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Original Article

Effect of *Camellia Sinensis*, *Punica Granatum*, and *Quercus Persica* Extracts on *in vitro* Fermentation Parameters

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Abstract

This experiment was conducted to study the effects of aqueous and alcoholic extracts of *Camellia sinensis* (L.) Kuntze leaves (green tea), *Punica granatum* (pomegranate-peel), and *Quercus persica* Jaub. & Spach (oak) at different concentrations on ruminal fermentation, dry matter and organic matter digestibility, methane production and protozoa population using gas production method. Experimental treatments were: control, 50, 100, and 200 µg/ ml aqueous and methanolic extract of *Camellia sinensis*, *Punica granatum*, and *Q. persica* (19 treatments in total). Cumulative gas production was recorded at 2, 4, 6, 8, 12, 24, 36, 48, 72, and 96 h after incubation. Dry matter digestibility (DMD), organic matter digestibility (OMD), metabolizable energy (ME), pH, and short-chain fatty acids (SCFA) were calculated after 24 h incubation. Gas production at different times, methane production, and protozoa population were also measured. DMD, OMD, and pH were decreased by adding extracts. Microbial mass production (MCP) and microbial mass production efficiency (EMCP) significantly increased at a low level (50 µg/ ml) and significantly decreased at high levels of extracts containing tannins (100 and 200 µg/ ml) ($P< 0.01$). The treatments also increased short-chain fatty acids (SCFA), reduced methane concentration, and reduced PF and protozoa populations only at the highest levels of extracts ($P<0.05$).

Keywords: Aqueous and alcoholic extracts, *Camellia sinensis*, *Punica granatum*, *Quercus persica*, Fermentation parameters, Methane, Protozoa

Introduction

Fermentation in the rumen causes wasting energy as methane and nitrogen as ammonia, which limits animal production performance and, on the other hand, releases environmental pollutants. Ruminant animals have been implicated as a significant source of enteric methane production to the greenhouse effect. In recent decades, additives such as antibiotics, ionophores, methane inhibitors, and antiprotozoal agents have been successfully used to reduce this wastage of energy and nitrogen in the rumen, increasing production efficiency and reducing metabolic disorders [1, 2]. However, due to the risk of antibiotic residues in meat and milk, as well as bacterial strain resistance, the use of these compounds has decreased in recent years. [3]. For this reason, many countries have banned their use since 2006 [3]. As a result, nutritionists are looking for better and less risky alternatives such as herbal extracts that improve ruminal fermentation with fewer side effects. Many studies have been done on using essential oils of various medicinal plants to manipulate and improve ruminal fermentation. Medicinal plants contain various chemical compounds such as saponins [4, 5], flavonoids [6], tannins [7-9], and essential oils [1, 10, 11]. These compounds have been isolated from a wide range of plant species and investigated for their effects on rumen fermentation and animal productivity improvement. Studies have shown that these compounds are useful for reducing methane production in ruminants [12]. Essential oils of medicinal plants have nutritional and environmental effects [13]. One of these is the anti-nutritional substances found in essential oils, such as enzymatic inhibitors, saponins, lectins, tannins, polyphenols, phytic acid, and oxalates. Compounds such as saponin, tannin, and lectin reduce protein digestibility and efficiency [14]. Tannins are polyphenols present in plants, foods, and beverages, and are of significant economic and ecological interest. They are water-soluble and with molecular weights ranging between 500 and 3000 Daltons [15]. One of the most

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well-known properties of phenolic compounds, including tannins, is their ability to bind to positively charged groups in the structure of proteins, amino acids, multivalent cations, and minerals such as iron, zinc, and calcium. The binding can result in very insoluble salts with poor bioavailability of minerals [16]. The tendency of tannins to form a protein complex in the rumen reduces protein degradation [17] and subsequently reduced ammonia-nitrogen production and increased intestinal ammonia nitrogen flow [17]. On the other hand, tannins decrease methane production by decreasing the population of methanogens and protozoa. Therefore, the use of tannin in ruminant feeds can also have a positive impact on the environment by reducing methane production and nitrogen oxide (These two gases, along with carbon dioxide, are considered the three major greenhouse gases) [18].

P. granatum is rich in tannic acid so that tannins make up 25% of the water-soluble constituents of *P. granatum* leaves [19]. *P. granatum* extract has a high antioxidant capacity due to its high phenolic compounds [20]. Using 1 to 4% of its extract improved the digestibility of crude protein, dry matter, and digestible fiber in neutral detergent and milk production in dairy cows [21]. The Food and Agriculture Organization (FAO) estimates the area of Iranian forests to be 12 million hectares, 55 percent of which are *Q. persica* (oak) species. So, approximately 6 million hectares of forests are covered by various *Q.* species, mainly dominated by *Q. persica*, *Q. infectoria*, and *Q. libani* species. *Q. persica* has been reported to contain high levels of hydrolyzable tannins [22] and also a high content of starch which makes it an alternative and cautionary feed for ruminants [23].

