Basrah Journal of Veterinary Research,Vol.17, No.3,2018
Proceeding of 6th International Scientific Conference,College of Veterinary Medicine
University of Basrah,Iraq

INFLUENCE OF GRADED LEVELS OF *NIGELLA SATIVA* SEEDS AND *ROSMARINUS OFFICINALIS* LEAVES ON *IN VITRO* RUMEN FERMENTATION AND APPARENT BIOHYDROGENATION

Kifah Jumaah Odhaib[*,**] , Awis Qurni Sazili[*,***,****]

[*]Department of Animal Science, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
[**] Department of Physiology, College of Veterinary Medicine, University of Basrah, 61004 Basra, Iraq
[***] Laboratory of Sustainable Animal Production and Biodiversity, Institute of Tropical Agriculture and Food Security, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
[****] Halal Products Research Institute, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

Keywords: y NS seeds, Fatty acids, *in vitro.*

Corresponding Author:kifahodhaib@gmail.com.

ABSTRACT

The results in present study are illustrated that the effects of different levels of NS seeds and RO leaves on *in vitro* gas production, rumen fermentation, fatty acids composition and the apparent biohydrogenation of oleic, linoleic and linolenic acids using rumen liquor from Dorper lambs. The NS seeds and RO leaves were supplemented at the rate of 0, 0.5, 1, 1.5 and 2% (w/w) DM of basal substrate [60% forage (urea treated rice straw) and 40% concentrate] and incubated for 24 h at 39°C. Substrates containing RO and NS had greater ($P<0.05$) gas production than the control substrates. The volume of gas produced increased as the levels of RO and NS increased up to 1.5% and decreased afterwards. Supplementation of RO and NS did

not affect (P>0.05) *in vitro* dry matter digestibility, *in vitro* organic matter digestibility, rumen pH, CH_4 and NH_3-N, total volatile fatty acids (VFA) and the molar proportion of acetate, propionate and butyrate. The RO supplements reduced the ruminal concentration of C18:0 and increased the ruminal concentration of C18:1n-9 in a dose dependent manner. The supplementation of RO leaves reduced (P<0.05) the apparent biohydrogenation of C18:1n-9 but had no effect (P>0.05) on the apparent biohydrogenation of C18:2n-6 and C18:3n-3.

INTRODUCTION

Herbs are used in animal feeds as the growth promoters. They play a major role as antibacterial, antioxidant, anthelmintic and anticoccidial. Majority of medicinal plants do not have the residual effects. It has been shown that phytochemicals and plant secondary metabolites could increase protein flow to the duodenum (1). The plants containing saponins have been found to suppress or eliminate protozoa from the rumen and decrease ammonia and methane production (2).

Plant polyphenols are commonly employed in ruminant nutrition and their usage could have positive and/or detrimental effects on rumen metabolism (3). The effects of plant polyphenols on rumen metabolism depends on the chemical nature of the polyphenols, the quantity in the feed, the abundance and diversity of rumen microbes and the response of rumen microbiota to the polyphenols (4;5). However, their primary sources are mainly the medicinal herbs (6).They are not always well characterized

because there are differences in their composition among various plant species and parts. Moderate concentration of these polyphenols in many plants may possibly reduce ruminal protein breakdown and increase duodenal protein flow (7). Nonetheless, when given to animal at higher doses they may also adversely affect the animal performance. Higher concentration in the diets has been known to cause a negative effect on digestibility of feed and nutrients in ruminants (8). This is due to their ability to bind nutrients particularly proteins and carbohydrates which in turn modifies the rate and extent of their digestion (9).

In some cases, presence of very high levels of polyphenols, particulary tannins can produce toxicity and eventually lead to death of the animals (10). Several feeding strategies have been introduced such as addition of antimicrobial constituents which modifies the rumen environment thereby improving animal's health and productivity these will increase yield and improve quality of products. Digestibility of herbs has been tested *in vitro* (11). Tannins have been used to reduce rate of biohydrogenation *in vitro* (12). Oil rich in polyunsaturated fatty acids (PUFA) have been supplemented in the diet of sheep (13) and alter to the rumen fermentation characteristics in cattle (14). However, there is no information on the use of the herbs on biohydrogenation of fatty acids.

There are new approaches to increase the effectiveness of digestion and metabolism of nutrients that will improve productivity and product quality and environment. One economical, sustainable and safer approach is the use of herbs such as *Nigella sativa* (NS) and *Rosmarinus officinalis* (RO) which contain diterpenoids, and polyphenols

such as flavonoids, tannins and essential oils (15; 16). These PSMs are among the other potent anti-microbial agents present in *Nigella sativa* seeds and *Rosmarinus officinalis* leaves such as alkaloids, saponine, thymo, luteolin , carnosic acid, and rosmarinic acid were the major phenolic compounds in these two herbs (17; 18). It is possible that the presence or combination of these plant bioactives could improve the effectiveness of nutrient utilization in ruminants. The *in vitro* study is a relatively simple, cheap and direct measurement of microbial activities *in vitro* and reflects all nutrients fermented. Thus, the purpose of this finding was to examine the suitable level of *Nigella sativa* seeds and *Rosmarinus officinalis* leaves from *in vitro* rumen metabolism using an *in vitro* gas production technique.

The objectives of this study were:

1- To determine the suitable level of *Nigella sativa* seeds and *Rosmarinus officinalis* leaves from *in vitro* gas production and rumen fermentation parameters.
2- To determine the suitable level of *Nigella sativa* seeds and *Rosmarinus officinalis* leaves from *in vitro* fatty acid composition and apparent biohydrogenation using rumen liquor from Dorper lambs.

MATERIALS AND METHODS

Animal welfare and source of medicinal herbs

The finding was carried out in line with the guidelines approved by the Universiti Putra Malaysia Institutional Animal Care and Use Committee. For the Care and Use of Animals Scientific Purposes (Research Policy, Universiti Putra Malaysia). *Nigella*

sativa seeds and *Rosmarinus officinalis* leaves were purchased from a local market in Selangor, Malaysia. The seeds and leaves were ground into powder, packaged in air-free polyethylene bags and stored in a cool, dry place prior to analysis.

