Effect of *Camellia sinensis*, *Punica granatum* Peel, 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* leaves (green tea), *Punica granatum* peel (pomegranate peel), and *Quercus persica* (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* peel, and *Quercus 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* peel, *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 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

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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].

*Punica granatum* is rich in tannic acid so that tannins make up 25% of the water-soluble constituents of *Punica granatum* leaves [19]. *Punica granatum* peel 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 *Quercus persica* (oak) species. So, approximately 6 million hectares of forests are covered by various *Quercus* species, mainly dominated by *Quercus persica*, *Quercus infectoria*, and *Quercus libani* species. *Quercus 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 *Camellia 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 *Camellia sinensis* leaves (green tea), *Punica granatum* peel, and *Quercus persica* on digestibility, gas production, and in vitro rumen fermentation parameters and protozoa population.

**Material and Methods**

**Sample collection**

*Camellia sinensis* samples were collected from Lahijan tea gardens, *Punica granatum* peel samples from Gonbad juices and oak fruit (*Quercus persica* sp.) were obtained from Minoodasht highlands in the north of Iran. *Camellia sinensis* leaves, *Punica granatum* peel, and *Quercus 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 (*Camellia sinensis*), GKU803921 (*Punica granatum*), and GKU803922 (*Quercus 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 g/ml aqueous extract of *Camellia sinensis*), 5 to 7 treatments (control + 50, 100 and 200 μg/ml methanol extract of *Camellia sinensis*) and 8 to 10 treatments (control + 50, 100, 200 μg/ml aqueous extract of *Punica granatum* peel), 11 to 13 treatments (control + 50, 100, 200 μg/ml methanol extract of *Punica granatum* peel), treatments 14 to 16 (control + 50, 100, 200 g/ml aqueous extract of *Quercus persica*) and 17 to 19 treatments (control + 50, 100, 200 g/ml of methanol extract of *Quercus 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].
\[ \text{OMD (\%)} = 14.88 + 0.889 \text{ GP} + 0.45 \text{ CP} + 0.0651 \text{ XA} \]
\[ \text{ME (MJ/kgDM)} = 2.20 + 0.136 \text{ GP} + 0.057 \text{ CP} + 0.0029 \text{ CF} \]
\[ \text{SCFA (mmol)} = 0.0222 \text{ GP} - 0.00425 \]
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].
\[ G_y = \frac{\text{GP}_{24}}{(0.5 - \text{Dry matter weight after oven drying})} \]
Where:
\[ G_y = \text{Gas production efficiency} \]
\[ \text{GP}_{24} = \text{the gas production after 24 h of incubation} \]
The microbial mass production was estimated using the following equation [32].
\[ \text{MCP (mg)} = (\text{GP} \times \text{PF}) - 2.2 \]
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]:
\[ \text{Methane Concentration} = \frac{\text{Net methane production}}{\text{Net gas production}} \times 100 \]
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
- \( \mu \): 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 *Camellia sinensis*, *Punica granatum* peel, and *Quercus 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 *Punica granatum* (treatment 2) and treatment with 200 µg / ml aqueous extract of *Punica granatum* peel (treatment 10) had the highest and lowest pH (6.55 and 6.01, respectively). Generally, except for treatments 2 and 3 (aqueous extracts of *Camellia sinensis* at levels of 50 and 100 g / ml), other treatments reduced pH compared to the control treatment (P<0.01).

**DMD and OMD**
The treatment containing 200 g / ml *Quercus 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 *Punica granatum* peel and *Quercus persica*) had the lowest, and treatment 14 (50 µg / ml of aqueous extract of *Quercus 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 *Quercus persica* (treatment 14) had the highest MCP, and EMCP and treatments with 200 µg / ml methanol and aqueous extracts of *Camellia sinensis*, and *Punica granatum* peel and methanol extract of *Quercus 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 *Camellia sinensis*, *Punica granatum* peel, and *Quercus 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 *Camellia sinensis* (treatment 2) and the treatment with 200 µg / ml methanol extract of *Camellia sinensis* (treatment 7) had the highest and lowest gas production potential, respectively. With increasing aqueous extract of *Camellia sinensis* and methanolic extract of *Punica granatum* peel, 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 g / ml aqueous extract of *Camellia sinensis* (treatment 4) had the highest ME, OMD, and SCFA concentration. Control, 8 and 17 treatments (50 µg / ml aqueous extract of *Punica granatum* peel and 50 µg / ml methanol extract of *Quercus persica*, respectively) had lower ME, OMD, and SCFA concentration than the others.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>pH</th>
<th>DMD2 (%)</th>
<th>OMD3 (%)</th>
<th>PF4 (mg/ml)</th>
<th>Gas yield524</th>
<th>MCP6 (mg)</th>
<th>EMCP7</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.43cd</td>
<td>0.79a</td>
<td>0.78a</td>
<td>2.98 bcde</td>
<td>314.11de</td>
<td>97.33d</td>
<td>0.26c</td>
</tr>
<tr>
<td>2</td>
<td>6.55a</td>
<td>0.73bcd</td>
<td>0.736c</td>
<td>2.98 bcde</td>
<td>347.38cd</td>
<td>110.73c</td>
<td>0.32b</td>
</tr>
<tr>
<td>3</td>
<td>6.46bc</td>
<td>0.69de</td>
<td>0.696cd</td>
<td>2.68 egef</td>
<td>351.91cd</td>
<td>65.70g</td>
<td>0.20ef</td>
</tr>
<tr>
<td>4</td>
<td>6.17h</td>
<td>0.63fg</td>
<td>0.64de</td>
<td>2.32 ikj</td>
<td>410.92b</td>
<td>31.63ji</td>
<td>0.10i</td>
</tr>
</tbody>
</table>
Effect of aqueous and alcoholic extracts of *Camellia sinensis, Punica granatum*, and *Quercus persica* on gas production parameters