The tea plant *C. sinensis* is grown in about 30 countries worldwide, and it's estimated that the area under tea cultivation in northern Iran is about 32,000 hectares. Tea has a high content of catechins and other polyphenols and exhibits powerful antioxidant activities [24]. According to our knowledge, no study has been conducted to compare the alcoholic and aqueous extracts of medicinal plants selected in this study and their effects on methane production and the protozoa population. Hence, the main aim of this study was to investigate the effect of adding different levels of aqueous and methanolic extracts of *C. sinensis* leaves (green tea), *P. granatum*, and *Q. persica* on digestibility, gas production, and *in vitro* rumen fermentation parameters and protozoa population.

Material and Methods

Sample Collection

C. sinensis samples were collected from Lahijan tea gardens, *P. granatum* samples from Gonbad juices and oak fruit (*Q. persica* sp.) were obtained from Minoodasht highlands in the north of Iran. *C. sinensis* leaves, *Punica granatum*, and *Q. persica* seeds (after removing the woody coating) were utilized in this experiment. All tested plant species were deposited at the Herbarium of the Botany Laboratory, Faculty of Agriculture, Gonbad Kavous University, under accession numbers GKU803923 (*C. sinensis*), GKU803921 (*P. granatum*), and GKU803922 (*Q. persica*).

Extraction Method

The samples were dried in an oven at 60 °C for 48 h. Samples were then ground using a grinding mill to pass a 1-mm screen. The extracts were prepared by soaking in distilled water and 95% methanol solvents. 100 ml of solvent was added to 10 g of powdered sample and the mixture was stirred for 24 h at room temperature with a magnetic stirrer. The solid part was then separated by filter paper [25]. The methanol extract was concentrated by rotary evaporation at 40 °C and stored in the refrigerator until use.

Determination of Condensed Tannins

Different concentrations of catechin solutions were prepared with water to measure condense tannins. The reaction is started by adding 0.5 ml of the test extract into the test tube and then 3 ml of butanol hydrochloric acid reagent and 100 ml of ferric ammonium sulfate reagent were added. The lid of the test tubes was closed with aluminum foils and metal clasps and placed in the oven at 100 °C for 1 hour. The tubes were then cooled and absorbed at 550 nm. To make the control solution, pour 0.5 ml methanol into a tube, then add 3 ml butanol chloride and 100 ml ammonium ferric sulfate [26].

Gas Production Procedure

Experimental treatments included: 1 or control treatment (without addition of extracts), 2 to 4 treatments (control + 50, 100, 200 µg/ ml aqueous extract of *C. sinensis*), 5 to 7 treatments (control + 50, 100 and 200 µg/ ml methanol extract of *C. sinensis*) and 8 to 10 treatments (control + 50, 100, 200 µg/ ml aqueous extract of *P. granatum*), 11 to 13 treatments (control + 50, 100, 200 µg/ ml methanol extract of *P. granatum*), treatments 14 to 16 (control + 50, 100, 200 µg/ ml aqueous extract of *Q. persica*) and 17 to 19 treatments (control + 50, 100, 200 µg/ ml of methanol extract of *Q. persica*). The gas production test was performed according to the method of [27]. The basal diet based on dry matter consisted of 34% barley grain, 30% corn, 8% soybean meal, 12% cotton seed meal, 5% sugar beet pulp, 10% wheat bran, 0.3% calcium carbonate, 0.2% salt and 0.5% mineral-vitamin supplement.

Three fistulated sheep of the Dalaq breed (average body weight of 45 ± 1 kg, SD) were used. The sheep were housed in individual cages, fed at the maintenance level (approximately 1.5 kg) with free access to drinking water. The fistulated sheep were fed daily in the morning and the afternoon (0800 and 1600). Ruminal fluid was obtained from fistulated sheep before morning feeding

and was filtered with a four-layer cloth. The filtered rumen fluid was bubbled with carbon dioxide and incubated at 38.6 °C to establish anaerobic conditions. Artificial saliva was prepared according to the method of Menke *et al*. and mixed with rumen fluid at a ratio of 2: 1 (rumen: buffer), and 30 ml of the mixture was added to glass vials containing 200 mg sample or control [28]. Immediately, each vial was bubbled with carbon dioxide for 10 s and sealed using rubber stoppers and aluminum cover, and incubated in a shaking water bath at 38.6 °C. Three blanks were used to correct the gas produced by the particles left in the rumen fluid. The produced gas was recorded at 2, 4, 6, 8, 12, 24, 36, 48, 72, and 96 hours after incubation. Cumulative gas production was determined according to [29]. Gas production parameters were estimated as described by [30]:

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

Where:

y: the gas produced at the time of incubation

b: gas production from an insoluble fermentable fraction

e: Euler's number

c: gas production rates for b

t: incubation time

The OMD, ME, and SCFA were calculated based on the following equations [28, 31].

OMD (%) =14.88+ 0.889 GP+ 0.45 CP + 0.0651 XA

ME (MJ/kgDM) = 2.20 + 0.136 GP + 0.057 CP + 0.0029 CF

SCFA (mmol) = 0.0222 GP- 0.00425

Where:

OMD: organic matter digestibility

ME: metabolizable energy

SCFA: short-chain fatty acids

GP: Net gas production after 24 hours (per 200 mg sample dry matter)

CP: crude protein (%)

XA: ash (%)

CF: crude fiber (%)

In vitro Digestibility of Dry Matter and Organic Matter

In regards to rumen fluid preparation, as well as the basal diet and treatments, this study was similar to the gas production test. After filtering with a 4-layer cloth under anaerobic circumstances, the rumen fluid was transferred to the laboratory and its pH was adjusted to 6.8 with a buffer. Then, 50 ml of artificial saliva was poured into glass vials containing 500 mg of a basal diet based on dry matter. The vials were then sealed with a plastic cap and aluminum cover and incubated in a water bath at 38.6 ° C for 24 hours. At the end of incubation, the pH of the samples was measured using a pH meter (Model 691, Metrohm Company). The contents of the bottles were filtered using a nylon cloth (42-mm pore size) to determine the disappearance of dry matter. The residue was dried in an oven at 60 °C for 48 hours and the apparent dry matter digestibility was calculated. Then the residual dry matter was placed into the oven at 550 °C for 5 h and ash content was calculated.

