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Determination of Chemical Composition, Degradability and Digestibility of Treated Walnut Hull by *Neurospora sitophila*

Research Article

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ABSTRACT

In this study, the nutritive values of treated Walnut hull by *Neurospora sitophila* were evaluated. The chemical composition of samples was evaluated by laboratory analysis. *In vitro* digestibility experiment was done to determine digestibility coefficients of dry matter, organic matter and also digestible organic matter in dry matter (DOMD) to estimate the metabolizable energy (ME) content of treated Walnut hull. In addition, organic matter (OM) and neutral detergent fiber (NDF) disappearance of samples were determined by *In-Situ* method at 0, 3, 6, 12, 24, 48 and 72 h after incubation and their kinetics were described using the equation $P= a + b(1-e^{-ct})$. The nutritive value index (NIV) of samples was calculated using the equation: NIV= a + 0.4b + 200c. The collected data was analyzed in a completely randomized design. The content of tannin and phenol in walnut hull decreased significantly by processing ($P<0.05$). No significant effect was seen on degradability parameters and nutritive value index. Digestibility coefficient of dry matter, organic matter, digestible organic matter in dry matter and metabolizable energy of treated walnut hull were significantly ($P<0.05$) lower than untreated Walnut hull.

KEY WORDS chemical composition, degradability, digestibility, *Neurospora sitophila*, Walnut hull.

INTRODUCTION

In the recent decade's population growth, economic and social development caused higher demand for livestock products in many developing countries. A large portion of agricultural by-products are being produced annually which has no direct human consumption but it can be used indirectly as human food. Effective usage of agricultural by-products as animal feed depends on some factors such as nutrients composition compare to animal needs (McDonald *et al*. 1995) and cost-effective processing of byproducts (Ammerman and Henry, 1991). Walnut hull is a lignocellulolytic product with low level of protein and high tannin content which is being produced after Walnut excoriation in some regions of Iran, so treating it with physical, chemical and biological methods increase its nutritive values for feedstuff.

One of the biological methods for treating agricultural byproducts is using fungi that with different microorganism's cultivation on agriculture by products can improve protein quality (Madadi Nouei *et al*. 1997) therefore in this study *Neurospora sitophila* was used. This fungus is of Ascomyceta species and has a good ability to grow on soluble and unsoluble carbohydrates. The aim of this study was to evaluate chemical composition and *in vitro* digestibility of treated walnut hull.

MATERIALS AND METHODS

Three ruminal cannulated rams weighing 50 ± 1.5 kg and consuming 1.2 ± 0.2 kg DM were used. Rams fed a total mixed ration consisting of 10% wheat barn, 60% alfalfa hay and 30% whole barley grain. The ration was fed twice daily one half at 08:00 and the other half at 16:00 h.

Inoculant preparation

Under completely sterilized condition, from original medium of fungi to each medium of PDA a loop of mycelium of fungi was inoculated and were kept in 30 ºC for 48 h and then refrigerated in 4 ˚C. A loop of mycelium of fungi was inoculated under completely sterilized condition to each medium of PDA and were kept in 30 ˚C for 48 h and then refrigerated in 4 ˚C.

The composition of preserving medium and inoculant in 1 liter were as below (Griffiths and Done, 1991): glucose, 10 g; yeast extract (medium) 2 g; potassium hydrogen phosphate (KH_2PO_4) 0.714 g; Urea 0.86 g; ammonium sulphate ($(NH_4)_2SO_4$); magnesium sulphate ($MgSO_4.7H_2O$) 0.2 g; calcium chloride 0.2 g; zinc sulphate ($ZnSO_4.7H_2O$) 4.4 g; boric acid (H_3BO_3) 0.144 mg; ammonium molybdate ($(NH_4)_6\ Mo_7O_{24}.4H_2O$) 0.48 mg; copper sulphate ($ZnSO_4.7H_2O$) 4.4 mg; magnesium chloride ($MnCl_2.4H_2O$) 0.144 mg; ferric chloride ($FeCl_3$) 3.2 mg. The preservative culture was made by taking 100 ml of medium, with above mentioned conditions were prepared, and transferred to a 250 mL Erlenmeyer flask. For maximum growth of fungi the pH was adjusted to be 5.5 and sterilized under 121 ˚C and 15 psi pressure for 15 min.