Treatments and experimental design

The experimental design was a completely randomized design, with three replications per treatment including triplicates of blank in three incubation runs. The basal substrate was 60% forage (urea treated rice straw) and 40% concentrate. The effect of NS seeds and RO leaves were examined in a separate incubation runs. The following diets were formulated:

1. Basal diet an additive (control).
2. Basal diet + 0.5% (w/w) DM NS seeds or RO leaves.
3. Basal diet + 1% (w/w) DM NS seeds or RO leaves.
4. Basal diet + 1.5% (w/w) DM NS seeds or RO leaves.
5. Basal diet + 2% (w/w) DM NS seeds or RO leaves.

Upon completion of the *in vitro* digestibility trial, the levels of NS and RO were increased to 2% (w/w) DM and for biohydrogenation following the procedures of Hassim *et al*. (19). The results generated through the *in vitro* studies were then used as baseline data for the conduct of the subsequent feeding trial.

Estimation of total phenol, tannins and non-tannin phenol

Determination of total polyphenol and tannins were executed following the procedure of Makkar *et al*. (21, 22). The phytochemical contents of the substrates are as presented in Table 3.1.

1.1.1 Proximate analyses

The (DM) dry matter, (CP, total nitrogen x 6.25) crude protein, crude fat and ash content of the treatments were evaluated in line with to the protocol of (23). The (NDF) Neutral detergent fiber were estimated according to the method of Van Soest *et al* (24) as well as and (ADF) acid detergent fiber. The nutrient compositions of the substrates are shown in Table 3.1.

Table .خطأ! لا يوجد نص من النمط المعين في المستند **1.Chemical composition of *in vitro* experimental diets**

Parameter (%) DM	Substrates								
	control	0.5% RO	1% RO	1.5% RO	2% RO	0.5% NS	1% NS	1.5% NS	2% NS
DM	91.95	92.23	91.87	91.92	91.67	92.98	92.36	92.06	92.37
Crude protein	16.81	16.96	16.66	16.53	16.73	16.72	16.85	17.04	16.78
Crude fat	3.39	3.46	3.44	3.27	3.39	3.53	3.24	3.24	3.35
Ash	8.08	8.43	8.35	8.18	8.37	8.06	8.06	8.32	8.45
ADF	27.93	27.66	27.81	28.94	28.07	28.21	28.60	29.58	29.30
NDF	58.75	56.49	58.01	57.83	58.28	56.80	57.40	57.04	56.18
Phytochemical compounds									
Total polyphenol (g/kg)	3.46	25.11	46.75	68.40	90.04	22.31	41.15	59.99	78.80
Non-tannin polyphenol (g/kg)	1.02	6.38	11.73	17.89	22.44	2.10	3.18	4.26	5.34
Tannin polyphenol (g/kg)	2.96	19.25	35.54	51.05	68.12	20.73	38.49	56.26	74.02

DM = dry matter. ADF=acid detergent fiber. NDF= neutral detergent fiber. RO = *Rosmarinus officinalis* leaves. NS=*Nigella sativa* seeds.

Rumen liquor collection

The rumen liquor was collected from three fistulated Dorper sheep fed diet containing 40% concentration and 60% forage (urea treated rice straw) for 2 weeks before feeding the animal in the morning into a thermo flask and continuously flushed with CO2 while the liquor was transported to the laboratory. The rumen liquor was pooled together and sieved with the aide of cheese cloth in four layers.

***In vitro* rumen fermentation of substrate**

The method of Menke and Steingass (25) was adhere to during the *in vitro* gas production experiment. The strained rumen liquor was included inside the media in a 1:2 (v/v) ratio under nonstop CO_2 flushing in a water bath at a temperature of 39 $^{\circ}$C. The media is made up of 200 mL macro mineral solution, 0.1 mL micro mineral solution, 200 mL buffer solution, 40 mL reducing solution, 1 mL Resazurin solution and 400 mL distilled H_2O

pH determination

After 24 h incubation the pH of the rumen fluid was measured using (Mettler- Toledo, Ltd England) pH meter.

***In vitro* dry matter digestibility (IVDMD)**

The IVDMD was measured in accordance with the method of Tilley and Terry (26).

Determination of volatile fatty acids (VFA)

After 24 h incubation, 25% metaphosphoric acids in the of ratio 4:1 (v/v) was added to the rumen fluid for the evaluation of the VFA profile with the aide of Gas Liquid Chromatography (Hewlett Packard 6890).

Determination of ammonia nitrogen (NH_3-N)

The NH3-N concentration of the treatment were evaluated with following the procedure of Solorzano (27).

Calculations

The Neway software program was employed to fit the data from gas production into the model V = a + b (1 – e^(-ct)) has explained by Ørskov and McDonald (28). Each of the variables in the model are detailed below.

V = Gas quantity at a specific period t, a = quantity of gas produced from the soluble portion, b = Gas quantity produced from the insoluble portion, c = rate of gas production for the insoluble portion and t = time of incubation. However, ME, IVOMD and methane were calculated using the formular below Menke and Steingass (25) and Moss *et al*. (2000)

ME=.0.0029.CF + 0.057.CP+ 0.136V + 2.20,

IVOMD%=.14.88+ 0.651AS +.0.45CP +.0.889 V

CH4=.0.45C2 -.0.275 C3 +.0.4 C4.

CF (crude fiber), CP (crude protein),.V total quantity of gas produced, AS (ash).

1.1.2 Determination of FA composition of substrates and rumen liquor

The mixture of chloroform: methanol (2:1, v/v) was employed as the extraction solvent for the total FA from each substrate diet (1 g) or rumen liquor (10 mL) in accordance with Folch *et al* (29) altered by Rajion *et al* (30). The fatty acid composition of the treatment are presented in Table 3.2.

Table .2 Fatty acid composition (% of total fatty acid) of substrates خطأ! لا يوجد نص من النمط المعين في المستند.

