^{bc}	0.95 ± 0.13^{a}	0.57 ± 0.06^{bc}	0.39 ± 0.09^{bcd}
Average	1.90	1.01	0.90	0.50	0.40
SEM	0.07	0.04	0.05	0.04	0.05
Signification	***	***	***	***	***

TP: Total phenols expressed as g equivalent tannic acid (TA)/100g of dry matter (DM), **NTP:** Non-tannic phenols expressed as eq TA/100g of DM, **TT**: Total tannins as g eq TA/100g of DM, **CT**: Condensed tannins expressed as g equivalent of leucocyanidin /100g of DM, HT: hydrolysable tannins expressed as g eq TA /100g of DM.

a, b, c, ⋯ : Within the same column, the values followed by the distinct letters are statistically different to 5% , **SEM**: Standard error of the mean. *** $P<0.001$, ** $P < 0.01$, * $P < 0.05$, NS (Not significant) $P>0.05$.

Concerning the condensed tannins, their evolution within the stage of harvest is quite different compared to the TP and TT. Indeed, the highest CT levels are obtained at the budding stage with an average content of 0.82

%DM followed by the vegetative stage with 0.54 %DM compared with only 0.14 %DM at flowering (figure 2). Moreover, the interaction between the factors (Stage x Ecotype) generates significant variations in terms of CT. Indeed, the maximum levels are obtained in the interaction between the budding stage and the ecotypes E1, E9 and E6 (with values of 1.29%, 1.05% and 0.90 %DM respectively, figure 3).

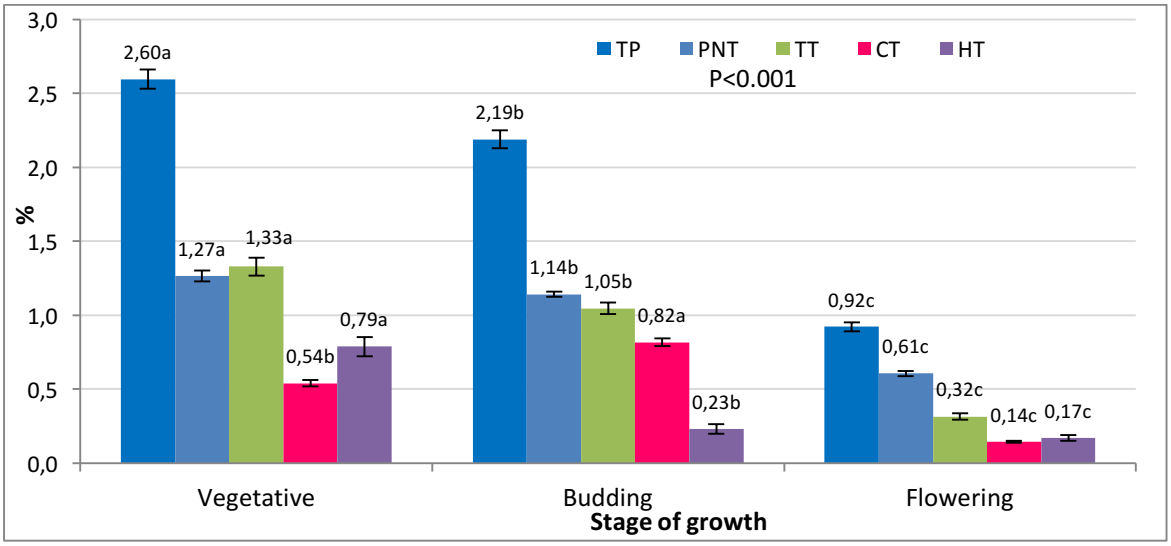


Figure 2: Evolution of the phenolic composition of *Hedysarum flexuosum* with the stage of growth

