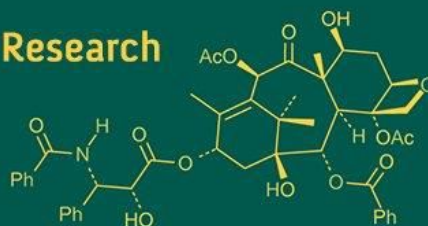
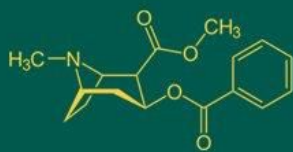


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Wisam Salim Al-Jumaili
 Department of Animal
 Production, College of
 Agriculture, Al-Qasim Green
 University, Iraq

Evaluation of dietary guava (*Psidium guajava* L.) leaf extract as a supplement for *in vitro* rumen gas production and fermentation of sheep

Wisam Salim Al-Jumaili

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Abstract

This study aims to evaluate the effect of guava leaf methanolic extract on *in vitro* rumen gas production, pH, NH₃-N, and VFA concentration. Guava leaves were harvested from the University of Putra Malaysia farms and used to prepare guava leaf methanolic extract (GLE). The experimental diets were Ctrl, T200, T300, and T600, which included 0, 200, 300, and 600 mg GLE/Kg of diet, respectively. Six replicates of 200 mg diet samples from each treatment were put in 100 ml syringes with 20 ml of incubation liquid and incubated at 38°C for 24 hours. Gas production was recorded at times 2, 4, 6, 8, 10, 12, and 24 hrs. of incubation, while the pH, NH₃-N, and VFAs were determined in the rumen liquor at the end of the incubation. Gas production significantly ($p < 0.05$) reduced in 2, 4, and 24 hrs of incubation and gas production rate. No significant differences were recorded in the total VFA's, Acetic, Propionic, acetic/ propionic ratio, Butyric, and the pH of rumen liquor. In contrast, a significant ($p < 0.05$) increase in the level of NH₃-N was noted in T300 compared to other treatments. With good availability of NH₃-N and no effect on different characteristics of the rumen, using 300 mg of guava leaf methanolic extract per Kg of sheep diet was the best result.

Keywords: Guava leaf, extract, *in vitro*, methane, gas production, rumen fermentation

Introduction

The shreds of evidence of the contribution of livestock to methane emission globally became an issue, with 71% of global methane emissions coming from ruminants (Gerber *et al.*, 2013) [17]. The contribution of ruminants to methane emissions is about 6% of global greenhouse anthropogenic emissions of gas (Beauchemin *et al.*, 2020) [6]. Furthermore, increasing methane production in ruminants is closely related to a decrease in the merit of feed conversion of rumen (Woghorn *et al.*, 2011) [38]. Methane production accounts for a 2-12% loss of dietary gross energy in ruminants (Johnson & Johnson, 1995) [21]. With a global warming potential 23 times higher than carbon dioxide, methane is considered a potent greenhouse gas in terms of the retention of warming (Bhatta *et al.*, 2015; IPCC, 2007) [9, 30]. Then, the concept of decreasing gas and methane emissions from ruminants is growing over time. To enhance the efficiency of feed energy utilization while decreasing rumen methanogenesis, it is necessary to distinguish feed additives that have the potential to modify rumen fermentation (Bhatta *et al.*, 2013) [10]. However, the byproducts of many plants, especially medical plant extracts, have the impact of manipulating rumen fermentation and reducing methane production (Beauchemin *et al.*, 2020) [6]. The reduction of methane production that occurs when adding a plant's secondary metabolites is associated with changes in the rumen environment because of its content of active substances that serve as an antibiotic as well as an antioxidant (Benchaar *et al.*, 2011; Rofiq *et al.*, 2021; Morales-Quintana *et al.*, 2022) [8, 32, 29]. Feed supplements reduce methane emissions in ruminants, which minimizes energy loss as methane emission increases energy utilization efficiency (Bueno *et al.*, 2015; Jayanegara *et al.*, 2015; Pinski *et al.*, 2015) [14, 20, 31]. According to their polyphenolic nature, some *in vitro* studies have manifested that plants' secondary metabolites can interfere with rumen methanogenesis (Jafari *et al.*, 2019; Buccioni *et al.*, 2015; Buccioni *et al.*, 2011) [19, 12, 11]. Guava leaf extract has gained interest for its potential effects on rumen characteristics in ruminant animals. Previous studies suggest that the bioactive compounds