	Substrates								
Parameter	Control	0.5 % RO	1% RO	1.5 %RO	2% RO	0.5 %NS	1% NS	1.5 %NS	2% NS
C14:0	0.45	0.56	1.26	0.24	0.21	1.12	0.470	0.24	0.43
C16:0	15.97	17.36	16.09	16.37	14.07	14.07	15.018	15.08	15.82
C16:1	0.55	0.65	0.29	0.55	0.43	0.81	0.322	0.43	0.66
C18:0	5.15	6.31	6.24	4.83	5.24	5.32	5.169	5.43	4.78
C18:1n-9	21.52	25.76	19.99	21.25	26.27	24.35	24.26	26.82	20.49
C18:2n-6	10..27	10.02	10.8	11.15	11.50	10.5	10.57	11.23	11.45
C18:3n-3	6.73	3.94	5.47	5.37	5.63	3.72	4.132	4.63	5.61
C20:4n-6	0.90	1.81	0.94	1.03	0.79	1.79	0.88	0.75	0.77
C20:5n-3	0.91	1.42	1.03	0.88	0.41	1.28	0.63	0.39	0.67
C22:5n-3	0.52	1.02	0.35	0.97	0.58	0.74	0.41	0.43	0.79
C22:6n-3	0.95	1.52	1.01	2.15	1.83	1.95	2.16	1.83	2.48
ΣSFA	21.58	24.25	23.60	21.45	19.53	20.51	20.65	20.76	21.04
ΣUFA	42.38	46.14	40.00	43.39	47.46	45.18	43.37	46.54	42.93
ΣMUFA	22.08	26.41	20.28	21.81	26.70	25.17	24.58	27.2	21.15
ΣPUFA	20.30	19.73	19.68	21.57	20.75	20.01	18.79	19.20	21.78
Σ n-3	9.12	7.90	7.88	9.39	8.46	7.71	7.340	7.29	9.56
Σn-6	11.17	11.82	11.79	12.18	12.29	12.30	11.45	11.98	12.22
n-6/n-3	1.22	1.49	1.49	1.29	1.45	1.59	1.55	1.64	1.27
UFA:SFA	1.96	1.90	1.70	2.02	2.43	2.20	2.10	2.24	2.04
PUFA:SFA	0.94	0.81	0.83	1.00	1.06	0.97	0.90	0.92	1.03

RO = *Rosmarinus officinalis* leaves. NS=*Nigella sativa* seeds. ΣSFA = (C14:0 + C16:0 + C18:0), ΣMUFA = (C16:1+ C18:1), ΣUFA = (C16:1+ C18:1+Σn-3 + Σn-6), ΣPUFA = (Σn-3 + Σn-6), Σn-3 = (C18:3n-3 + C20:5n-3 + C22:5n-3 + C22:6n-3), Σn-6 = (C18:2n-6 + C20:4n-6) n-6:n-3 = (C18:2n-6 + C20:4n-6) ÷ (C18:3n-3 + C20:5n-3 + C22:5n-3 + C22:6n-3).

1.1.3 Rate of biohydrogenation

The rate of disappearance or apparent biohydrogenation of oleic acid (C18:1n-9) linolenic acid (C18:3n-3) and linoleic acid (C18:2n-6) were calculated from the difference in the concentration of the fatty acids between 0 h and 24 h *in vitro* incubation as: Apparent biohydrogenation (%) = [100*[(CFA)i – (CFA)f]/ (CFA)i]

Where (CFA)i= % concentration of unsaturated fatty acid at 0 h incubation,

(CFA) f = % concentration of unsaturated fatty acid at 24 h incubation (19).

1.1.4 Statistical analysis

The gas production parameter was examined in a completely randomized design using the MIXED procedure of SAS (31) with sampling time as a repeated measure. Data for fatty acid composition, VFA and rumen fermentation were analyzed in a completely randomized design using the generalized linear model (GLM) procedure of SAS (SAS, 2003). For all parameters, differences between treatments means were separated by Duncan Multiple Range test. Mean differences were considered significant at ($P< 0.05$).

Results

1.1.5 *In vitro* gas production and rumen fermentation parameters

The *in vitro* gas production and rumen fermentation parameters of substrates supplemented with graded levels of *Rosmarinus officinalis* leaves and *Nigella sativa* seeds are as presented in Tables 3.3, 3.4 and 3.5. Supplementation of graded levels of RO and NS influenced ($P<0.05$) gas production throughout the 24 h incubation (Table 3.3). Substrates containing RO and NS had greater ($P<0.05$) gas production than the control substrates. There were significant differences ($P<0.05$) among substrates

containing RO and NS. The volume of gas produced increased as the levels of RO and NS increased up to the 1.5% and decreased afterwards (Table 3.3).

Table .خطأ! لا يوجد نص من النمط المعين في المستند **3** ***In vitro*** **gas production of substrates containing graded levels of** ***Rosmarinus officinalis*** **and** ***Nigella sativa*** **seeds during 24 h incubation**

Treatment	3 h	6 h	9 h	12 h	15 h	18 h	21 h	24 h	SEM	P value	Diet*Time
0% RO	11.95^{cz}	14.62^{cz}	17.84^{cy}	22.05^{by}	26.24^{bx}	28.66^{bx}	32.27^{bw}	34.95^{bw}	1.039	<.0001	
0.5% RO	20.62^{aby}	21.63^{bxy}	25.80^{abxy}	28.95^{abwx}	32.70^{abwx}	34.77^{abw}	37.34^{abv}	39.39^{abv}	1.76	<.0001	0.99
1% RO	21.40^{aby}	32.25^{abxy}	27.20^{abxy}	31.40^{awx}	35.39^{aw}	38.96^{aw}	41.44^{aw}	43.50^{av}	3.31	0.001	
1.5% RO	24.03ay	27.92^{axy}	30.63^{axy}	34.56^{awx}	37.54^{aw}	39.72^{aw}	41.54^{av}	43.04^{abv}	2.07	<.0001	
2% RO	15.10^{bcy}	18.58^{bcy}	22.83^{bcxy}	30.24^{axy}	32.16^{abwx}	35.40^{abwx}	38.79^{abw}	40.99^{abv}	1.73	<.0001	
P value	0.012	0.004	0.004	0.037	0.023	0.031	0.107	0.162			
0% NS	11.95^{dz}	14.62^{cz}	17.84^{cy}	22.05^{dy}	26.24^{cx}	28.66^{cx}	32.27^{cw}	34.95^{bw}	1.039	<.0001	
0.5% NS	19.30^{bcy}	21.81^{aby}	24.70by	28.81^{bcy}	31.35^{bxy}	34.13^{abwx}	36.86^{abcw}	38.48^{abv}	0.96	<.0001	0.88
1%NS	24.03^{az}	26.13^{ayz}	29.07^{ayz}	32.83^{abxy}	35.92^{awx}	37.69^{aw}	39.99^{aw}	41.22^{av}	1.92	<.0001	
1.5%NS	21.93^{abx}	26.12^{ax}	30.01^{awx}	35.71^{aw}	35.12^{axw}	37.18^{aw}	39.27^{abv}	40.39^{av}	2.36	0.0003	
2%NS	15.89^{cdy}	18.40^{bcy}	22.05^{bw}	25.50^{cdw}	29.47^{bcw}	32.47^{bcw}	34.80^{bv}	37.00^{abv}	0.55	<.0001	
P value	0.001	0.001	0.0003	0.0008	0.0003	0.003	0.024	0.080			

RO = *Rosmarinus officinalis* leaves. NS=*Nigella sativa* seeds.
^{abcd} Means there is significant difference between treatment.
^{vwxyz} Means there is significant difference between gas production.