Estimates of gas production efficiency were calculated based on the following equation [32].

$G_y = GP_{24}$/ (0.5- Dry matter weight after oven drying)

Where:

G_y= Gas production efficiency

GP_{24}= the gas production after 24 h of incubation

The microbial mass production was estimated using the following equation [32].

MCP (mg) = (GP× PF) - 2.2

Where:

MCP= Microbial mass production

GP= pure gas Production after 24 hours (ml)

PF= Partitioning factor (mg of organic matter digested/ ml of pure gas volume)

The efficiency of microbial protein was estimated using the following equation (Reference).

Microbial mass production efficiency = MCP/ disappeared organic matter

Measurement of methane production

Methane gas production was estimated according to Fievez *et al*. method [33]. Solutions and samples were prepared according to the gas production test method, but 120 ml bottles and 125 mg samples were used. The gas production was recorded after 24 hours. Then, 2 ml of 10N NaOH was injected into the bottles with a syringe. Again, the amount of gas production in each bottle was recorded. Finally, methane production was estimated at 24 hours. Net methane was calculated by the differences of the methane in the test syringe and the corresponding blank. Methane concentration was obtained using the following equation [34]:

$$\text{Methane Concentation} = \frac{\text{Net methane production}}{\text{Net gas production}} \times 100$$

Protozoa Counting Method

Methyl green-formalin-saline (MFS) solution was prepared for protozoa counting. MFS solution consisted of 10 ml of 35% formaldehyde, 90 ml of distilled water, 0.06 g methyl green, and 0.8 g sodium chloride. Four ml of this solution was added to 1 ml of filtered rumen fluid, and after 30 minutes, the protozoa were counted by a light microscope with a Neobar slide [35].

Statistical Analysis

The data were analyzed in a completely randomized design using the GLM procedure [36]. The least significant difference (LSD) test was used to compare the means. The statistical model was as follows:

$Y_{ij} = \mu + T_i + e_{ij}$

Where:

Y_{ij}: the dependent variable

μ: the overall mean

T_i: main effect of treatments

e_{ij}: experimental error

Results

The effect of adding different levels of aqueous and methanol extracts of *C. sinensis*, *P. granatum*, and *Q. persica* on pH, dry matter, and organic matter digestibility and fermentation parameters are shown in Table 1.

pH

Treatment with 50 µg / ml aqueous extract of *P. granatum* (treatment 2) and treatment with 200 µg / ml aqueous extract of *P. granatum* (treatment 10) had the highest and lowest pH (6.55 and 6.01, respectively). Generally, except for treatments 2 and 3 (aqueous extracts of *C. sinensis* at levels of 50 and 100 µg/ ml), other treatments reduced pH compared to the control treatment (P<0.01).

DMD and OMD

The treatment containing 200 g/ ml *Q. persica* methanol extract (treatment 19) and the control treatment (treatment 1) exhibited the lowest and maximum dry matter and organic matter digestibility, respectively. However, there was a significant difference between treatments containing extract with control treatment for dry matter and organic matter digestibility (p <0.05).

PF, MCP, and EMCP

Treatments 4, 7, 9, 10, 12, 13, 18, and 19 significantly decreased the partitioning factor (PF; P<0.01). Treatments 13 and 19 (200 µg / ml of methanolic extracts of *P. granatum* and *Q. persica*) had the lowest, and treatment 14 (50 µg / ml of aqueous extract of *Q. persica*) had the highest PF (1.99 vs. 3.50, respectively).

All treatments (except for 2, 11, and 14 that increased MCP and EMCP) significantly decreased the MCP and EMCP (P<0.01). Treatment with 50 µg / ml aqueous extract of *Q. persica* (treatment 14) had the highest MCP, and EMCP and treatments with 200 µg / ml methanol and aqueous extracts of *C. sinensis*, and *P. granatum* and methanol extract of *Q. persica* (treatments 4, 7, 10, 13 and 19) had the lowest microbial production efficiency.

Gas Production and Gas Production Parameters

The effects of adding different levels of aqueous and methanolic extracts of *C. sinensis*, *P. granatum*, and *Q. persica* on the gas production parameters are shown in Table 2 and on the total gas production in Table 3. The treatment containing 50 µg / ml aqueous extract of *C. sinensis* (treatment 2) and the treatment with 200 µg / ml methanol extract of *C. sinensis* (treatment 7) had the highest and lowest gas production potential, respectively. With increasing aqueous extract of *C. sinensis* and methanolic extract of *P. granatum*, gas production potential decreased. In the other treatments, the potential of gas production increased with increasing the tannins extract level. There were significant differences among the experimental treatments regarding estimated gas production parameters (p< 0.05). In this regard, the treatment with 200 µg/ ml aqueous extract of *C. sinensis* (treatment 4) had the highest ME, OMD, and SCFA concentration. Control, 8 and 17 treatments (50 µg/ ml aqueous extract of *P. granatum* and 50 µg/ ml methanol extract of *Q. persica*, respectively) had lower ME, OMD, and SCFA concentration than the others.