Corresponding Author:
 Wisam Salim Al-Jumaili
 Department of Animal
 Production, College of
 Agriculture, Al-Qasim Green
 University, Iraq

present in guava leaves, including tannins and flavonoids, can influence microbial populations, fermentation processes, and the biohydrogenation of the rumen (Akinbode *et al.*, 2024) [2]. Therefore, current study objective is to evaluate the effect of guava leaf methanolic extract (GLE) on *in vitro* rumen gas production, pH, NH₃-N, and VFA concentration.

Materials and Methods

Guava leaves were harvested from the farms of the University of Putra Malaysia. These leaves were minced (2-3) cm and then relocated to the lab. The leaf samples were desiccated in a 50°C oven for (72) hours and then passed to a 1-mm sieve. By using a 10-volume (v/w) of 80% methanol, 250 g of the powder of guava leaves has extracted, and this process was thrice repeated (Lee *et al.*, 2009) [26].

Six Kacang-crossbred goats (rumen-fistulated) with a weight of 38± 0.90 kg were used to collect rumen fluid. The University of Putra Malaysia Animal Care and Use Committee approved all animal management and sampling procedures. The diet that was fed to the animals (twice daily) contained equal amounts of concentrate and alfalfa hay (50:50, w/w). Guava leaves were harvested from the University of Putra Malaysia farms and used to extract the (GLE). The gas production (*In vitro*) was estimated following the method explained by Fievez *et al.* (2005) [16], as a measured 30 mL of rumen liquor buffered solution was divided into each 100 mL plastic syringe. Each syringe contained 200 mg of feed sample containing equal amounts of concentrate and alfalfa hay (50:50, w/w). The experimental treatments were designed according to (Al-Jumaili *et al.*, 2017; Anyanji *et al.*, 2013;) [3, 4] with different levels of GLE: 0, 200, 300, and 600 mg/kg DM of feed, and the treatments named Ctrl, T200, T300, and T600, respectively. 6 Replicates/treatment were used for the

incubation - *in vitro*-. At (39 °C), all syringes were incubated for 24 hours. For the calibration of the layout of gas production -*in vitro*-, rumen-buffered liquid was only incubated at the same run. The movement of the piston of each syringe by the incubation time was used to record the gas production at 2, 4, 6, 8, 10, 12, and 24 hours.

For the analysis of CH₄, a chromatograph of gas (Agilent 5890 Series Gas Chromatograph, Wilmington, DE, USA) which equipped to a thermal-conductive detector was used by transferring a sample of the gas from the headspace of each syringe at the end of incubation time. Using an electrode of (Mettler-Toledo Ltd. England), the pH of each syringe content was recorded at the end of incubation. The concentrate values of the VFAs (Acetic, Propionic, and Butyric acid) were determined according to Barnett & Reid (1957) [5], explained, and modified by Wang *et al.* (2020) [40]. The concentrations of NH₃-N (mg/dL) have been determined by following the method described by Roy *et al.* (2014) [33]. The recorded data analyses, were managed by SAS (version 9.4; SAS Institute Inc., Cary, NC) using a (Completely Randomized Design). The repeated measures (ANOVA) were used to analyze gas production, sampling at various incubation times. The values (*p*<0.05) were considered significant. To check data for normality, SAS ver. 9.4 (PROC UNIVARIATE) has been used.

