



Determination of Nutritive Value of Citrus Tree Leaves for Sheep Using *In vitro* Gas Production Technique

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ABSTRACT : The nutritive values of leaves of *Citrus grandis*, *Citrus aurantium*, *Citrus oranges*, *Citrus limon*, and *Citrus deliciosa* were evaluated by chemical composition and *in vitro* gas production techniques. There were significant ($p < 0.001$) differences among citrus species in terms of chemical composition. Crude protein (CP) contents ranged from 123.0 to 148.3 g/kg DM. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) contents were varied with species in the range 219.4-355.4 and 215.0-278.8 g/kg DM respectively. Condensed tannin (CT) contents were ranged from 5.9 to 10.2 g/kg DM. The PEG addition significantly ($p < 0.001$) increased the gas production and some estimated parameters of citrus tree leaves. However, species showed variable responses to polyethylene glycol (PEG) treatment. There were also significant ($p < 0.001$) differences among species in terms of gas production and estimated parameters. The OMD and ME contents of citrus leaves without PEG supplementation were ranged from 66.5 to 73.3% and 9.8 to 10.9 MJ/kg DM respectively. The improvement in gas production, organic matter digestibility (OMD) and metabolizable energy (ME) with PEG emphasized the negative effect of tannins on digestibility. The increase (%) in the estimated OMD and ME contents ranged from 5.5 to 9.8% and 5.7 to 10.2% respectively. All citrus tree leaves studied in this experiment have potential nutritive values indicated by high crude protein content, OMD, ME and low fiber values. (**Key Words :** Citrus Leaves, Condensed Tannin, Digestibility, Metabolizable Energy, PEG)

INTRODUCTION

Shrub and tree leaves are an important component of diets for ruminant animals in the most part of animal (Holechek, 1984; Papachristou and Nastis, 1996) and play an important role in the nutrition of grazing animals in areas where few or no alternatives are available (Meuret et al., 1990). *Citrus grandis*, *Citrus aurantium*, *Citrus oranges*, *Citrus limon* and *Citrus deliciosa* are evergreen species. The annual pruning of citrus trees produce considerable amount of stem and leaves although exact quantities are very difficult to estimate. These cheap wastes obtained after pruning were used as alternative ingredients in ruminant diets with some success in some farms (Hernandez et al., 1998). The presence of tannins and other phenolic compounds in a large number of nutritionally important shrubs and tree leaves hampers their utilization as animal feed (Tolera et al., 1997). High levels of tannins in leaves

restrict the nutrient utilization and decrease voluntary feed intake, nutrient digestibility and N retention (Kumar and Vaithiyanathan, 1990; Silanikove et al., 1996a, 2001)

However the citrus leaves were undervalued mainly because of insufficient knowledge about their potential feeding value. Chemical composition, in combination with *in vitro* digestibility and ME content, can be considered useful indicators for preliminary evaluation of the potential nutritive value of previously uninvestigated shrub and tree leaves (Ammar et al., 2005). Current chemical analysis techniques do not reflect the biological effects of tannin, and therefore the use of *in vitro* techniques has been proposed to supplement the chemical analysis (Nsahlai et al., 1994). The gas production technique has been proved to be efficient in determining the nutritive value of feeds containing anti-nutritive factors (Rubanza et al., 2003; Evitayani et al., 2004; Fujihara et al. 2005; Ozkan and Sahin, 2006).

The aim of this study was to screen citrus tree leaves from five species grown in the southern Turkey to (1) quantify chemical compositions and level of condensed tannin contents of citrus leaves, (2) assess the effect of tannin activity on feed digestibility and nutrient availability

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in vitro using a polyethylene glycol (PEG) tannin bio-assay.

MATERIALS AND METHODS

Leave samples

Leaves from *Citrus grandis*, *Citrus aurantium*, *Citrus oranges*, *Citrus limon* and *Citrus deliciosa* from the annual pruning were used. Citrus tree leaves from at least 10 different trees in three experimental plots in 2005 in the southern Turkey were dried at 50-52°C using a forced air oven. Dried leave samples were ground to pass through 1 mm sieve for subsequent analysis.