Short Chain Fatty Acids and Methane

Short-chain fatty acids increased significantly in treatments 2, 3, 4, 6, 7, 10, 11, 12, 13, 15, 16, and 19 compared to the control (P<0.01). Researchers have shown various patterns of ruminal SCFA that depend on the inclusion rate of dietary tannins.

The effect of adding different levels of aqueous and methanolic extracts of *C. sinensis*, *P. granatum*, and *Q. persica* on methane production and its reduction is shown in Table 3. All treatments decreased methane production compared to the control treatment (P< 0.05). Treatment 18 (100 µg/ ml methanol extract of *Q. persica*) had the most considerable reduction in methane production.

Protozoa Population

The addition of aqueous and methanolic extracts of *C. sinensis*, *P. granatum*, and *Q. persica* significantly reduced the total population of protozoa (Table 4). Treatments 4, 7, and 13 (200 µg/ ml aqueous and methanolic extracts of *C. sinensis* and methanolic extracts of *P. granatum*, respectively) significantly decreased the total protozoa population (P<0.05).

Discussion

pH

The decrease in pH resulting from the addition of saponins and tannins has also been observed in other studies [37,38]. The use of olive pulp and leaf as a source of condensed tannin in sheep's diet also reduced the pH [39]. Studies by Min *et al*. (2002) showed that using different levels of *Lotus corniculatus* (3.2% condense tannin) decreased the rumen pH of sheep compared to controls [40]. Also, rumen pH decreased in sheep fed dried *Elaeis guineense* supplementation diet 5 h after feeding [41]. In contrast to our findings, Bhatta *et al*. (2009) reported increasing *in vitro* pH with six plant sources of hydrolyzable or condensed tannins [42]. On the other hand, using tannins had no significant effect on the ruminal pH of sheep fed with various levels of the *Q. persica* leaf [43] and *P. granatum* extract [44]. The use of *P. granatum* extract [45] and soybean meal treated with various amounts of tannins extracted from pistachio hulls [46] did not affect the rumen pH of dairy cows and Holstein bulls. One of the reasons for the decrease in pH is the change in the pattern of rumen bacteria, especially cellulolytic bacteria [47]. The amount of rumen pH also depends on the time of consumption of tannin-containing feed and volatile fatty acids [48].

Table 1 Effect of aqueous and alcoholic extracts of *C. sinensis* (L.) Kuntze, *P. granatum* L., and *Q. persica* Jaub. & Spach on pH, digestibility, and fermentation parameters

Treatments1	pH	DMD2 (%)	OMD3 (%)	PF4 (mg/ml)	Gas yield5 24	MCP6 (mg)	EMCP7
1	6.43 cd	0.79 a	0.78 a	2.98 bcde	314.11 de	97.33 d	0.26 c
2	6.55 a	0.73 bcd	0.73 bc	2.98 bcde	347.38 cd	110.73c	0.32 b
3	6.46 bc	0.69 de	0.69 cd	2.68 egef	351.91 cd	65.70 g	0.20 ef
4	6.17 h	0.63 fg	0.64 de	2.32 ikj	410.92 b	31.63 ji	0.10 i
5	6.51 ab	0.72 cd	0.72 bc	2.82 cdef	334.24 cde	78.76 f	0.23 de
6	6.23 gh	0.69 de	0.71 bc	3.03 bcd	321.36 de	61.53 g	0.16 gh
7	6.21 h	0.61fg	0.64 ed	2.38 igjh	413.00 b	35.73 hi	0.11 i
8	6.23gh	0.71 cd	0.72 bc	2.85 bcdef	335.10 cde	86.50 e	0.25 cd
9	6.19 h	0.72 cd	0.73 bc	2.62 igh	365.34c	59.66 g	0.17 gh
10	6.01i	0.71 cd	0.72 bc	2.37 ijk	404.51b	33.93 ji	0.10 i
11	6.33 ef	0.77 ab	0.80 a	3.12 bc	307.30 ef	127.33 b	0.34 b
12	6.22 gh	0.66 ef	0.62 ef	2.45 igjh	412.63 b	62.63 g	0.20 ef
13	6.18 h	0.56 hi	0.55 g	1.99 k	440.16 b	26.83 j	0.10 i
14	6.31 ef	0.74 abc	0.75 ab	3.50 a	270.49 f	139.43 a	0.39 a
15	6.21 h	0.74 abc	0.73 bc	3.17 b	301.88 ef	96.33 d	0.27 c
16	6.22 gh	0.69 de	0.70 c	2.72 dgef	348.68 cd	63.06 g	0.19 gf
17	6.34 ef	0.69 de	0.70 c	2.87 bcdef	329.20 cde	87.10 e	0.26 c
18	6.38 de	0.59 hg	0.58 hg	2.18 jk	421.73 b	41.04 h	0.15 h
19	6.28 gf	0.54 i	0.56 g	1.99 k	485.67 a	29.36 ji	0.11 i
P-value	0<0001	0<0001	0<0001	0<0001	0<0001	0<0001	0<0001
SEM*	0.025	0.015	0.016	0.117	13.446	2.601	0.009