Supplementation of RO did not affect (P>0.05), metabolizable energy, pH, IVOMD, IVDMD, CH4 and NH4-N, total VFA and the molar proportion of acetate, propionate and butyrate (Table 3.4). The volume of gas produced from soluble fraction was greater in the control substrate compared with the substrates supplemented with 0.5% RO and 2% RO. The value of gas production from the soluble fraction in 1% RO and 1.5% RO did not differ (P>0.05) from that of other treatments. The value of NGP, b, a+b and c did not differ (P>0.05) among the treatments. The molar proportion of propionate in the control substrate did not differ (P>0.05) from those supplemented with graded levels of RO. The 2% RO had lower (P<0.05) ratio of acetate to propionate (A:P) compare with the control, 0.5% and 1% RO substrates.

Table .خطأ! لا يوجد نص من النمط المعين في المستند**.4** ***In vitro*** **fermentation, pH, and concentration of volatile fatty acids in substrates containing graded levels of** ***Rosmarinus officinalis*** **during 24 h incubation**

	Substrates						
Parameter	0% RO	0.5% RO	1% RO	1.5% RO	2% RO	SEM	*P value*
a (mL)	33.53^{x}	30.90^{y}	32.51^{xy}	31.81^{xy}	28.05^{z}	0.75	0.017
b (mL)	69.68	86.89	76.26	62.83	77.54	8.15	0.36
c (mL/h)	0.040	0.023	0.033	0.043	0.036	0.005	0.23
a + b (mL)	103.21	117.79	108.77	94.64	106.59	8.29	0.43
NGP (mL)	37.25	40.50	43.50	43.16	41.66	1.75	0.16
pH (unit)	6.90	6.90	6.76	6.80	6.83	0.035	0.073
ME	7.76	8.23	8.57	8.45	8.02	0.105	0.086
IVOMD	58.78	62.21	64.46	63.53	60.75	0.840	0.119
IVDMD	52.83	50.83	54.00	54.83	58.50	2.98	0.871
CH_4	21.74	22.06	22.93	24.03	22.54	0.443	0.318
NH_4N	11.92	11.99	12.65	11.34	12.70	0.296	0.364
Total VFA (mmol/L)	78.97	78.82	82.74	87.33	82.83	2.80	0.24
Acetate (A)	55.56	55.89	58.38	61.66	58.14	2.01	0.28
Propionate (P)	16.44	16.21	17.40	18.76	18.09	0.69	0.12
Butyrate	3.15	3.41	3.60	3.62	3.37	0.15	0.26
A:P	3.38^{xy}	3.44^{x}	3.35^{xy}	3.29^{yz}	3.21^{z}	0.025	0.003

RO= *Rosmarinus officinalis* a=volume of gas produced from soluble fraction; b =volume of gas produced from insoluble fraction; c=gas production rate constant from the insoluble fraction; NGP= net gas production; pH= ruminal pH. ^{xyz} Means there is significant difference

Supplementation of NS did not affect (P>0.05), metabolizable energy, pH, IVOMD, IVDMD, CH4, NH4-N, total VFA and the molar proportion of acetate, propionate and butyrate (Table 3.5).

Table .خطأ! لا يوجد نص من النمط المعين في المستند**.5** ***In vitro*** **fermentation, pH, and concentration of volatile fatty acids in substrates containing graded levels of** ***Nigella sativa*** **after 24 h incubation**

	Substrates						
Parameter	0% NS	0.5%NS	1% NS	1.5% NS	2% NS	SEM	*P value* ns.
a (mL)	33.53	32.10	32.48	30.05	32.22	1.23	0.43
b (mL)	69.68	68.59	75.74	54.95	79.30	7.91	0.30
c (mL/h)	0.040	0.033	0.036	0.056	0.026	0.006	0.07
a + b (mL)	103.21	100.69	108.23	85.00	111.52	7.51	0.19
NGP (mL)	37.25	39.83	41.83	41.50	38.75	1.12	0.07
pH (unit)	6.90	6.89	6.82	6.86	6.87	0.025	0.29
ME	7.76	8.33	8.87	8.83	8.54	0.203	0.159
IV0MD	58.78	62.67	66.38	66.23	64.37	1.303	0.183
IVDMD	52.83	51.83	54.17	56.16	57.00	3.376	0.961
CH_4	21.74	22.61	22.19	22.00	21.38	0.636	0.944
NH_4-N	11.92	12.71	12.32	12.48	13.01	0.167	0.949
Total VFA (mmol/L)	78.97	78.82	82.74	87.33	82.83	4.41	0.92
Acetate	55.56	58.64	57.95	57.01	55.40	3	0.91
Propionate	16.44	18.42	18.32	17.95	17.54	1.32	0.82
Butyrate	3.15	3.22	2.88	3.20	3.20	0.2	0.77
A:P	3.38	3.20	3.18	3.18	3.16	0.08	0.53

NS=*Nigella sativa* seeds. a=volume of gas produced from soluble fraction; b =volume of gas produced from insoluble fraction; c=gas production rate constant from the insoluble fraction; NGP= net gas production pH= ruminal pH. ns= not significant.

1.1.6 *In vitro* biohydrogenation and fatty acid composition of rumen liquor

The fatty acid composition of rumen liquor supplemented with different levels of RO are presented in Table 3.6. The concentration of C12:0 and C14:0 were lower (P<0.05) in 2% RO compared to other substrates. The concentrations of C15:0, C15:1, C16:0 and C16:1n-7 did not differ (P>0.05) among the treatments. The 2% RO had greater (P>0.05) concentrations of C16:1n-9 and C18:1n-9 and lower concentration of C18:0 compared with other treatments. The concentrations of C18:0 in 1% and 1.5% RO did not differ but was greater (P<0.05) than that of the control and 0.5% RO substrates. The concentrations of C18:2n-6, C18:3n-3, CLAc9t11, CLAc12t10, C20:4n-6 and C22:6n-3 did not differ (P>0.05) among the substrates. The total UFA was greater (P< 0.05) while the total SFA was lower (P< 0.05) in 2% RO compared with other substrates. The rate of biohydrogenation of C18:1n-9 decreased (P<0.05) as the level of RO increased in the substrate. Supplementation of RO had no significant effect on the apparent biohydrogenation of C18:2n-6 and C18:3n-3 in the substrate.