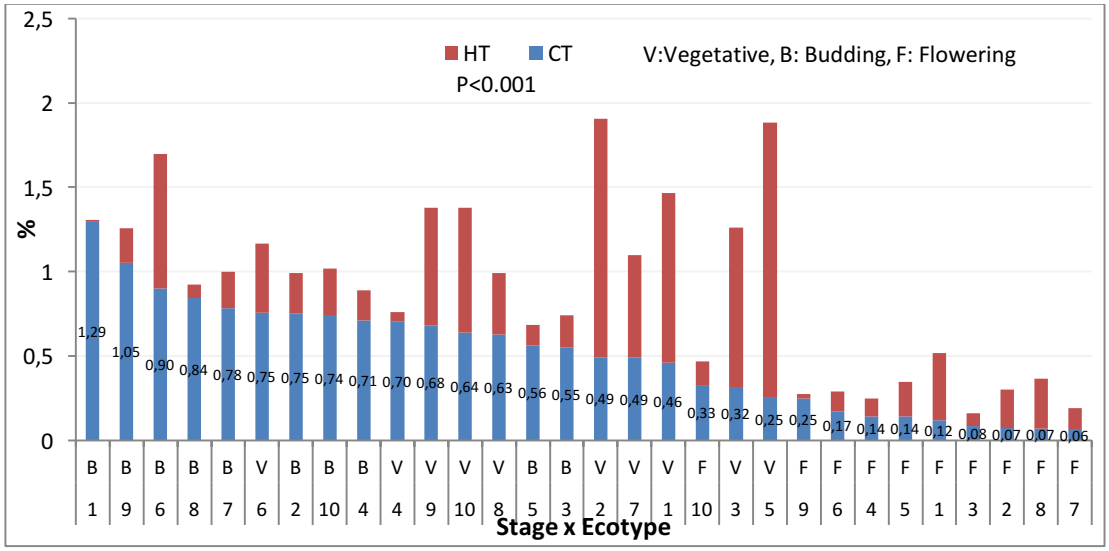


Figure 3: Variation of the tannin content (CT and HT) with the interaction between the ecotypes (1, 2 ... 10) and the stage of growth (V, B and F).

Depending upon the ecotypes of H. *flexuosum*, the average CT content was 0.50% (5 g leucocyanidin equivalent / Kg DM), with a variation between 0.32% and 0.66 %DM (table 2).
Concerning the levels of hydrolyzable tannins, the mean value is 0.40 %DM with a variation from 0.11% to 0.65 %DM (table 1). Like TP and TT, HT decreases progressively with the maturation of the plant (0.79% against 0.23% and 0.17 %DM recorded for the vegetative stage, budding and flowering respectively, figure 2).
Indeed, the highest concentrations of HT are obtained when the harvest is done at the vegetative stage of the plant, the ecotypes E5, E2 and E1 recorded the maximum HT levels (1.63%, 1.42% and 1.01 %DM respectively figure 3).

4. Discussion

This study revealed a diversity within the ecotypes of *Hedysarum flexuosum* in terms of phenolic compounds concentration, which also varies significantly with the stage of growth.

4.1. Total Phenols

In comparison with *Hedysarum coronarium*, Aissa et al. (2015) [11] reported a total phenol concentration of 1.30 %DM, which is much lower than the recorded results at the vegetative and the budding stage (2.60% and

2.19 %DM respectively). A comparable level of TP for the same specie (*H. Coronarium*) to that at the budding stage for *H. flexuosum* was reported by Molle et al. (2004) [12] (2.25 %DM for the April harvest). However, these authors recorded an increase in total phenols concentration in the second period (May) with a concentration of 3.04 %DM, which is inconsistent with the evolution of this parameter for the studied ecotypes. Indeed, the TP concentration is higher at the vegetative stage for all the ecotypes.

Cabiddu et al. (2009) [13] also reported relatively higher concentrations (3.21%, 3.55% and 3.42 %DM recorded for *H. coronarium* at the beginning of May, mid-May and early June respectively). However, Molle et al. (2004) [12] recorded a decrease in TP with the harvest date of *Lolium Rigidum* (1.28% and 0.53 %DM for samples harvested in April and May respectively). In comparison with the studied ecotypes, these levels are much lower, especially for the first two stages of harvest. These differences might be explained by environmental conditions especially, under favorable conditions when the synthesis of the phenolic compounds is less important, knowing that water stress and luminous intensity (sunlight) are positively correlated with the polyphenol content of the plant [25].

4.2. The Condensed Tannins

Concerning the condensed tannins, the obtained results are much lower than those reported by several authors. In fact, Sulla is known as a tannin legume, with values ranging generally from 2 to 4 %DM [15]. These tannins are considered to be of great interest for milk quality and animal performance, according to Tava et al. (2005) [16], amongst the given reasons for this beneficial effect arises the structure of Sulla's condensed tannins, composed essentially of cyanidin and delphinidin monomers.