1Treatments: 1. Control (without the addition of extracts) 2. Aqueous extract of *C. sinensis* (50 µg/ ml) 3. Aqueous extract of *C. sinensis* (100 µg/ ml) 4. Aqueous extract of *C. sinensis* (200 µg/ ml) 5. The methanol extract of *C. sinensis* (50 µg/ ml) 6. The methanol extract of *C. sinensis* (100 µg/ ml) 7. The methanol extract of *C. sinensis* (200 µg/ ml) 8. Aqueous extract of *P. granatum*-peer (50 µg/ ml) 9. Aqueous extract *P. granatum*-peer (100 µg/ ml) 10. Aqueous extract of *P. granatum*-peer (200 µg/ ml) 11. The methanol extract of *P. granatum*-peer (50 µg/ ml) 12. The methanol extract of *P. granatum*-peer (100 µg/ ml) 13. The methanol extract of *P. granatum*-peer 200 (µg/ ml) 14. Aqueous extract of *Q. persica* (50 µg/ ml) 15. Aqueous extract of *Q. persica* (100 µg/ ml) 16. Aqueous extract of *Q. persica* (200 µg/ ml) 17. The methanol extract of *Q. persica* (50 µg/ ml) 18. The methanol extract of *Q. persica* (100 µg/ ml) 19. The methanol extract of *Q. persica* (200 µg/ ml).

2 DMD: Dry matter digestibility (%)

3 OMD: Organic matter digestibility (%)

4 PF: Partitioning factor (mg/ml)

5 Gas yield24: The amount of gas production after 24 hours of incubation (ml)

6 MCP: Microbial crude protein (mg)

7 EMCP: Efficiency of Microbial crude protein

a,b Averages within a column with non-identical letters were significantly different (P <0.05)

* SEM: Standard error of the mean

DMD and OMD

In this experiment, the addition of extracts reduced the digestibility of dry matter and organic matter compared to the control treatment. Consistent with the results of this experiment, Motamedi *et al*. reported that the use of tannin-degrading bacteria (*Klebsiella pneumonia*) increases DMD and OMD, which indicates tannins reduce the digestibility of DM and OM [49]. On the other hand, Nunez-Hernandez *et al*. reported an increase in dietary tannin (25% or 50% mountain mahogany) had no effect on organic matter intake in sheep and goats [50]. Also, *P. granatum* extract (20, 25, and 30% on a DM basis) as the tannin source did not affect the apparent digestibility of DM and OM [44]. In some studies, the in vitro DMD was affected only at greater levels of tannin consumption (Over 20%) [51]. Tannins inhibit the activity of microbial enzymes by forming protein complexes with bacterial cell wall enzymes. In this way, it reduces the digestibility of carbohydrates, especially structural carbohydrates, by cellular microbes. Therefore, through this mechanism, they affect the digestion of whole foods [17]. The inconsistencies in the results of different experiments may be due to differences in the type and concentration of tannins and dietary components.

Table 2 Effect of aqueous and alcoholic extracts of *C. sinensis* (L.) Kuntze, *P. granatum* L. and *Q. persica* Jaub. & Spach on gas production parameters

Treatments1	(a+ b)2	C2	SCFA3 (mmol)	ME4 (mj/kg)	OMD5 (%)
1	328.8 ± 6.51	0.0650±0.0039	1.10 h	8.98 i	59.68 i
2	384.2 ±3.98	0.0629±0.0019	1.31 abcd	10.27 abc	68.22 abc
3	375 ±6.38	0.0675±0.0035	1.30 abcd	10.20 abcd	67.77 abcd
4	369.8 ±4.79	0.0784±0.0032	1.36 a	10.58 a	70.32 a
5	320.9 ±4.05	0.0765±0.0030	1.15 gh	9.29 ghi	61.78 ghi
6	325 ±8.70	0.0903±0.0056	1.35 ab	10.54 ab	70.02 ab
7	363.8 ±6.96	0.1278±0.0119	1.27 bcde	10.04 bcde	66.72 bcde
8	298.5 ±5.49	0.0805±0.0047	1.10 h	8.95 i	59.53 i
9	305 ±5.56	0.0841±0.0049	1.13 gh	9.18 hi	61.03 hi
10	309.6 ±8.17	0.1094±0.0098	1.20 gef	9.61 ghef	63.88 efgh
11	346.3 ±9.31	0.0837±0.0073	1.26 cde	9.52 cde	66.12 cde
12	335 ±7.66	0.0869±0.0065	1.21 gef	9.70 defg	64.47 defg
13	330 ±5.19	0.0948±0.0084	1.23 cdf	9.81cdef	65.22 cdef
14	326.1 ±5.48	0.0730±0.0036	1.17 fgh	9.38 ghif	62.38 fghi
15	328.1 ±8.81	0.0871±0.0048	1.20 efg	9.59 ghef	63.73 efgh
16	347.7 ±6.26	0.0926±0.0055	1.32 abc	10.31 abcd	68.52 abc
17	309.2 ±6.54	0.0738±0.0049	1.09 h	8.93 i	59.38 i
18	330.6 ±6.80	0.0774±0.0050	1.16 gh	9.36 ghif	62.23 ghif
19	338 ±7.62	0.0831±0.0060	1.19 efg	9.54 ighf	63.43 efgh
SEM *	-	-	0<0001	0<0001	0<0001
P-value	-	-	0.029	1.018	0.180