Chemical analysis

Dry matter (DM) was determined by drying the samples at 105°C overnight and ash was determined by igniting the samples in muffle furnace at 525°C for 8 h. Nitrogen (N) content was measured by the Kjeldahl method (AOAC, 1990). Crude protein was calculated as N×6.25. Cell wall contents (NDF and ADF) of leaves were determined using the method described by Van Soest et al. (1991). Total condensed tannin was determined by butanol-HCl method as described by Makkar et al. (1995). Mimosa tannin was used as an external standard. All chemical analyses were carried out in duplicate in the laboratory of Department of Animal Science, Faculty of Agriculture, Kahramanmaraş Sutcu Imam University.

In vitro gas production

In the absence and presence of PEG (1 g, MW, 6000; Sigma, UK), the leave samples (0.200 g DM) were incubated *in vitro* with diluted rumen fluid (10 ml rumen fluid+20 ml culture medium) in calibrated glass syringes of 100 ml following the procedures of Menke and Steingass (1988). The aim of PEG addition was to determine the adverse effect of CT on the gas production and estimated parameters. All incubations were carried out in triplicate. Rumen fluid was obtained from two fistulated sheep fed a daily ration of 800 g alfalfa hay and 250 g concentrates divided into two equal meals at 8:00 and 16:00 h daily. The sheep had free access to water throughout the experiment. Rumen samples was collected before the morning meal in the thermos flasks and taken immediately to the laboratory where it was strained through 4 layers of cheesecloth and kept at 39°C.

Readings of gas production were recorded 0, 3, 6, 12, 24, 48, 72 and 96 h after incubation. Total gas values were corrected for blank gas production. The *in vitro* gas production kinetics was estimated fitting cumulative gas production data to the model suggested by Orskov and McDonald (1979);

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

Where,

y = gas produced at time 't'

a = the gas production from the rapid soluble fraction (ml)

b = the gas production from the slowly degradable fraction (ml)

c = the gas production rate constant for the slowly degradable fraction (h)

t = incubation time (h)

The metabolizable energy (MJ/kg DM) contents and OMD (%) values of citrus tree leaves were calculated using equations suggested by Menke et al. (1979);

$$ME \text{ (MJ/kg DM)} = 2.20 + 0.136 \text{ GP} + 0.057 \text{ CP}$$

$$R^2 = 0.94$$

$$OMD \text{ (\%)} = 14.88 + 0.889 \text{ GP} + 0.45 \text{ CP} + 0.00651 \text{ XA}$$

$$R^2 = 0.92$$

Where GP is 24 h net gas production (ml/200 mg DM)

CP: Crude protein (%)

XA: Ash content (%)

In vitro gas production measurements were carried out in the laboratory of Department of Animal Science, Faculty of Agriculture, Bursa Uludag University.

Statistical analysis

Data on chemical composition was subjected to the one way of ANOVA using GLM of SPSS for windows (2002), and were analyzed based on the statistical model: $Y_{ij} = \mu_{ij} + S_i + e_i$. Where, Y_{ij} = the general mean common for each parameter under investigation, S_i the *i*th effect of citrus tree species on the observed parameters, e_i the standard error term.

Data on *in vitro* gas production kinetics, OMD and ME contents of citrus tree leaves were subjected to the two way of ANOVA using GLM of SPSS for windows (2002), and were analyzed based on the statistical model: $Y_{ij} = \mu_{ij} + S_i + P_j + (S \times P)_{ij} + e_i$. Where, Y_{ij} is the general observation on *in vitro* gas production kinetics, OMD and ME contents, S_i the *i*th effect of citrus species on the observed parameters and P_j the *j*th effect of PEG on the observed parameters. The $(S \times P)_{ij}$ term represents *i*th and *j*th interaction effects of species and PEG on gas production and *in vitro* digestibility, and e_i the standard error term common for all observations. Significance between individual means was identified using the Duncan test. Mean differences were considered significant at $p < 0.05$. Standard errors of means were calculated from the residual mean square in the analysis of variance.