Then few pieces of purified mycelium were transferred into flask containing preservative culture and were shaked for 24 h in 35 ˚C. Finally inoculated culture kept refrigerated in 4 ˚C until use.

Treating walnut hull

Walnut hull was dried in the sun and sieved through sieves with 10 and 18 meshes. The amount of DM and pH of Walnut hull were determined 93% and 3.4 respectively. To bring the pH to 5.5 (suitable pH for production of proteins within a single cell) 0.6 mL per 10 g Walnut hull sieved through a sieve with 10 mesh and 0.7 mL per 10 g of sample sieved through a sieve with 18 mesh was added to the sample. One milliliter of inoculated liquid was added per 10 g of dried Walnut hull.

Twenty gram of Walnut hull sieved by 18 mesh sieve was added to each of the two 250 mL Erlenmeyer flask and the other two 250 mL flasks, each 20 grams of Walnut hull sieved through a sieve with 10 mesh and then 53.2 mL of mixture of ammonia and water was added to each of the flasks.

In order to investigate the effect of sample volume on increasing the protein percentage in each flask, 40 g of sample sieved with 40 mesh sieve was added to a separate Erlenmeyer flask and 10 g of sample sieved with 10 and 18 were added to two other flasks. Appropriate amount of mixture of water and ammonia was added to achieve 75% moisture content and 5.5 pH. After sterilization of the flasks and their contents, 1 mL of fungi culture medium per 10 g of Walnut hull was inoculated under hood in sterile conditions. Then, the flasks were transferred to the incubator for 120 h at 35 ˚C. After incubation samples within the flasks were transferred to Petri dishes. Those samples were dried at a temperature of 45-50 ˚C to prevent decreasing the quality of protein due to high temperature (Shojaosadati *et al.* 1999). After complete drying, samples were grounded and mixed and their protein content was determined.

Chemical composition

Dry matter, organic matter, crude protein and ether extract, acid detergent fiber, neutral detergent fiber, total phenol and tannin were determined by AOAC (1990), Van soest (1994) and Makkar (2003) method respectively.

Digestibility

In vitro dry matter digestibility was determined according to Tilley and Terry (1963) two stages method. In the first, 250 mL of ruminal liquor were obtained from a rumen cannulated ram. The rumen liquor was strained through two layers of muslin into tubes, and CO_2 was passed into the flask to displace air from above the rumen liquor. A 0.5 g of dried sample is incubated in buffered solution. The flask was then kept at 38 ˚C in a water bath for 48 h. The second stage involves digestion with pepsin-HCl for 48 hours at 38 ˚C. The OMD is calculated as the difference between the organic matter in the original sample and in the residue.

ME (MJ/kg DM) content, digestibility of dry matter (DMD), organic matter (OMD) and organic matter in dry matter (DOMD) by in vitro method, were calculated using equations of Tilley and Terry (1963) and AFRC (1993) as follows:

$$DMD = [a - [(b-c) - (d-c)] / f] \times 100$$
$$OMD = [(a-e1) - [(b-c-e2) - (d-e3)] / f - e1] \times 100$$
$$DOMD = [(a-e1) - [(b-c-e2) - (d-c-e3)] / f] \times 100$$
$$ME\ (MJ/Kg\ DM) = 0.016\ DOMD$$

Where:
a= initial sample weight.
b= weight of filter paper and the digested residue.
c= dry weight of filter paper.
f= dry weight of initial sample.
d= net weight of the sample and filter paper.

e_1= weight of sample´s ash.
e_2= residual ash weight.
e_3= net sample ash weight.