1 Treatment: 1. Control (without the addition of extracts) 2. Aqueous extract of *C. sinensis* (50 µg/ ml) 3. Aqueous extract of *C. sinensis* (100 µg/ ml) 4. Aqueous extract of *C. sinensis* (200 µg/ ml) 5. The methanol extract of *C. sinensis* (50 µg/ ml) 6. The methanol extract of *C. sinensis* (100 µg/ ml) 7. The methanol extract of *C. sinensis* (200 µg/ ml) 8. Aqueous extract of *P. granatum*-peer (50 µg/ ml) 9. Aqueous extract *P. granatum*-peer (100 µg/ ml) 10. Aqueous extract of *P. granatum*-peer (200 µg/ ml) 11. The methanol extract of *P. granatum*-peer (50 µg/ ml) 12. The methanol extract of *P. granatum*-peer (100 µg/ ml) 13. The methanol extract of *P. granatum*-peer 200 (µg/ ml) 14. Aqueous extract of *Q. persica* (50 µg/ ml) 15. Aqueous extract of *Q. persica* (100 µg/ ml) 16. Aqueous extract of *Q. persica* (200 µg/ ml) 17. The methanol extract of *Q. persica* (50 µg/ ml) 18. The methanol extract of *Q. persica* (100 µg/ ml) 19. The methanol extract of *Q. persica* (200 µg/ ml).

2 a: quickly degradable fraction, b: slowly degradable fraction, c: constant rate of degradation

3 SCFA: Short-chain fatty acid (mmol)

4 ME: Metabolizable energy (MJ/kg)

5 OMD: Organic matter digestibility (%)

a,b Averages within a column with non-identical letters were significantly different (P <0.05)

* SEM: Standard error of the mean

PF, MCP, and EMCP

Similar to the results of this experiment, Hundal *et al*. (2020) revealed that PF decreased (5.8%) in the herbal feed additives containing saponins in comparison to the unsupplemented feeds [52]. Contrary to these results, the use of three types of tannin and saponins-rich plants had higher PF (from 3.35 to 4.86) compared to the basal substrate hay (PF = 3.11) or concentrate-hay mixture (PF = 3.16) [53]. Similar results were reported by Fagundes *et al*. (2020), who recorded high levels of PF during incubation of herbage containing tannins [54]. The inclusion of tannins and saponins did not affect the PF, according to Castro-Montoya *et al*. [55]. A higher PF indicates that more nitrogen from degraded OM is converted to microbial biomass. The PF, which indicates a proportion of the substrate organic matter that leads to the production of gases, short-chain fatty acids, and microbial mass in a closed system such as the gas production technique, could be used to evaluate nutrient separation. Increasing the PF indicating increased efficiency of microbial mass production [53]. The effect of tannin on PF was dependent on the concentration of extract, as the aqueous and methanolic extract of *C. sinensis*, *P. granatum*, and *Q. persica* reduced PF only at high concentrations (100 and 200 µg/ ml).

The addition of saponin-containing plants (*Quillaja saponaria*, *Yucca schidigera, Acacia auriculoformis*, and *quillaja*) increased the EMCP [55, 56]. The higher N content, as a result of the lower protozoan count, was attributed as the primary determinant of these effects of saponins [55]. Makkar *et al*. (1998) found that *yucca*, *quillaja*, and *acacia* saponins improved microbial protein synthesis efficiency [56]. According to Makkar's report, a significant increase in the incorporation of ^{15}N into

microbes in the presence of both tannins and saponins, and EMPS was higher for both tannins and saponins [13]. Although tannins decrease nutrient availability, both condense and hydrolyzable tannins have a moderating role in the efficiency of microbial protein synthesis and protect the protein from rumen degradation. Tannins change the path of nutrient utilization so that a greater proportion of nutrients are consumed by microbial and less used by short-chain fatty acids [13]. Low levels of tannins have the potential to modulate rumen fermentation to maximize microbial protein synthesis. The decrease in the rate of digestion of feeds by tannins could help to synchronize the release of different nutrients, which might be responsible for the increase in microbial efficiency [14]. Tannins have been shown to improve the synchronization of nutrients released during fermentation, and this, together with their ability to eliminate bacteriophages and protozoa, indicates that tannins may have the potential to improve the EMCP [55]. A lower energy content may also have restricted the use of plant protein in the synthesis of microbial protein [57] . As a result, high tannin concentrations in treatments 4, 7, 10, 13, and 19 (200 µg/ ml) may reduce microbial protein synthesis, whereas treatments 2, 11 and 14 with a lower amount of extract (50 µg/ ml) improved microbial protein synthesis efficiency.