Table .خطأ! لا يوجد نص من النمط المعين في المستند.**6 Fatty acid composition (% of total fatty acids) of rumen fluid and rate of biohydrogenation at 24 h incubation of substrates containing graded levels of *Rosmarinus officinalis***

Fatty acids	Diet						
	0% RO	0.5% RO	1% RO	1.5% RO	2% RO	SEM	*P-value*
C12:0	1.15^{a}	1.10^{a}	1.20^{a}	0.84^{ab}	0.58^{b}	0.14	0.04
C14:0	1.60^{a}	1.99^{a}	1.50^{ab}	0.96^{bc}	0.79^{c}	0.17	0.005
C15:0	4.21	3.77	5.21	4.04	4.09	0.31	0.06
C15:1	0.87	0.65	0.29	0.63	0.17	0.25	0.18
C16:0	22.39	25.90	28.80	23.78	18.02	3.37	0.29
C16:1n-7	1.06	0.77	0.67	0.63	0.53	0.11	0.09
C16:1n-9	1.40^{b}	1.25^{b}	1.83^{b}	1.93^{b}	2.93^{a}	0.27	0.01
C18:0	29.32^{ab}	32.78^{a}	22.04^{c}	24.98^{bc}	15.65^{d}	1.76	0.0004
Trans-11 C18:1	3.59	4.28	2.89	2.49^{ab}	2.28	0.48	0.08
C18:1n-9	13.20^{c}	10.37^{c}	21.52^{bc}	26.67^{b}	43.58^{a}	4.66	0.003
C18:2n-6	13.60	7.68	8.40	6.84	4.50	1.81	0.060
C18:3n-3	2.72	2.07	2.58	2.66	2.67	0.4	0.78
CLAc9t11	0.92	2.15	0.55	0.55	0.80	0.40	0.09
CLAc12t10	1.37	1.60	0.78	0.55	1.08	0.28	0.14
C20:4n-6	1.10	1.26	1.07	1.13	0.39	0.25	0.20
C22:6n-3	1.43	2.31	1.32	0.97	1.79	0.4	0.24
ΣSFA	58.68^{b}	65.55^{a}	58.76^{b}	54.61^{b}	39.14^{c}	3.85	0.007
ΣUFA	41.32^{b}	34.44^{b}	41.23^{b}	45.38^{b}	60.86^{a}	3.85	0.007
ΣMUFA	20.15^{b}	17.34^{b}	26.51^{b}	32.67^{b}	49.62^{a}	4.87	0.006
UFA:SFA	0.67^{b}	0.52^{b}	0.82^{b}	0.80^{b}	1.55^{a}	0.08	0.0001
Apparent biohydrogenation (%)							
C18:1n-9	68.21^{a}	64.43^{b}	61.23^{C}	54.78^{d}	52.11^{d}	3.67	0.04
C18:2n-6	83.04	84.34	86.03	80.23	80.45	4.79	0.21
C18:3n-3	89.20	89.00	90.25	85.44	86.56	4.85	0.10

RO = *Rosmarinus officinalis* leaves. ΣSFA = (C14:0 + C16:0 + C18:0), ΣMUFA = (C16:1+ C18:1+ C18:1 trans-11), ΣUFA = (C16:1+ C18:1+Σn-3 + Σn-6), Σn-6 = (C18:2n-6 + C20:4n-6) n-6:n-3 = (C18:2n-6 + C20:4n-6) ÷ (C18:3n-3 + C20:5n-3 + C22:5n-3 + C22:6n-3). SFA= saturated fatty acids; UFA= unsaturated fatty acids; MUFA= monounsaturated fatty acids.

The fatty acid composition of rumen liquor supplemented with graded levels of NS after 24 h incubation is as presented in Table 3.7. Supplementation of graded levels of NS had no significant effect on the fatty acid composition of rumen liquor after 24 h incubation.

The apparent biohydrogenation of C18:1n-9, C18:2n-6 and C18:3n-3 was not influenced by the supplementation of NS.

Table .خطأ! لا يوجد نص من النمط المعين في المستند.7 Fatty acid composition (% of total fatty acids) of rumen fluid and rate of biohydrogenation at 24 h incubation of substrates containing graded levels of *Nigella sativa* seeds

Fatty acids	Substrate						
	0% NS	0.5% NS	1 % NS	1.5% NS	2% NS	SEM	*P value* ns.
C12:0	1.15	1.22	1.64	1.32	0.78	0.17	0.07
C14:0	1.60	1.93	2.20	1.62	1.26	0.64	0.86
C15:0	4.21	4.90	5.04	4.53	3.46	0.47	0.21
C15:1	0.87	1.27	1.28	0.21	1.55	0.80	0.79
C16:0	22.39	24.40	27.25	25.25	26.68	1.70	0.33
C16:1n-7	1.06	0.81	0.90	0.88	0.72	0.11	0.44
C16:1n-9	1.40	2.56	1.19	1.51	1.99	0.37	0.15
C18:0	29.32	23.19	31.45	28.96	22.61	3.10	0.24
Trans-11 C18:1	3.59	2.90	3.88	4.34	1.81	0.69	0.16
C18:1n-9	13.20	22.72	7.46	14.61	25.81	5.94	0.25
C18:2n-6	13.60	6.57	12.05	8.20	7.06	1.78	0.06
C18:3n-3	2.72	2.80	0.91	1.76	2.31	0.80	0.47
CLAc9t11	0.92	0.97	0.74	1.41	0.37	0.30	0.26
CLAc12t10	1.37	0.96	0.84	0.81	0.51	0.24	0.24
C20:4n-6	1.10	1.27	0.33	1.73	1.36	0.4	0.25
C22:6n-3	1.43	1.48	2.80	2.76	1.69	0.47	0.15
ΣSFA	58.68	55.65	67.59	61.69	54.80	4.53	0.33
ΣUFA	41.32	44.34	32.40	38.30	45.19	4.53	0.33
ΣMUFA	20.15	30.27	14.71	21.58	31.88	5.29	0.19
UFA:SFA	0.67	0.86	0.48	0.64	0.83	0.12	0.34
Apparent biohydrogenation (%)							
C18:1n-9	69.00	67.25	65.12	66.08	66.21	0.83	0.21
C18:2n-6	78.21	80.23	76.21	75.32	75.17	0.24	0.11
C18:3n-3	86.15	87.23	87.45	87.24	88.16	0.95	0.09

NS=*Nigella sativa* seeds. ΣSFA = (C14:0 + C16:0 + C18:0), ΣMUFA = (C16:1+ C18:1+ C18:1 trans-11), ΣUFA = (C16:1+ C18:1+Σn-3 + Σn-6); Σn-6 = (C18:2n-6 + C20:4n-6) n-6:n-3 = (C18:2n-6 + C20:4n-6) ÷ (C18:3n-3 + C20:5n-3 + C22:5n-3 + C22:6n-3); SFA= saturated fatty acids; UFA= unsaturated fatty acids; MUFA= monounsaturated fatty acids. ns=not significant.