The average condensed tannin content of the selected ecotypes of *H. flexuosum* is 0.50 %DM marqued by an increase from the vegetative stage to the budding (from 0.54% to 0.82 %DM), followed by a significant decrease at the flowering (0.14 %DM). In fact, the decrease in condensed tannins observed between the last two stages might be explained by a decrease in the leaf-stem ratio in the whole plant biomass at maturation. In fact, condensed tannins concentrations in leaves are higher than those in stems, which consequently explain the decrease in CT concentration at the advanced stages of maturation. Actually, Borreani et al. (2003) [17] noted a decrease from 2.70% to 1.60 %DM for *H. coronarium* during its complete cycle of maturation.

Cabiddu et al. (2009) [13] reported much higher levels of condensed tannins (2.50% to 2.74 %DM) for *H. coronarium*. In addition, the latter authors noted a slight increase in CT with the maturation of the plant. Di Trana et al. (2015) [18] also reported higher levels for *H. coronarium* (1.99 %DM). On the other hand, Aissa et al. (2015) [11] recorded a low content (0.37 %DM) which is comparable to the average levels recorded for the two *H. flexuosum* ecotypes (0.32% and 0.33 %DM respectively for E3 and E5).

Compared with alfalfa *(Medicago sativa)*, Niezen et al. (2002) [19] noted that this forage legume is extremely poor in condensed tannins compared to the Sulla (0.06% vs 3.13 %DM).

While the maximal value of condensed tannins content (1.29 %DM) recorded for the ecotype E1 at the budding stage is close to that reported by Tzamaloukas et al. (2005) [20] for *H. coronarium* (1.58 %DM), these authors underlined that the sheep feeding by Sulla for 14 days did not affect the parasitic load of *T. circumcincta,* and specified, in particular, that one of the main reasons for this result is owing to the low concentration of condensed tannins in *Hedysarum*, knowing that a concentration of condensed tannins below 2% has no effect on voluntary forage intake [3]. For that reason, we could assume that the incorporation of *H. flexuosum* ecotypes into the goat diet would have no adverse effect on the animal's health.

In addition, according to Pomroy et al. (2006) [21], Sulla contains 2.6% free condensed tannins, 1.8% protein-bound condensed tannins and 0.1% fiber-bound condensed tannins. In this case, the study was carried out only on free condensed tannins. Indeed, a more detailed study on extractable and non-extractable tannins would be of great interest to tackle the in vitro digestibility of organic matter and proteins.

Finally, Sulla's condensed tannins concentration varies greatly within the Mediterranean conditions, especially in terms of varietal diversity, climatic conditions and soil quality [22]. Mansion et al. (1997) [23] recorded various profiles of evolution of condensed tannins for *Lotus uliginosus* depending on soil quality. Moreover, the plant's biotic stress generated by the herbivores or pathogens attack induces the synthesis and storage of condensed tannins in the plant (by defense mechanism). Even the proportions of CT in it's free form or bounded to fibers and proteins are also influenced by the pedoclimatic environment and nutrient stress of the plant [24].

Conclusion

This study underlines that the *Hedysarum flexuosum* ecotypes of northwestern Morocco have relatively low levels of total phenols and condensed tannins. The concentration of these compounds depends closely on the harvest stage and on the ecological origin of the seeds.

We were able to observe the same evolution of the studied parameters for all the ecotypes. Indeed. The contents of total phenols, total tannins and hydrolyzable tannins are more important at the vegetative stage, and the concentration of these compounds decreases with plant maturation. On the other hand, the content of condensed tannins is maximum at the budding stage. However, it is noted that the obtained levels remain below the threshold influencing the digestibility. Several factors may be responsible for this variation, namely the reduction in the leaf-stem ratio between the budding stage and the flowering, the soil quality, the climatic conditions and even the drying conditions of the samples. The maximum levels of condensed tannins are obtained at the budding stage (between 0.90% and 1.29 %DM).
According to several authors, the phenols, especially the condensed tannins, have certainly a clear effect on the production and the quality of milk and meat. However, the determination of condensed tannin levels for *H. flexuosum* that might influence the production performances and the quality of the ruminant products is of great interest regarding the fields of animal nutrition, zootechnics and agri-food.