Table 1. Chemical composition (g/kg DM) of selected citrus tree leaves

| Species | CP | NDF | ADF | Ash | CT |
|-------------------------|--------|--------|---------|--------|-------|
| <i>Citrus grandis</i> | 148.3b | 355.4c | 278.8d | 122.2b | 6.9a |
| <i>Citrus aurantium</i> | 129.6a | 295.8b | 253.7bc | 104.5a | 5.9a |
| <i>Citrus oranges</i> | 146.2b | 219.4a | 240.6b | 147.8d | 10.2b |
| <i>Citrus limon</i> | 132.5a | 316.1b | 268.9cd | 131.7c | 10.1b |
| <i>Citrus deliciosa</i> | 123.0a | 219.8a | 215.0a | 170.8e | 7.4ab |
| SEM | 3.54 | 10.06 | 6.27 | 2.13 | 0.83 |
| Sig. | *** | *** | *** | *** | *** |

Column means with common superscript did not differ ($p>0.05$), SEM: Standard error of mean, CP: Crude protein, NDF: Neutral detergent fibre, ADF: Acid detergent fibre, CT: Condensed tannin, Sig: significance level, *** $p<0.001$.

RESULTS AND DISCUSSION

Chemical composition

The chemical compositions of leaves of *Citrus grandis*, *Citrus aurantium*, *Citrus oranges*, *Citrus limon* and *Citrus deliciosa* are given in Table 1. There were significant ($p<0.001$) differences among citrus tree leaves. The CP contents of citrus tree leaves ranged from 123.0 to 148.3 g/kg DM, NDF from 219.4 to 355.4 g/kg DM, ADF from 215.0 to 278.8 g/kg DM and CT content from 7.4 to 10.2 g/kg DM.

The CP contents of *Citrus grandis* and *Citrus oranges* were significantly ($p<0.001$) higher than those of *Citrus aurantium*, *Citrus limon* and *Citrus deliciosa*, whereas the NDF content of *Citrus grandis* was significantly ($p<0.001$) higher than those of *Citrus oranges*, *Citrus aurantium*, *Citrus limon* and *Citrus deliciosa*. The ADF content of *Citrus grandis* was significantly ($p<0.001$) higher than those of *Citrus aurantium*, *Citrus oranges* and *Citrus deliciosa*. The condensed tannin contents of *Citrus oranges* and *Citrus limon* were significantly ($p<0.001$) higher than those of *Citrus grandis*, *Citrus aurantium* and *Citrus deliciosa*. On the other hand the ash content of *Citrus deliciosa* was significantly ($p<0.001$) higher than those of *Citrus grandis*, *Citrus aurantium*, *Citrus oranges* and *Citrus limon*.

Variation in chemical composition among species could be partly due to genotypic factors that control accumulation of foliage nutrients (Rubanza et al., 2005). The chemical composition of *Citrus limon* was comparable with that reported by Hernandez et al. (1998) who found that the CP, NDF, ADF, ash contents were 112, 259, 169 and 165 g/kg DM respectively. The CP contents of citrus tree leaves were considerably higher than those of widely used tree leaves such as *Arbutus andrachne*, *Pistica lentiscus* and *Juniperus communis* reported by Kamalak et al. (2005a) who found that CP ranged from 74.1 to 102.4 g/kg DM. The CP content of citrus tree leaves was sufficiently high to warrant consideration of their use as protein supplement to low quality diets. Hernandez et al. (1998) reported that leaves of