DISCUSSION

Rumen fermentation and gas production has close association, therefore gas-measuring techniques were believed to be used as a routine method for feed evaluation (32). Many studied have reported a high correlation between gas production *in vitro* and *in vivo* apparent digestibility (33; 34). It has been proved that the inclusion of NS and RO increased the cumulative *in vitro* gas production over the 24 h incubation period.

The *in vitro* gas production was greater in substrates containing 1% and 1.5% concentrations of NS and RO than the substrate containing higher concentration of NS and RO (2%). This observation suggests that the effects of NS and RO on *in vitro* gas production were concentration-dependent. This was particularly true in RO supplements when where in the 2% RO had lower value of a (gas produced from soluble fraction) than other supplements (table 3.4). This observation could be due to the higher concentration of polyphenols present in the supplements. This finding is in agreement with the results of Soycan-Önenç (35) who observed that the supplementation of *Vitex agnus-castus* improved cumulative *in vitro* gas production up to 8 h, and then the GP-reducing effect occurred from 8 to 12 h of incubation in all the treatments compared with the control, it was shown that, the concentrate feed with *Vitex agnus-castus* addition reduced degradation of storage polymers such as starch. Contrarily, Moujahed *et al*, (36) found that rosemary (*Rosmarinus officinalis*) EOs at doses (0, 5, 10, 20, and 40 µl/50 ml of buffered rumen fluid) did not affect cumulative *in vitro* gas production, while thyme (*Thymus capitatus*) EOs decreased ($P<0.0001$) gas production starting from 10 µl dose.

In spite of the changes in the cumulative gas production, net gas production and gas produced from the insoluble fraction were not affected by the supplementation of RO and NS after 24 h incubation. This observation could be responsible for the lack of significant changes observed

among the supplements for IVDMD, IVOMD, pH, total and individual VFA. Natural feed additives such as RO and NS and their product can be regarded has been beneficial in ruminant nutrition when there is a positive impact on the propionic acid and total VFA by increasing their production and less impact by reducing the acetic/propionic ratio (37; 38). Many findings have attested to the damaging impact of this compound on the total VFA production along with a decrease in the digestion of feed, particularly with high concentration. Some plant extract have been examined which include (cove, anise, oregano, fenugreek, yucca, cinnamon, garlic, bud, ginge and tea tree) included at varied quantity for 24 h in vitro ruminal fermentations (39). The author of this research noticed a reduced total VFA production in nearly all the treatment as the quantity increased in the feed and this could be an indication of low feed digestion. In addition, Moujahed et al, (36) reported that rosemary (Rosmarinus officinalis) EOs at doses (0, 5, 10, 20, and 40 µl/50 ml of buffered rumen fluid) rosemary EO increased NH3-N concentration ($P<0.001$) when administered at the doses of 20 and 40, while there is no significantly diffirent of VFA accumulation. Cobellis et al. (40) indicated that rosemary (Rosmarinus officinalis L.) and oregano (Origanum vulgare L.) essentials oil at doses (0, 0.5, 1, 1.5, 2 g/L) decreased total VFA by oregano but was unaffected by rosemary, while the yield of ammonia was greatly decreased among the treatments with exclusion of rosemary at the minimum quantity. Also nearly all the treatment neutral detergent fiber and dry matter degradability was greatly decreased.

Even though, IVDMD, IVOMD, total and individual VFA did not differ statistically ($P>0.05$), the slightly higher values observed in the (1% and 1.5%) in both NS and RO could partly be due to the concentration of polyphenols at accepted level to improve rumen fermentation (41; 42). Hence, the concentration of total polyphenols observed in this study, could have altered the

fermentation characteristics (9). It is due to the presence of those polyphenol (43; 41; 44) which could be responsible for the slightly higher dry matter, organic matter digestibility, total and individual VFA as compared to the control diet.

This study demonstrated that, supplementation of RO reduced the concentration of C12:0 and C14:0 in a dose dependent manner. This observation could be attributed to the phenolic compounds such as triterpenes, phenolic acids, and flavonoids (45; 46) in the supplements, which reduced the activities of lipogenic enzymes responsible for the synthesis of medium chain fatty acids (47). Supplementation of RO increased the concentration of C18:1n-9 and reduced the concentration of C18:0 in a dose dependent manner. This observation is consistent with the reduced biohydrogenation of C18:1n-9 in substrates containing RO. Similarly, supplementation of Andrographis paniculata leaves reduced the concentration of C18:0 and increased the concentration of C18:1n-9 in rumen liquor from goats. Supplementation of RO reduced the apparent biohydrogenation of C18:1n-9 but no impact was noticed in the biohydrogenation of C18:2n-6 and C18:3n-3. The decrease in the apparent biohydrogenation of C18:1n-9 could be due to the presence of polyphenolic compounds, which selectively inhibit the activities of microbes responsible for the biohydrogenation of C18:1n-9.

Contrarily to the observations in RO supplements, the supplementation of NS did not have a significant effect on the fatty acid composition of rumen liquor. Nonetheless, there were numerical changes in fatty acid composition similar to the trend observed in the RO supplements. Regardless of the supplement, the apparent biohydrogenation of C18:3n-3 was greater than that of C18:2n-6, which was in turn greater than that of C18:1n-9. Similar trends were observed in in vitro (48; 47) rumen fatty acid composition in goats.