References

1. J.L. Burke, G.C. Waghorn, I.M. Brookes, *Proceedings of the New Zealand Society of Animal Production.* 62 (2002) 152-156.
2. G.C.Waghorn, M.H. Tavendale, D.R. Woodfield, *Proc. N.Z. Grassland Assoc.* 64 (2002) 167-171.
3. R. Aerts, T. Barry, W.C. McNabb, *Agric. Ecosyst. Env.* 75(1-2) (1999) 1-12.
4. B.R. Min, J.M. Fernandez, T.N. Barry, W.C. McNabb, P.D. Kemp, *Anim. Feed Sci. Technol.* 92 (2001) 185-202.
5. O. Tibe, L.P. Meagher, K. Fraser, D.R.K. Harding, *J. Agric. Food Chem.* 59 (2011) 9402-9409.
6. L. R. McMahon, T. A. McAllister, B. P. Berg, W. Majak, S. N. Acharya, J. D. Popp, B. E. Coulman, Y. Wang, K.J. Cheng, *Can. J. Plant Sci.* 80 (3) (2000) 469-485.
7. D.S. Seigler, S. Seilheimer, J. Keesy, H.F. Huang, *J. Sci. Food Agric.* 29 (1986) 778-794.
8. H.P.S. Makkar, M. Bluemmel, N.K. Borowi, K. Becker, *J. Sci. Food Agric.* 61 (1993) 161-165.
9. H.P. Makkar, *A laboratory Manuel FAO/IAEA, Vienna, Austria*. (2003) 49- 53.
10. SAS Institute, SAS Version 9.0. *SAS Inst. Inc. Cary, NC, USA*. (2002).
11. A. Aissa, F. Manolaraki, H. Ben Salem, K. Kraiem, H. Hoste, *Int. J. Agron. Agri. Res.* 7 (4) (2015) 103-110.
12. G. Molle, M. Sitzia, M. Decandia, N. Fois, A. Cabiddu, G. Scanu, S. Ligios, In : H. Ben Salem (ed.), A. Nefzaoui (ed.), P. Morand-Fehr (ed.). *CIHEAM.* 59 (2004) 35-40.
13. A. Cabiddu, G. Molle, M. Decandia, S. Spada, M. Fiori, G. Piredda, M. Addis, *Livest Sci.*123(2009) 230-240.
14. H. Makkar, M. Blümmel, K. Becker, *Br. J. Nutr.* 73(1995) 897-913.
15. T.N. Barry, In: J.V. Nolan, R.A. Leng, D.I. Demeyer (Eds.), *University of New England Publishing Unit, Armidale, Australia*. (1989) 153-167.
16. A. Tava, M.G. De Benedetto, D. Tedesco, G. Di Miceli, G. Piluzza, *Proc. 20th Int. Grassland Congr, Dublin, Ireland. (*2005) 271.
17. G. Borreani, P.G. Peiretti, E. Tabacco, *Agronomie.* 23 (2003) 193-201.
18. A. Di Trana, A. Bonino, S. Cecchini, D. Giorgio, A. Di Grigoli, S. Claps, *J. Dairy Sci.* 98 (2015) 1-10.
19. J.H. Niezen, W.A.G. Charleston, H.A. Robertson, D. Shelton, G.C. Waghorn, R. Green, *Vet. Parasitol.* 105 (2002) 229-245.
20. O. Tzamaloukas, S. Athanasiadou, I. Kyriazakis, F. Jackson, R.L. Coop, *Int. J. Parasitol.* 35 (2005) 329-335.
21. W.E. Pomroy, B.A. Adlington, *Vet. Parasitol.* 136 (2006) 363-366.
22. G. Pilluzza, S. Bullita, M. Deroma, M. Odoardi, *Cah. Opt. Mediterr.* 45 (2000) 199-202.
23. G. Mansion, S. Blaise, J.P. Briane, A. Lacoste, *Acta bot. Gallica.* 144 (4) (1997) 443-448.
24. P. Frutos, G. Hervas, G. Ramos, F.J. Giraldez, A.R. Mantecon, *Anim. Feed Sci. Technol.* 95 (2002) 215-226.
25. G.L. Lees, C.F. Hinks, N.H. Suttill, *J. Sci. Food Agric.* 65 (1994) 415-421.
26. P. Schofield, D.N. Mbugua, A.N. Pell, *Anim. Feed Sci. Technol.* 91 (2001) 21-40.