***In situ* ruminal degradability of OM and NDF**

Three ruminally fistulated Kermani male sheep weighting 50 ± 1.5 kg and consuming 1.2 ± 0.2 kg DM were used. The sheep were fed a total mixed ration containing alfalfa hay (60%) and concentrate (40%) twice daily at 08:00 and 16:00 h. The *in situ* technique (Orskov and McDonald, 1979) was used to measure the kinetics of OM and NDF degradation of untreated and treated walnut hull samples. Dried samples (2 g) were weighted into 5 cm × 13 cm nylon bags (50 µ pore size), and 9 bags were prepared for each sample and each incubation time. Ruminal incubation times were 0, 3, 6, 12, 24, 48 and 72 h. The bags were removed after incubation in the rumen of sheep and washed in cold running water until the washing ran became clear and colorless. Zero time disappearance was obtained by washing unincubated bags in a similar way. All washed bags were dried in a forced-air oven at 60 ˚C for 48 h. The OM and NDF disappearance were calculated using the equation:

$$P= a + b(1-e^{-ct})$$

Where:
P: disappearance rate at time t.
a: rapidly degradable OM or NDF fraction.
b: slowly degradable OM or NDF fraction in the rumen.
c: rate constant of degradation of b.
t: time of incubation.

The effective degradability values of OM and NDF were calculated using the equation:

$$P= a + [(b\times c) / (c+r)]$$

Where:
P: effective degradability of nutrients.
a: water-soluble fraction.
b: potentially degradable fraction.
c: degradation rate of parameter.
r: passage rate of the digest out of the rumen at 0.02 h−1, which is an average value for animals fed at approximately maintenance level (AFRC, 1993).

The nutritive value index (NIV) of each nutrient for samples was calculated using the equation of Orskov and McDonald (1979) as:

$$NIV= a + 0.4b + 200c$$

Where:
a: water-soluble fraction.
b: potentially degradable fraction.
c: degradation rate of parameter.

Statistical analysis

Data from chemical analysis, degradability and digestibility parameters were subjected to analysis of variance as a completely randomized design and treatment means were compared by the Tukey test. All statistical analysis was performed using the SAS (2005) procedure.

RESULTS AND DISCUSSION

Chemical composition

Chemical compositions of untreated and treated walnut hull by *Neurospora sitophila* are given in Table 1.

Table 1 Chemical compositions of untreated and treated walnut by *Neurospora sitophila*

Chemical composition	Walnut hull		SEM	P-value
	Untreated	Treated		
DM (%)	92.95	98.04	1.596	NS
OM (%)	94.55	94.21	0.480	NS
CP (%)	5.78	6.19	0.413	NS
NDF (%)	37.44	37.95	0.260	NS
ADF (%)	29.22	28.73	0.314	NS
EE (%)	7.1	5.57	1.218	NS
Total tannin (%)	3.438[a]	0.358[b]	0.159	0.005
Total phenol (%)	5.976[a]	1.114[b]	0.177	0.002

DM: dry matter; OM: organic matter; CP: crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber and EE: ether extracts.
SEM: standard error of the means and NS: non significant.

There were no significant differences between chemical compositions of untreated and treated walnut hull except total tannin and total phenol. The total tannin and phenol contents decreased significantly (P<0.05) by treating by *Neurospora sitophila*. The processing pistachio peel by *Neurospora sitophila* caused a significant reduction in total amount of phenolic and tannin compounds (Vahabzadeh, 2011). Moreover, Ghiasi (2010) reported a reduction in the total average of phenolic compounds and extractable tannin of treated grape pomace, which might be related to the use of phenolic compounds and tannins or breaking down of tannin-protein complex or polysaccharides by fungi (Shojaosadati, 1999). For biological degradation of tannins, white fungi such as mushrooms was used and with culturing *Sporotrichum pulverulentum* decreased the total and condensed tannin (Makkar *et al*. 1993).

Dashti *et al*. (2009) reported that treating beet pulp by *Neurospora sitophila* has led to decrease the DM and NDF content significantly in comparison to untreated beet pulp, while ADF and CP amounts were increased by treating.

Since the structural and non-structural hydrocarbon compounds were used as an energy source by fungi, NDF and ADF content of product reduced (Shojaosadati *et al*. 1999). These findings are confirmed with the results of Nazem *et al*. (2008). Increase in fungal biomass during fermentation process causes the increase of crude protein, because fungi use easily fermentable and lignocellulosic materials by its extracellular enzymes and produce energy, protein and carbon dioxide (Shojaosadati *et al*. 1999). Also, the fungal biomass of *Neurospora sitophila* has about 45 percent crude protein.