<table>
<thead>
<tr>
<th>Treatments</th>
<th>(a+ b)</th>
<th>C2</th>
<th>SCFA3 (mmol)</th>
<th>ME4 (mj/kg)</th>
<th>OMD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>328.8 ± 6.51</td>
<td>0.0650±0.0039</td>
<td>1.10h</td>
<td>8.98i</td>
<td>59.68i</td>
</tr>
<tr>
<td>2</td>
<td>384.2 ±3.98</td>
<td>0.0629±0.0019</td>
<td>1.31abcd</td>
<td>10.27abc</td>
<td>68.22abc</td>
</tr>
<tr>
<td>3</td>
<td>375 ±3.38</td>
<td>0.0675±0.0035</td>
<td>1.30abcd</td>
<td>10.20abc</td>
<td>67.77abcd</td>
</tr>
<tr>
<td>4</td>
<td>369.8 ±4.79</td>
<td>0.0784±0.0032</td>
<td>1.36a</td>
<td>10.58a</td>
<td>70.32a</td>
</tr>
<tr>
<td>5</td>
<td>320.9 ±4.05</td>
<td>0.0765±0.0030</td>
<td>1.15gh</td>
<td>9.29ghi</td>
<td>61.78ghi</td>
</tr>
<tr>
<td>6</td>
<td>325 ±8.70</td>
<td>0.0903±0.0056</td>
<td>1.35ab</td>
<td>10.54ab</td>
<td>70.02ab</td>
</tr>
</tbody>
</table>

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

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 *Camellia sinensis, Punica granatum* peel, and *Quercus 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 *Quercus persica*) had the most considerable reduction in methane production.

Protozoa population

The addition of aqueous and methanolic extracts of *Camellia sinensis, Punica granatum* peel, and *Quercus persica* significantly reduced the total population of protozoa (Table 4). Treatments 4, 7, and 13 (200 µg/ml aqueous and methanolic extracts of *Camellia sinensis* and methanolic extracts of *Punica granatum* peel, respectively) significantly decreased the total protozoa population (P<0.05).
In contrast to our findings, a averages within a column with non-identical letters were significantly different (P <0.05) 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].

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 pneumoniae) 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, Punica granatum peel 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 3 Effect of aqueous and alcoholic extracts of Camellia sinensis, Punica granatum-peer and Quercus persica on
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 *Camellia sinensis*, *Punica granatum* peel, and *Quercus persica* reduced PF only at high concentrations (100 and 200 μg/ml).

The addition of saponin-containing plants (*Quillaja saponaria*, *Yucca schidigera*, *Acacia auriculiformis*, 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 15N 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 4 Effect of aqueous and alcoholic extracts of Camellia sinensis, Punica granatum-peer and Quercus persica on rumen protozoa counts (10³/ml)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>genus</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Holotrichia</td>
<td>Entodinium</td>
</tr>
<tr>
<td>1</td>
<td>230ab</td>
<td>75</td>
</tr>
<tr>
<td>2</td>
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<tr>
<td>3</td>
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<td>13</td>
<td>130ab</td>
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<tr>
<td>SEM*</td>
<td>56.97</td>
<td>24.52</td>
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<tr>
<td>P-value</td>
<td>0.0003</td>
<td>0.79</td>
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</table>


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 Camellia sinensis pulp increased in vitro ruminal gas production, and it is attributed to good fermentation of Camellia 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

Contrary to the results of this experiment, with the addition of oak acorn (*Quercus 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 *Quercus incana* and *Dichostachys cinerea* also decreased the production of SCFAs [13]. On the other hand, Abarghuei et al. observed that concentrations of total SCFA and molar proportions of individual SCFA were not affected by *Punica granatum* peel extract [45]. Also, the use of soybean meal treated with various amounts of tannins extracted from pistachio hulls did not affect the SCFAs 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 H2 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 *Quercus 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 *Punica granatum* peel 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 *Camellia sinensis* and methanol extract of *Camellia sinensis* and *Punica granatum* peel reduced the population of protozoa only at the concentration of 200 g/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 *Camellia sinensis*, *Punica granatum* peel, and *Quercus 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 *Punica granatum* peel and *Quercus persica* waste in farm animal feed not only reduces pollution, but also improves animal nutrition by reducing ruminal pH, methane emissions, and protozoa population.

References


68. Benchaar C., McAllister T., Chouinard P. Digestion, ruminal fermentation, ciliate protozoal populations, and milk production from dairy cows fed cinnamaldehyde, quebracho condensed tannin, or Yucca schidigera saponin extracts. J Dairy Sci. 2008;91;4765-4777.