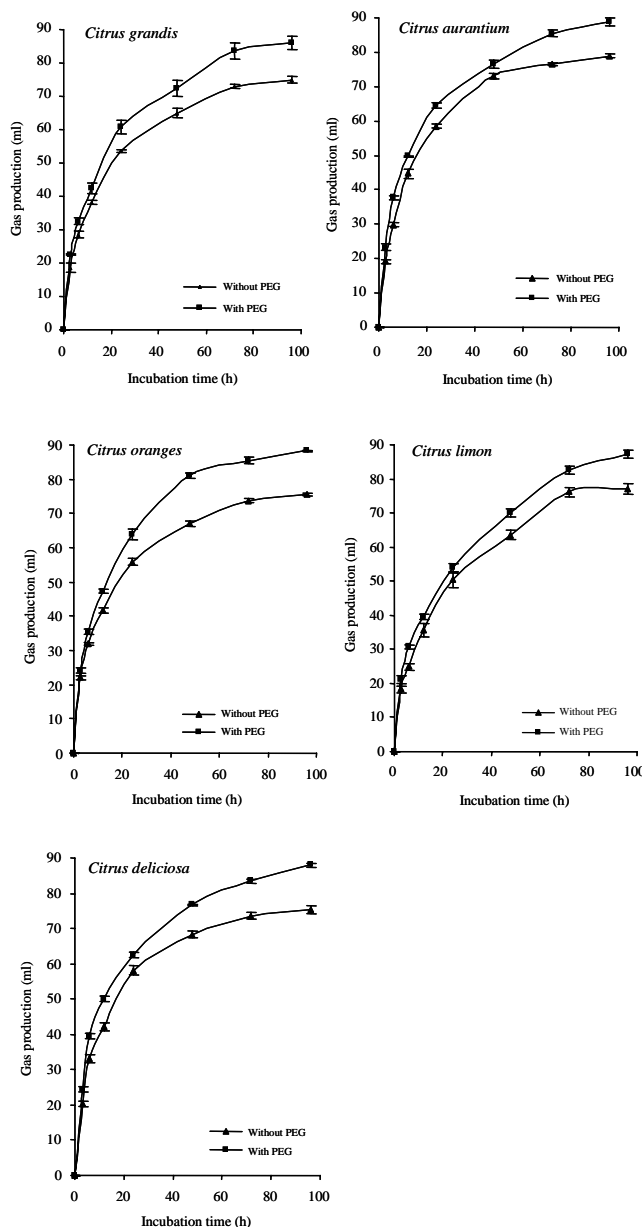


Figure 1. The effect of polyethylene glycol (PEG) on the gas production of selected citrus tree leaves.

Citrus limon can be incorporated into forage diets at high levels as a substitute for NH_3 -treated barley straw with no adverse effect on intake or digestibility. Hernandez et al. (1998) also reported that the cost of *Citrus limon* was lower than that of the ammonia treated straw.

El-Shatnawi and Mohawesh (2000) suggested that ewes require 7-9% CP for maintenance and 10-12% for lactation. It seems to be likely that citrus tree leaves studied in this experiment will meet the CP requirements of ewes for maintenance and lactation since the CP content of citrus tree leaves studied in this experiment higher than those requested for maintenance and lactation of sheep.

However, tannins in leaves may form a less digestible

complex with dietary proteins and may bind and inhibit action of the endogenous protein, such as digestive enzymes (Kumar and Singh, 1984). Tannin can thus adversely affect the microbial and enzyme activities (Singleton, 1981; Lohan et al., 1983; Barry and Duncan, 1984; Makkar et al., 1989, Silanikove et al., 1994, 1996b). In tree leaves tannins are present in NDF and ADF in significant amounts which are tightly bound to the cell wall and cell protein and seem to cause decrease in digestibility (Reed et al., 1990). However in ruminants, dietary condensed tannins (20-30 g/kg DM) have been shown to have beneficial effects because they reduce the protein degradation in the rumen by formation of a protein-tannin complex (Barry, 1987). Citrus tree leaves selected in this study had low CT contents which are ranged from 5.9 to 10.2 g/kg DM. On the other hand the CT contents of citrus tree leaves obtained in this experiment were considerably lower than those of widely used tree leaves such as *Arbutus andrachne*, *Pistacia lentiscus* and *Juniperus communis* reported by Kamalak et al. (2005a) who found that the CT ranged from 136.2 to 187.5 g/kg DM. Therefore optimal utilization of CP in browsable leaves could not be limited by low levels of condensed tannin due

to tannin activity through the chemical binding with dietary nutrients.

In vitro gas production

The *in vitro* gas production of citrus tree leaves in the absence and presence of PEG are given in Figure 1. Gas production in the presence of PEG was considerably higher than those obtained in the absence of PEG irrespective of citrus tree leaves at all incubation times. However citrus species showed variable responses on increase in gas production. Leaves of *Citrus oranges* and *Citrus deliciosa* had the highest increase in the gas production at 96 h incubation times.

The gas production kinetics of citrus tree leaves incubated in the presence and absence of PEG are given in Table 2. Although the PEG supplementation had no effect on the gas production rate (c), there are significant differences in the gas production rate among citrus trees. *Citrus deliciosa* had the highest gas production rate in the citrus tree leaves in the absence and presence of PEG. The species and PEG supplementation resulted in increase in the gas production (a) from soluble fraction, gas production (b)