CONCLUSION

Irrespective of the level, the inclusion of RO leaves and NS seeds in the diets indicate no deleterious effects on rumen fermentation but reduced rumen gas production at 2%. From this result, it is believed that the supplementation of RO leaves and NS seeds up to 1% DM of substrate had no deleterious effect on *in vitro* ruminal fermentation and improve the digestibility. Generally, the results from the present study suggested that RO leaves and NS seeds can be efficiently utilized to manipulate rumen fermentation characteristics without adversely affecting or compromising the production of VFAs, dry matter digestibility and some desirable fatty acids in the product.

REFERENCES

1- Brown, W.F., and Pitman, W.D. (1991). Concentration and degradation of nitrogen and fibre fractions in selected tropical grasses and legumes. *Tropical Grassland*, 25, 305-312.

2- Kamra, D.N., Singh, R., Agarwal, N. and Pathak, N.N. (2000). Soapnut (Reetha) as natural defaunating agent its effect on rumen fermentation and in sacco degradability of jowar hay in buffaloes. *Buffalo Journal*, 16, 99-104.

3- Wanapat, M., Cherdthong, A., Pakdee, P., and Wanapat, S. (2008). Manipulation of rumen ecology by dietary lemongrass (*Cymbopogon citratus* stapf) powder supplementation. *Journal of Animal Science*, 86(12), 3497–3503.

4- McSweeney, C. S., Palmer, B., Mcneill, D. M., and Krause, D. O. (2001). Microbial interactions with tannins : nutritional consequences for ruminants. *Animal Feed Science and Technology*, 91, 83-93.

5- Ali, M.F., Saleh M.S., Eweedah N.M., and Mohmoud S.A. (2005). Effect of using chamomile (*Mtricaria chamomilla*) flowers as feed additives on performance of growing

lambs under desert farming systems. *Egypt Journal of Nutrition and feeds*, 8, 127-137.

6- Okuda, T. (2005). Systematics and health effects of chemically distinct tannins in medicinal plants. *Phytochemistry*, 66,2012- 2031.

7- Carulla, J.E., Kreuzer, M., Machmüller, A. and Hess, H. (2005). Supplementation of Acacia mearnsii tannins decreases methanogenesis and urinary nitrogen in forage-fed sheep. *Australian Journal of Agricultural Research*, 56, 961–970.

8- Elsa, L., Harshadrai, R., Florian, J. S., Fernando, C.S., Ana, F., Costa, A.R., Antunes, C., André, M.A., Coelho, A.V.,and Sales-Baptista, E. (2011). The Effect of Tannins on Mediterranean Ruminant Ingestive Behavior: The Role of the Oral Cavity. *Molecules* 16, 2766-2784.

9- Kariuk I.W., and Norton B.W. (2008). The digestion of dietary protein bound by condensed tannins in the gastro-intestinal tract of sheep. Animal Feed Science and Technology, 142, 197-209.

10- Garg SK , Makkar H.P , Nagal K.B , Sharma S.K , Wadhwa D.R , Singh B. (1992). Oak (*Quercus incana*) leaf poisoning in cattle. *Veterinary and Human Toxicology*, 34, 161-164.

11- Mir Ishtiyak, A., Kumar, R., Sharma, R.K. and Barman, K. (2010). Effect of addition of herbs on *in vitro* digestibility of feed with rumen liquor of goat. *Indian Journal of veterinary Resesrch*. 19(1), 13-18.

12- Vasta, V., Mele, M., Serra, A., Scerra, M., Luciano, G., Lanza, M., and Priolo, A. (2009). Metabolic fate of fatty acids involved in ruminal biohydrogenation in sheep fed concentrate or herbage with or without tannins. *Journal of Animal Science*,87, 2674–2684.

13- Váradyová, Z., Kišidayová, S., Siroka, P., and Jalč, D. (2007). Fatty acid profiles of rumen fluid from sheep fed diets supplemented with various oils and effect on the rumen ciliate population. *Czech Journal of Animal Science*, 52:(11),399–406.

14- Jitkamol, T., Surasak, J., Somkiat K., and Theera R. (2010). Fatty acid profile of ruminal fluid, plasma and milk fat of dairy cows fed soybean and sunflower oil-rich diets, without effects on milk production. *Kasetsart Journal (Natural Science)*, 44, 837-849.

15- Piras, A, Rosa, A., Marongiu, B., Porcedda, S., Falconieri, D., Dessì, M.A., Ozcelik, B, Kocae, U. (2013). Chemical composition and *in vitro* bioactivity of the volatile and fixed oils of *Nigella sativa* L. extracted by supercritical carbon dioxide. *Industrial Crops and P*roducts 46, 317– 323.

16- Sahraei, M., Pirmohammadi, R., and Payvastegan, S. (2014). The effect of rosemary (*Rosmarinus officinalis L.*) essential oil on digestibility, ruminal fermentation and blood metabolites of Ghezel sheep fed barley-based diets. *Spanish Journal of Agricultural Research*, 12:(2), 448-454.

17- Venkatachallam, S.K.T., Pattekhan, H., Divakar S., and Kadimi U.S. (2010). Chemical composition of *Nigella sativa L.* seed extracts obtained by supercritical carbon dioxide. *Journal Food Science and Technology*, 47:(6), 598–605.

18- Sarker, M. R., Mazumder, M. E., and Rashid H. (2011). *In vitro* enhancement of polyclonal IgM production by ethanolic extract of *Nigella sativa L* . Seeds in whole spleen cells of female BALB / c Mice. *Bangladesh Pharmaceutical Journal*, 14(1), 73–77.

19- Hassim, H.A., Lourenço, M., Goh, Y.M., Baars, J.J., and Fievez, V. (2012). Rumen degradation of oil palm fronds is improved through pre-digestion with white rot fungi but

not through supplementation with yeast or enzymes. *Canadian Journal of Animal Science*, 92(1): 79-87.

20- Barman K. (2004). *Biodegradation of tanniniferous feeds and their influence on nutrient utilization and productivity of the dairy animals*. Ph.D. The National Dairy Research Institute; Karnal, India.

21- Makkar, H. P. S., Blummel, M., Borowy, N. K., and Becker, K. (1993). Gravimetric Determination of Tannins and their Correlations with Chemical and Protein Precipitation Methods. *Journal of the Science of Food and Agriculture*, 61, 161–165.

22- Yusuf, A. L., Ebrahimi M., Goh, Y. M., Samsudin, A. A., Idris A.R., Alimon, A. R. and Sazili, A. Q. (2012). *In vitro* Digestibility of Diets Containing Different Parts of Andrographis paniculata Using Rumen Fluid from Goats. *Journal of animal and veterinary advances,* 11:(21), 3921-3927.