Results and Discussion

The effect of GLE on gas production is shown in Table (1). There were no significant effects on gas production except the significant (*p*<0.05) reduction at times 2, 4, and 24 hours of incubation and the gas production rate in the groups of treatment compared to the Ctrl group. Furthermore, a significant (*p*<0.05) reduction was noted in methane production for treatment groups compared to the Ctrl group.

Table 1: Effect of GLE on *in vitro* gas (mL/25 mg DM) production (mean ± SE)

Incubation Time \hrs	Treatment				
	Ctrl	T200	T300	T600	Significance
2	3.333 ±0.56 a	2.500 ±0.72 a	1.667 ±0.17 b	2.667 ±0.71 a	*
4	8.000 ±0.86 ab	8.500 ±0.34 a	5.333 ±1.15 c	5.833 ±0.31 bc	*
6	10.500 ±0.72	10.167 ±0.17	9.333 ±0.88	9.500 ±0.22	N.S
8	13.000 ±0.93	13.000 ±0.58	10.833 ±0.91	11.167 ±0.48	N.S
10	15.167 ±1.05	15.000 ±0.58	12.667 ±1.15	13.167 ±0.48	N.S
12	16.000 ±1.21	15.667 ±0.71	13.500 ±0.85	14.167 ±0.40	N.S
24 (Total)	27.500 ±1.86 a	25.500 ±1.06 ab	22.000 ±1.18 b	23.000 ±0.86 b	*
Rate (mL/h)	1.148 ±0.08 a	1.062 ±0.04 ab	0.915 ±0.05 b	0.958 ±0.04 b	*
CH ₄ (mL/100ml gas)	7.596±0.245a	6.025±0.260b	5.591±0.278b	4.241±0.300c	*

Different letters in each row mean significant differences (*p*<0.05); N.S - not significant.

Similar to the results shown in this study, many previous studies revealed that plant secondary metabolites decreased the production of gas and methane emissions in ruminants Ku-Vera *et al.*, (2020) [24]. Reducing methane emissions and total gas production reveals evident improvement in the performance of ruminants (Vyas *et al.*, 2018; Ungerfeld, 2018) [37, 36]. By decreasing energy loss in gases, the inhibited CH₄ emission may induce the worthiness of converting digestible energy to metabolizable energy (Johnson & Johnson, 1995) [21]. H₂ is the source of the energy of the rumen

microflora; most of the H₂ in the rumen is lost in CH₄ emission, and the energy savings by reducing CH₄ come from saving an additional quantity of H₂ in the rumen Beauchemin *et al.*, 2020 [6].

Reduced rumen CH₄ production in this study could be linked to the role of bioactive components of GLE, which could reduce CH₄ because they inhibit the degradation of fiber and reduce the populations of methanogenic and protozoa (Bhatta *et al.*, 2012) [11]. Protozoa can synergistically provide hydrogen as a source of electrons to the methanogenic archaea, and hence, antiprotozoal effects of different PSM may expected to reduce CH₄ emission connected to protozoa activity (Bhatta *et al.*, 2013) [10]. About 36% of CH₄ emission was reduced by using the methanolic extract of Mango leaves at a level (of 0.5 ml/30 ml) in a study by Kumar *et al.* (2011) [25], in which, they assigned their results to the content of polyphenols in the samples leaf. Recently, studies interested in using plant extracts enriched with PSM as an additive of feed to

dominate production due to their efficiency as antimicrobial and antiprotozoal (Wischer *et al.*, 2013; Kamra *et al.*, 2012; Halim *et al.*, 2011)^[43, 22, 18].