Table 2. The effect of polyethylene glycol (PEG) and species on the gas production kinetics

| Species | c | | a | | b | | a+b | |
|---------------------|------------------|-------|-------|-------|-------|-------|-------|---------|
| | PEG | | PEG | | PEG | | PEG | |
| | - | + | - | + | - | + | - | + |
| <i>C. grandis</i> | 5.8b | 5.5b | 4.4bc | 5.7a | 68.3a | 77.3a | 72.7a | 83.5ab |
| <i>C. aurantium</i> | 6.7c | 6.9d | 3.1a | 5.3a | 73.8c | 79.1a | 76.9b | 84.4abc |
| <i>C. oranges</i> | 6.9cd | 6.3c | 5.2c | 5.6a | 67.5a | 80.4b | 72.7a | 86.1c |
| <i>C. limon</i> | 4.3 ^a | 4.1a | 5.4c | 7.7b | 71.6b | 78.01 | 77.0b | 85.8bc |
| <i>C. deliciosa</i> | 7.4d | 7.3d | 3.8b | 5.7a | 68.9a | 77.1a | 72.7a | 82.8a |
| SEM | 0.194 | 0.156 | 0.339 | 0.373 | 0.499 | 0.651 | 0.559 | 0.733 |
| Sig. | *** | *** | *** | *** | *** | *** | *** | *** |
| Species | | *** | | *** | | *** | | *** |
| PEG | | NS | | *** | | *** | | *** |
| Species×PEG | | NS | | NS | | *** | | *** |

Column means with common superscript did not differ ($p>0.05$), a: the gas production from the rapid soluble fraction (ml), b: the gas production from the insoluble fraction (ml), c: the gas production rate (ml/h) for the slowly degradable fraction (b), a+b: potential gas production (ml), SEM: Standard error of mean, Sig: significance level, *** $p<0.001$, NS: Non-significant.

Table 3. The effect of Polyethylene glycol (PEG) and species on the organic matter digestibility (OMD) and metabolizable energy (ME)

| Species | OMD | | | ME | | |
|---------------------|-------|--------|------------|------------------|--------|------------|
| | PEG | | % Increase | PEG | | % Increase |
| | - | + | | - | + | |
| <i>C. grandis</i> | 70.0b | 76.3b | 8.9 | 10.3b | 11.3b | 9.3 |
| <i>C. aurantium</i> | 73.3c | 78.6c | 7.1 | 10.9c | 11.7c | 7.3 |
| <i>C. oranges</i> | 72.1c | 79.2c | 9.8 | 10.6c | 11.7c | 10.2 |
| <i>C. limon</i> | 66.5a | 69.6a | 6.9 | 9.8 ^a | 10.3a | 7.4 |
| <i>C. deliciosa</i> | 73.2c | 77.2bc | 5.5 | 10.8c | 11.4bc | 5.7 |
| SEM | 0.608 | 0.683 | 1.182 | 0.09 | 0.104 | 1.213 |
| Sig. | *** | *** | NS | *** | *** | NS |
| Species | | *** | | | *** | |
| PEG | | *** | | | *** | |
| Species×PEG | | * | | | NS | |

Column means with common superscript did not differ ($p>0.05$), PEG: Polyethylene glycol OMD: Organic matter digestibility (%), ME: Metabolizable energy (MJ/kg DM), SEM: Standard error of mean, Sig: significance level *** $p<0.001$; NS: Non-significant.

Table 4. Correlation coefficient (r) relationship of chemical composition with gas production at 24 h our incubation and some estimated parameters

| Parameters | Chemical constituents | | | | |
|--------------------|-----------------------|---------------------|---------------------|---------------------|----------------------|
| | CP | NDF | ADF | Ash | CT |
| Gas _{24h} | -0.354 ^{NS} | -0.539* | -0.662* | 0.141 ^{NS} | -0.574* |
| c | -0.177 ^{NS} | -0.666* | -0.739** | 0.362 ^{NS} | -0.410 ^{NS} |
| a | 0.423 ^{NS} | 0.018 ^{NS} | 0.252 ^{NS} | 0.229 ^{NS} | 0.814*** |
| b | -0.518* | 0.315 ^{NS} | 0.210 ^{NS} | -0.621* | -0.362 ^{NS} |
| a+b | -0.386 ^{NS} | 0.352 ^{NS} | 0.342 ^{NS} | -0.578* | -0.037 ^{NS} |
| ME | -0.234 ^{NS} | -0.515* | -0.610* | 0.111 ^{NS} | -0.561* |
| OM | -0.220 ^{NS} | -0.546* | -0.636* | 0.158 ^{NS} | -0.530* |

from slowly degradable fraction and the potential gas production (a+b). The potential gas production of *Citrus aurantium* and *Citrus limon* obtained in the absence of PEG were significantly ($p < 0.001$) higher than those of *Citrus grandis*, *Citrus oranges* and *Citrus deliciosa*.