Ghiasi (2010) observed that DM and OM content of grape pomace did not change by processing whereas CP and Ash increased significantly. During processing of grape pomace, fungi used structural carbohydrates as a source of carbon, and nitrogen sources were used for protein synthesis, which means that grape pomace through bio-conversion method is being enriched (Ghiasi, 2010).

Processing date tops fronds by *Neurospora sitophila* decreased DM (Dayani *et al*. 2013). Fungus released extracellular enzymes during growth on the substrate surface, as a result hydrocarbon bonds break, fungal cell mass grew and carbon dioxide gas released, so this phenomenon reduced DM content (Shojaossadati, 1999). The phenomenon of weight loss during the fermentation process has been confirmed by various researchers, but it depends on the type of fungus and the substrate (type of chemical bonds; Dashti *et al*. 2009).

Totally treating had no significant effect on chemical composition, it might be due to non proper growth of fungus on walnut hull. The methanol extract of walnut hull had inhibitory effects on four species of fungi like *Microsporum canis*, *Trichophyton mentagrophytes*, *Epidermophyton floccosum* and *Candida albicans* and stopped their growth up to 60% (Salamat *et al*. 2006). Shadzi *et al*. (1991) studied the effect of hydrocarbon extract of walnut hull and leaves on some saprophytic fungi, yeasts and dermatophytes. Result showed that the extract had antifungal effect on dermaphytes and fungus but its effect was not significant on saprophytic fungi.

In situ degradability

The results for degradation parameters, effective degradability and Nutritive value index (NIV) of OM and NDF of samples are given in Table 2 and 3. Walnut hull OM and NDF degradability coefficients were not affected by processing. OM and NDF degradation during 48 h of incubation in untreated walnut hull was higher than treated walnut hull ($P<0.05$). OM and NDF degradation of treated walnut hull at 0, 3, 6, 12, 24 and 72 h of incubation in the rumen were not different from untreated walnut hull. Percentage of insoluble but degradable in the rumen (b) of OM in walnut hull was increased after processing, but the changes were not significant. Rate of degradation (c) of OM of treated walnut hull decreased significantly ($P<0.05$) whereas, parameter c of NDF of untreated walnut hull did not change by processing.

Table 2 Organic matter degradability of untreated and treated walnut hull by *Neurospora sitophila*

	Walnut hull		P-value	SEM
	Untreated	Treated		
Degradability				
0	22.52	22.159	1.640	NS
3	42.478	41.778	0.888	NS
6	45.194	44.552	0.534	NS
12	54.517	48.698	3.583	NS
24	65.887	57.714	2.940	NS
48	77.656	66.247	0.898	0.001
72	76.795	73.969	1.154	NS
Degradability parameters				
A	22.520	22.160	1.640	NS
B	57.500	65.786	4.108	NS
a + b	80.020	87.946	3.802	NS
C	0.050	0.020	0.007	0.041
Effective degradability				
% K= 2	67.566	65.966	0.866	NS
% K= 4	61.233	63.866	2.568	NS
% K= 6	57.600	60.866	5.605	NS
% K= 8	55.466	56.866	10.406	NS
NIV	55.646	52.601	1.741	NS

SEM: standard error of the means and NS: non significant.
NIV: nutritive value index.

Table 3 Neutral detergent fiber degradability of untreated and treated walnut hull by *Neuorospora sitophila*

	Walnut hull		P-value	SEM
	Untreated	Treated		
Degradability				
0	0.000	0.692	0.336	NS
3	3.475	6.454	1.549	NS
6	8.107	7.604	2.114	NS
12	18.373	7.990	3.788	NS
24	29.605	16.082	6.779	NS
48	46.594	25.389	1.425	0.0005
72	44.050	41.577	0.888	NS
Degradability parameters				
A	0.000	0.693	0.334	NS
B	50.233	58.100	8.897	NS
a + b	50.233	58.790	8.658	NS
C	0.047	0.025	0.010	NS
Effective degradability				
% K= 2	32.866	30.633	2.411	NS
% K= 4	24.266	22.533	2.661	NS
% K= 6	19.033	18.333	2.665	NS
% K= 8	15.500	16.233	2.608	NS
NIV	29.660	29.065	2.522	NS

SEM: standard error of the means and NS: non significant.
NIV: nutritive value index.