23- AOAC. (2007). *Official Methods of Analysis of the Association of Official Analytical Chemists* (18th Ed). Association of Official Analytical Chemists, Washington D.C., USA.

24- Van Soest, P. J., Robertson, J. B., and Lewis, B. A. (1991). Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *Journal of Dairy Science*, 74(10), 3583–3597.

25- Menke, K.H. and Steingass, H. (1988). Estimation of the energetic feed value obtained by chemical analysis and *in vitro* gas production using rumen fluid. Animal Research Development, 28, 7–55.

26- Tilley J.M., and Terry R.E. (1963). A two-stage technique for in vitro digestion of forage crops. *Grass and Forage Science,* 18, 104-111.

27- Solorzano L. (1969). Determination of ammonia in natural waters by the

phenolhypochlorite method. *imnology and Oceanography,*14, 799-801.

28- Ørskov, E., and McDonald, I. (1979). The estimation of protein degradability in the rumen from incubation measure- ments weighted according to rate of passage. The Journal of Agricultural Science, 92, 499–503.

29- Folch, J., Lees, M., and Sloane-Stanley, G. A. (1957). Simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry, 226, 497–509.

30- Rajion, M. A., Mclean, J. G., and Cahill, R. N. P. (1985). Essential fatty acids in the fetal and newborn lamb. Australian Journal of Biological Sciences, 38, 33–40.

31- SAS. (2003). Statistical Analysis System package (SAS) Version 9.2 software. SAS Institute Inc., Cary, NC, USA.

32- Getachew, G., Blummel, M., Makkar, H.P.S., and Becker, K. (1998). In vitro gas measuring techniques for assessment of nutritional quality of feeds: a review. Animal Feed Science and Technology, 72, 261-281.

33- Blummel, M., Steingass, H., and Becker, K. (1997). The relationship between in vitro gas production, in vitro microbial biomass yield and 15N incorporation and its implications for the prediction of voluntary feed intake of roughages. British Journal of Nutrition, 77, 911-921.

34- Blummel, M., and Becker, K. (1997). The degradability characteristics of fifty-four roughages and roughage neutral-detergent fibre as described by in vitro gas production and their relationship to voluntary feed intake. British Journal of Nutrition, 77, 757-786.

35- Soycan-önenç, S. (2016). Effect of Vitex agnus-castus on in vitro digestibility in ruminant. African Journal of Agricultural Research, 11(23), 2058–2063.

36- Moujahed, N., Bouaziz, Y., and Khelfa, A. (2013). Effects of essential oils from Rosmarinus officinalis and Thymus capitatus on in vitro rumen fermentation in sheep. In Feeding and management strategies to improve livestock productivity, welfare and product quality under climate change. H. Ben Salem and A. López Francos (eds). Zaragoza: CIHEAM/INRAT/OEP/IRESA/FAO. Options Méditerranéennes, Se ries A: Mediterranean Seminars (Vol. 107, pp. 35-38).

37- Calsamiglia, S., Busquet, M., Cardozo, P. W., Castillejos, L., and Ferret, A. (2007). Invited Review : Essential oils as modifiers of rumen microbial fermentation. Journal of Dairy Science, 90(6), 2580–2595.

38- Medjekal, S., Bodas, R., Bousseboua, H., and López S. (2017). Evaluation of three medicinal plants for methane production potential, fiber digestion and rumen fermentation in vitro. Energy Procedia 119, 632–641.

39- Busquet, M., Calsamiglia, S., Ferret, A. and Kamel, C. (2006). Plant extracts affect in vitro rumen microbial fermentation. Journal of Dairy Science, 89(2), 761–771.

40- Cobellis, G., Acuti, G., Forte, C., Menghini, L., De Vincenzi, S., Orrù, M., A. Valiani A., Pacetti, D., and Trabalza-Marinucci, M. (2015). Use of Rosmarinus officinalis in sheep diet formulations: Effects on ruminal fermentation, microbial numbers and in situ degradability. Small Ruminant Research, 126, 10-18.

41- Venkatachallam, S.K.T., Pattekhan, H., Divakar S., and Kadimi U.S. (2010). Chemical composition of Nigella sativa L. seed extracts obtained by supercritical carbon dioxide. Journal Food Science and Technology, 47(6), 598–605.

42- Sasaki, K., El, A., Kondo, S., Han, J., and Isoda, H. (2013). *Rosmarinus officinalis* polyphenols produce anti-depressant like effect through monoaminergic and cholinergic

functions modulation. *Behavioural Brain Research*, 238, 86–94.

43- Michel, C. G., El-Sayed, N. S., Moustafa, S. F., Ezzat, S. M., Nesseem, D. I., and El-Alfy, T. S. (2011). Phytochemical and biological investigation of the extracts of Nigella sativa L. seed waste. Drug Test Analysis, 3: 245–254.

44- Vatansev, H., Ciftci, H., Ozkaya, A., Ozturk, B., Evliyaoglu, N., and Kiyici, A. (2013). Chemical composition of Nigella sativa l. seeds used as a medical aromatic plant from east Anatolia region, turkey. Asian journal of chemistry, 25(10), 5490.

45- Bai, N., He, K., Roller, M., Lai, C.-S., Shao, X., Pan, M. H., and Ho, C. T. (2010). Flavonoids and phenolic compounds from Rosmarinus officinalis. Journal of Agricultural and Food Chemistry, 58(9), 5363–5367.

46- Borrás-Linares, I., Pérez-Sánchez, A., Lozano-Sánchez, J., Barrajón-Catalán, E., Arráez-Román, D., Cifuentes, A., Micol V., and Carretero, A. S. (2015). A bioguided identification of the active compounds that contribute to the antiproliferative/cytotoxic effects of rosemary extract on colon cancer cells. Food and Chemical Toxicology, 80, 215–222.

47- Yusuf, A. L. (2014). Growth performance, rumen fermentation and meat quality of Boer goats fed diets containing different parts of king of bitters (Andrographis paniculata (Burm.f.) Wall. Ex nees). PhD thesis, Universiti Putra Malaysia. Retrieved on 25 January 2015.

48- Adeyemi, K. D., Ebrahimi, M., Samsudin, A. A., Alimon, A. R., Karim, R., Karsani, S. A., and Sazili, A. Q. (2015). Influence of Carotino oil on in vitro rumen fermentation, metabolism and apparent biohydrogenation of fatty acids. Animal Science Journal, 86(3), 270–278.