Table 2: Effect of GLE on *in vitro* fatty acids (mM) production (mean $\pm$ SE)

Fatty Acids	Treatment				Significance
	Ctrl	T200	T300	T600	
Acetic	46.156 $\pm$ 1.38	49.823 $\pm$ 1.46	50.168 $\pm$ 1.56	49.657 $\pm$ 1.69	N.S
PROPI ONIC	30.738 $\pm$ 0.68	30.151 $\pm$ 0.72	29.600 $\pm$ 0.77	30.154 $\pm$ 0.83	N.S
Ac/Prop	1.511 $\pm$ 0.09	1.659 $\pm$ 0.09	1.731 $\pm$ 0.10	1.658 $\pm$ 0.11	N.S
Butyric	12.760 $\pm$ 0.39	11.453 $\pm$ 0.41	11.659 $\pm$ 0.44	11.823 $\pm$ 0.48	N.S
Total	930.757 $\pm$ 68.15	889.372 $\pm$ 72.28	1028.32 $\pm$ 77.27	933.596 $\pm$ 83.47	N.S

Different letters in each row mean significant differences ($p < 0.05$); N.S - not significant.

Table 2 shows the effect of GLE on *in vitro* fatty acid production. The addition of GLE to the diet had no significant effects.

In this study, the reduction of gas and methane did not affect the availability of VFAs, which are considered an energy source of rumen microflora (Wang *et al.*, 2020)^[40]. The

volatile fatty acids are defined as an end product of rumen microbic fermentation that provides ruminants with an essential supply of (ME) metabolizable energy; therefore, the reduction of their production might be disadvantageous for the ruminant fermentation process (Busquet *et al.*, 2006)^[15].

Table 3: Effect of GLE on *in vitro* rumen characteristics (mean $\pm$ SE)

Rumen Characteristics	Treatment				Significance
	Ctrl	T200	T300	T600	
pH	6.887 $\pm$ 0.124	6.820 $\pm$ 0.131	7.067 $\pm$ 0.140	6.983 $\pm$ 0.152	N.S
NH ₃ -N (mg/dl)	22.444 $\pm$ 0.224ab	21.781 $\pm$ 0.237b	23.086 $\pm$ 0.253a	22.051 $\pm$ 0.274b	*

Different letters in each row mean significant differences ($p < 0.05$); N.S - not significant.

The effect of the GLE on *in vitro* rumen characteristics is shown in Table (3). Using GLE did not affect the pH of the rumen liquor, whereas there was a significant ($p < 0.05$) increase in NH₃-N of T300 compared to the other treatments and Ctrl Group. NH₃-N is regarded as the rumen's most important provider of nitrogen for the synthesis of microbial protein (Wanapat, *et al.*, 2008)^[39]. In the study of Al-Jumaili (2017)^[3], the concentration of NH₃-N was not affected by the supplement of tannic acid. Conversely, the inclusion of oil palm leaf extract in the incubation *-in vitro* decreased the concentration of NH₃-N (Aiman-Zakaria *et al.*, 2017)^[1], and these results were attributed to the formation of the tannin-protein complex. Wei *et al.* (2012)^[42] have revealed; the addition of tea-saponin, the leaf extract of mulberry, and Ecdysterone didn't cause any differences of fermentation traits such as pH, NH₃N, total and individual VFAs, and acetic/propionic acid ratio at 28 h of *in vitro* incubation. Previous studies reported that including hydrolyzable tannin as a feed supplement (< 20 g/kg DM) didn't affect the ruminants' production performance (Śliwinski *et al.*, 2002; Krueger *et al.*, 2010; Toral *et al.*, 2011)^[34, 23, 35].

Conclusions and Recommendation

According to the results of decreasing gas and methane emission, and non-significant effect on VFA concentration by using GLE as a supplement. In addition to the increase in the availability of NH₃-N as a nitrogen source for microbial protein synthesis. It can be concluded that using GLE as a dietary supplement in ruminants could be an appropriate solution to solve the problems of losing energy in the rumen due to methane and gas emissions. Further research is recommended to study the effect of GLE on the rumen microflora population and on ruminant production performance.

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Novelty statement

The inclusion of plant extracts as a feed supplement as a solution to reduce methane and gas production and to production performance has been conducted recently. The whole guava leaves have been used and evaluated for the same purpose. The extract of Guava leaves has been tested for medical issues. However, Guava leaf extract has never been evaluated before as a feed supplement in ruminants as a solution to reduce methane emission and save the rumen-produced energy.

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