Passage rate from the rumen (k) is affected by the amount of feed, and by increasing the level of feed intake, this amount will increase. With an increase in the value of k

from 2 to 8% of the time, the percentage of effective degradability of OM and NDF decreased that was consistent with Ghiasi (2010), Rashidian (2011) and Dashti *et al.* (2009) results.

Nutritive value index of OM and NDF did not change by processing. This finding was confirmed by Rashidian (2011). Ghiasi (2010) reported that processing of grape pomace increased water soluble fraction (a) but fraction with slow degradation rate (b) and degradation rate of fraction b were reduced.

The reason of increasing soluble fraction (a) might be because of high quantity of crude fiber and soluble compounds which are being used by fungal enzyme systems during processing and converted into soluble material. By increasing water-soluble materials, more energy can be available for growth of rumen microorganisms and feed materials degradation will increase (Orskov, 1992; McDonald *et al.* 1995).

Rashidian (2011) observed that processing date tops fronds by *Neurospora sitophila* decreased NDF degradability at 48 and 72 h after incubation.

In vitro digestibility

In vitro digestibility of untreated and treated walnut hull by *Neurospora sitophila* are shown in Table 4.

Table 4 *In vitro* digestibility of untreated and treated walnut hull by *Neurospora sitophila*

Digestibility parameter	Walnut hull		SEM	P-value
	Untreated	Treated		
Dry matter (%)	63.88	56.68	0.507	< 0.0001
Organic matter (%)	59.43	46.90	0.581	< 0.0001
Organic matter in dry matter (%)	51.96	37.03	0.505	< 0.0001
Metabolisable energy (MJ/Kg DM)	8.15	5.81	0.079	< 0.0001

SEM: standard error of the means.

DM, OM and DOMD digestibility of untreated walnut hull was higher than treated walnut hull significantly (P<0.05). This finding is supported by the data of Ghiasi (2010), this reduction might be related to an increase in lignin content. Processing has reduced the percentage of cell wall but lignin was increased. However, there is a negative correlation between the amount of lignin and feed digestibility. In other words, by increasing the amount of lignin in a food, its digestibility will decrease (Nazem *et al.* 2008; Durand *et al.* 1988). Digestion coefficients show that *Neurospora sitophila* is not able to break down the lignin and digestion coefficients were reduced (Ghiasi, 2010).

Dashti *et al.* (2009) found that DM, OM and DOMD digestibility of beet pulp increased by processing by *Neurospora sitophila*. An increase in DM, OM and DOMD digestibility of lemon and orange pulp treated by *Neurospora sitophila* was observed by Nazem *et al.* (2008).

Metabolisable energy of untreated walnut hull decreased significantly by treating by *Neurospora sitophila* (P<0.05). There is an inverse relationship between NDF content and digestibility (Durand, 1988). When the amount of NDF increases, digestibility decreases. Treating by *Neurospora sitophila* caused to an increase in ME of lemon and orange pulp (Nazem *et al.* 2008).

The ME values of 2.5 and 3.1 (MJ/kg DM) for untreated and treated beet pulp expressed by Dashti *et al.* (2009), respectively. Vahabzadeh (2011) indicated that treating by *Neurospora sitophila* had no significant effect on ME of pistachio peel.

CONCLUSION

In this study, treating by *Neurospora sitophila* did not appear to affect the nutritive value of walnut hull as neither the chemical composition nor the OM and NDF degradability and digestibility were changed as compared with untreated walnut hull, so it can be concluded that *Neurospora sitophila* did not grow very well on this substrate. Walnut hull can be treated by other methods and other fungi species. Additional *in vivo* experiments can be conducted to further evaluate this fungus for treatment of walnut hull as the dietary ingredient for ruminants. Moreover development of *Neurospora sitophila* in substrate depends on several factors such as type of substrate, storage temperature, moisture content, presence of oxygen, and gaseous composition as well as other colonized microorganisms. Therefore, it seems necessary to test *Neurospora sitophila* species and substrates to find the best situation.

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