Table 3 Effect of aqueous and alcoholic extracts of *C. sinensis* (L.) Kuntze, *P. granatum* L. and *Q. persica* Jaub. & Spach on Total gas production (mL/125 mgDM), methane production, and MRP

Treatments1	Total gas production (ml/125 mgDM)	Methane (%)	MRP2 (%)
1	49 abc	24.50 a	-
2	45 d	19.39 cd	32.41 ab
3	50.5 bcd	19.13 cd	23.48 ab
4	63.5 a	22.67 ab	9.28 c
5	49 bcd	20.08 abc	19.67 abc
6	51 bcd	21.25 bc	15.00 bc
7	56 b	21.54 abc	13.84 bc
8	48 cd	21.82 abc	12.72 bc
9	49 bcd	21.12 bc	15.51 bc
10	49 bcd	20.41 bcd	18.33 abc
11	50 bcd	20.49 bcd	18.03 abc
12	63 a	20.19 bcd	19.23 abc
13	70 a	21.88 abc	12.50 bc
14	51 bcd	21.98 abc	12.06 bc
15	55 bc	21.48 bcd	14.06 bc
16	69 a	21.56 bc	13.75 bc
17	53 bc	22.08 abc	11.66 bc
18	47 cd	17.46 d	30.14 a
19	54 bc	20.4 cd	19.85 abc
SEM *	2.22	0.901	3.67
P-value	<0.0001	0.0153	0.041

1Treatments: 1. Control (without the addition of extracts) 2. Aqueous extract of *C. sinensis* (50 µg/ ml) 3. Aqueous extract of *C. sinensis* (100 µg/ ml) 4. Aqueous extract of *C. sinensis* (200 µg/ ml) 5. The methanol extract of *C. sinensis* (50 µg/ ml) 6. The methanol extract of *C. sinensis* (100 µg/ ml) 7. The methanol extract of *C. sinensis* (200 µg/ ml) 8. Aqueous extract of *P. granatum*-peer (50 µg/ ml) 9. Aqueous extract *P. granatum*-peer (100 µg/ ml) 10. Aqueous extract of *P. granatum*-peer (200 µg/ ml) 11. The methanol extract of *P. granatum*-peer (50 µg/ ml) 12. The methanol extract of *P. granatum*-peer (100 µg/ ml) 13. The methanol extract of *P. granatum*-peer 200 (µg/ ml) 14. Aqueous extract of *Q. persica* (50 µg/ ml) 15. Aqueous extract of *Q. persica* (100 µg/ ml) 16. Aqueous extract of *Q. persica* (200 µg/ ml) 17. The methanol extract of *Q. persica* (50 µg/ ml) 18. The methanol extract of *Q. persica* (100 µg/ ml) 19. The methanol extract of *Q. persica* (200 µg/ ml).

2MRP: Methane production reduction potential

a,b Averages within a column with non-identical letters were significantly different (P <0.05)

* SEM: Standard error of the mean

Table 4 Effect of aqueous and alcoholic extracts of *C. sinensis* (L.) Kuntze, *P. granatum* L. and *Q. persica* Jaub. & Spach on rumen protozoa counts (103/ ml

Treatments1	genus				Total
	Holotrichia	Entodinium	Diplodinium	Ophryoscolex	
1	230 ab	75	0	0	305 abc
2	135 ab	85	5	0	225 abc
3	165 ab	40	5	0	210 abc
4	75 b	25	5	0	105 c
5	125 ab	55	5	0	185 abc
6	155 ab	40	5	0	200 abc
7	85 b	25	0	0	110 c
8	135 ab	30	10	0	175 abc
9	65 b	60	10	0	135 bc
10	170 ab	55	0	0	225 abc
11	215 ab	60	15	0	290 abc
12	290 a	10	0	0	300 abc
13	130 ab	40	5	0	175 abc
14	225 ab	70	25	0	320 abc
15	160 ab	60	0	0	220 abc
16	225 ab	55	0	0	280 abc
17	310 a	30	5	0	345 ab
18	260 ab	40	10	0	310 abc
19	310 ab	80	0	0	390 a
SEM*	56.97	24.52	7.34	0	66.42
P-value	0.0003	0.79	0.72	0	0.02

1Treatments: 1. Control (without the addition of extracts) 2. Aqueous extract of *C. sinensis* (50 µg/ ml) 3. Aqueous extract of *C. sinensis* (100 µg/ ml) 4. Aqueous extract of *C. sinensis* (200 µg/ ml) 5. The methanol extract of *C. sinensis* (50 µg/ ml) 6. The methanol extract of *C. sinensis* (100 µg/ ml) 7. The methanol extract of *C. sinensis* (200 µg/ ml) 8. Aqueous extract of *P. granatum*-peer (50 µg/ ml) 9. Aqueous extract *P. granatum*-peer (100 µg/ ml) 10. Aqueous extract of *P. granatum*-peer (200 µg/ ml) 11. The methanol extract of *P. granatum*-peer (50 µg/ ml) 12. The methanol extract of *P. granatum*-peer (100 µg/ ml) 13. The methanol extract of *P. granatum*-peer 200 (µg/ ml) 14. Aqueous extract of *Q. persica* (50 µg/ ml) 15. Aqueous extract of *Q. persica* (100 µg/ ml) 16. Aqueous extract of *Q. persica* (200 µg/ ml) 17. The methanol extract of *Q. persica* (50 µg/ ml) 18. The methanol extract of *Q. persica* (100 µg/ ml) 19. The methanol extract of *Q. persica* (200 µg/ ml).