The estimated OMD and ME contents of citrus tree leaves incubated in the presence and absence of PEG are given in Table 3. There were significant ($p < 0.001$) differences in the estimated OMD and ME contents of leaves incubated in the presence and absence of PEG among citrus species. The species and PEG supplementation had a significant ($p < 0.001$) effect on the estimated OMD and ME contents. The estimated OMD and ME contents of *Citrus aurantium* and *Citrus oranges* were significantly ($p < 0.001$) higher than those of *Citrus grandis* and *Citrus limon*. The PEG supplementation increased the estimated OMD and ME contents but increase in OMD and ME contents is not statically significant ($p > 0.05$) among citrus trees. Species showed variable responses on increase in OMD and ME. The increase in the estimated OMD and ME contents due to PEG supplementation ranged from 5.5 to 9.8% and 5.7 to 10.2% respectively. The OMD of *Citrus limon* obtained in this experiment was consistent with that reported by Hernandez et al. (1998) who found that OMD of *Citrus limon* for goat was 68.5%.

Correlation coefficient (r) relationship of chemical composition with gas production at 24 h our incubation and some estimated parameters are given in Table 4. Gas production at 24 h incubation, gas production rate (c), ME and OMD were negatively correlated with NDF, ADF and CT contents of citrus tree leaves. This result was in agreement with finding of Abdulrazak et al. (2000), Kamalak et al. (2005b).

The increase in the gas production and estimated parameters with PEG emphasizes the negative effect of tannins on digestibility. PEG, a non-nutritive synthetic polymer, has a high affinity to tannins and makes tannins inert by forming tannin PEG complexes (Makkar et al., 1995). The PEG can liberate protein from the preformed tannin-protein complexes (Barry et al., 1986). Some studies clearly showed that PEG supplementation increased the gas production and volatile fatty acid production (Getachew et

al. 2002; Seresinhe and Iben, 2003). The adverse effects of tannin on digestibility of *Acacia* spp. and *Dichrostachys* spp. have been demonstrated by Makkar et al. (1995) and Getachew et al. (2000). The increase in the gas production, their kinetics, OMD and ME in the presence of PEG is possibly due to an increase in the available nutrients to rumen micro-organisms, especially carbohydrates and nitrogen. McSweeney et al. (1999) showed that addition of PEG caused a significant and marked increase in the rate and extent of ammonia production. It was also shown that supplementation of PEG at a level of 25 or 50 g per day to goat fed lentils leaves and concentrate markedly increased *in vivo* DM, OMD and protein digestibility (McSweeney et al., 1999). The leaves of citrus tree did not give the same response for PEG supplementation possible due to differences in the tannin structure and function of citrus trees. The reason why leaves of tree give the different response is the differences in chemical composition of tannins, the variation in tannin anti nutritive activity between foliage species, the nature of tannin and chemical structure (Dalzell and Kerven, 1998) and degree of polymerization (Schofield et al., 2001). The effect of PEG also depends on the level of proteins in diet. The higher the level of proteins the lesser is the effect of PEG (Makkar and Becker, 1996). The improvements in OMD and ME were lower than those found by Rubanza et al. (2003, 2005).

In tree leaves CT can be found in the soluble and bound form (Kamalak et al., 2005a). The CT of citrus tree leaves possibly is in the bound form. Therefore there is less soluble condensed tannin to bind with rapid soluble fraction of citrus leaves. As a result positive correlation between CT and gas production (a) from rapid soluble fraction could have been obtained in the current study.

IMPLICATIONS

Citrus tree species have potential nutritive values indicated by high crude protein content, OMD, ME and low fiber values. Citrus tree leaves had the low level of condensed tannin content which may be beneficial for ruminant nutrition although the improvement in gas production, digestibility and metabolizable energy contents

of citrus tree leaves due to addition of PEG suggest a negative effect of condensed tannin on digestibility and represent recovered feed nutrients. However, before large scale implementation of PEG, further investigations are recommended to determine the effect of CT on feed intake, animal performance and profitability of supplementation. The results of the present study should be valuable to farmers because it provides information concerning the chemical composition, OMD and ME contents of citrus tree leaves so that farmers can make full use of citrus tree leaves.

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