a,b Averages within a column with non-identical letters were significantly different (P <0.05)

* SEM: Standard error of the mean

Gas production and Gas production Parameters

Many studies have reported a reduction in gas production due to tannins [13] and saponins [58]. In studies using polyethylene glycol (PEG) to investigate the biological effect of tannin on fermentation processes *in vitro*, increased gas production was observed following the addition of PEG, indicating that tannins have a negative effect on ruminal fermentation [59]. *In vitro* digestibility studies using purified tannins from various sources showed a reduction in gas production [13]. Also, the use of tannin-degrading bacteria (*Klebsiella pneumoniae*) increases gas production, estimated ME, total SCFA, and molar proportion of acetate [49]. On the other hand, some studies have been shown that saponin-containing feeds at high concentrations increase gas and SCFA production [60]. Kondo *et al*. reported that silage of *C. sinensis* pulp increased *in vitro* ruminal gas production, and it is attributed to good fermentation of *C. sinensis* pulp which improves nitrogen absorption without increasing ammonia nitrogen in the rumen [61]. Since the diets contain approximately the same amount of protein, carbohydrates, and fat, and their main difference is in the amount and type of phenolic extracts and substances in them, it seems that the main factor affecting gas production is the phenolic content treatments, especially tannins. Tannins bind to proteins and restrict the access of microorganisms to the protein. As a result, the growth of microorganisms is restricted and gas production is reduced.

Short Chain Fatty Acids and Methane

Contradictory to the results of this experiment, with the addition of oak acorn (*Q. persica*) a noticeable decrease in ammonia and SCFA concentration was observed, and adding PEG to the diets increased these values even higher than those of control [62]. Purified tannins from *Q. incana* and *Dichostachys cinerea* also decreased the production of SCFA [13]. On the other hand, Abarghuei *et al*. observed that concentrations of total SCFA and molar proportions of individual SCFA were not affected by *P. granatum* extract in the diet [45]. Also, the use of soybean meal treated with various amounts of tannins

extracted from pistachio hulls did not affect the SCFA of Holstein bulls [46]. As SCFAs are the end products of rumen microbial fermentation and represent the main supply of energy for the ruminant [63], variation in results among studies may have been due to the type or source of tannin and basal diets.

Intake of tannin-containing compounds has been shown to reduce methane emissions [62,64]. Denninger *et al*. reported that a significant decline in methane production was detected 20 min after starting supplementation with tannins [64]. Stewart *et al*. (2019) also found that tannin-containing hays have the potential to reduce enteric methane emissions from beef cattle, especially when the animals' consumption is limited [57]. The most significant source of greenhouse gas emissions was found to be mature cows, and enteric methane was the primary source of these emissions [65]. Ruminant methane emissions from enteric fermentation contribute to around 17% of total global anthropogenic methane emissions [54]. Animal enteric methane emissions are influenced by the amount and composition of feed intake, as well as the rumen microorganisms and fermentation process [66]. The decrease in methane proportion in the present experiment could be attributed to the inhibitory effect of tannins on the protozoa population [62]. Morgavi *et al*. (2010) also showed a strong relationship between some genera of protozoa and methane emission in the rumen. For instance, they were introduced *Entodinium caudatum* as a significant methane producer. Also, most fibrolytic bacteria produce H_2 as a main fermentation end product which is rapidly used by rumen methanogens, and therefore the reduction in their number can reduce methane production [67].

Protozoa Population

Consistent with the results of this experiment, the total protozoa population decreased in the rumen of sheep fed *Q. persica* (oak acorn), and the addition of PEG increased the protozoa population [62]. Soybean meal was treated with various amounts of tannins extracted from pistachio hulls [46], and *P. granatum* extract [45] decreased the total protozoa population. Motamedi *et al*. (2019) also reported that the use of tannin-degrading bacteria increases the total protozoa population and the *Isotricha* subfamily compared with the control. On the other hand, some studies have shown that tannin and saponins-containing compounds did not affect the protozoa population [68,69]. The antiprotozoal effect of treatments was most likely due to the phenolic structure of active compounds (i.e., tannins and saponins). This structure may disrupt the protozoa membrane, deactivate the protozoa enzymes and deprive of the essential substrates and metal ions for cell metabolism [1]. It seems that the effect of tannin on the total number of protozoa was dependent on the concentration of extract, as the aqueous extract of *C. sinensis* and methanol extract of *C. sinensis* and *P. granatum* reduced the population of protozoa only at the concentration of 200 μg/ ml (treatments 4, 7 and 13). In agreement with the observations of this experiment, some other work indicated that the effects of saponins on ruminal fermentation and protozoan populations were generally significant only at high application rates [70]. Accordingly, the discrepancies in observations may be due to the level and type of tannin, diet type, animal variability, and sampling methods [39].

Conclusion

Using different levels of aqueous and methanolic extracts of *C. sinensis*, *P. granatum*, and *Q. persica* reduced pH, DMD, OMD, methane production, and decreased the total number of protozoa, MCP, and EMCP only at a high level of concentrations. Therefore, the effects of these plants on fermentation parameters, methane production, and protozoa population are dose-dependent. The inclusion of *P. granatum* and *Q. persica* waste in farm animal feed not only reduces pollution, but also improves animal nutrition by reducing ruminal pH, methane emissions, and protozoa population.

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