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3 Running head: Effect of hazel leaves on physiology of sheep

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5 # Effect of supplementation of pelleted hazel (*Corylus avellana*) leaves on
6 blood antioxidant activity, cellular immune response and heart beat
7 parameters in sheep[1]

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24 **ABSTRACT**

25 Hazel leaves (*Corylus avellana*) fed to sheep resulted in decreased methane emissions
26 without negatively affecting feed intake, and were found to have antioxidant properties *in*
27 *vitro*. The objective of this study was to evaluate effects of hazel leaves, rich in tannins, on
28 blood antioxidant activity, cellular immune response and heart beat parameters in sheep. Four
29 experimental pellets were produced by mixing alfalfa and hazel leaves in different proportions,
30 including alfalfa alone as a control, 30% and 60% of hazel leaves, the latter also with 3.8%
31 polyethylene glycol (PEG). Six adult, non-pregnant, non-lactating female sheep (71 ± 5.7 kg
32 of body weight) were allocated to four treatments in a 6 × 4 crossover design with four 18 d
33 periods. The diet consisted of experimental pellets and ryegrass-dominated hay (ratio 80% to
34 20% in dry matter), resulting in hazel leaf proportions of approximately 0, 25 and 50% in the
35 total diet. Blood samples were collected at the end of each period to determine plasma total
36 phenol concentration and markers of oxidative status as well as peripheral blood mononuclear
37 cells (PBMC) activation and proliferation response *in vitro*. Heart rate (HR) and HR
38 variability parameters were measured for two consecutive days in each period, during
39 different activities (i.e., eating pellets or hay, or lying). Treatments were compared with
40 multiple comparisons and contrast analysis was used to test for linear and quadratic relations.
41 Compared to control, feeding a high dosage of hazel leaves enhanced ($P = 0.006$) the plasma
42 total antioxidant capacity, which linearly ($P = 0.016$) increased with increasing level of hazel
43 leaves in the diet. The total phenol concentration and activities of the antioxidant enzymes
44 superoxide dismutase, catalase and glutathione reductase in the plasma were not different (P
45 ≥ 0.23) among the treatments; however, the latter slightly increased linearly ($P = 0.047$) with
46 increasing hazel leaves proportion. No differences were observed in the activation and
47 proliferation of PBMC among treatments. The HR decreased linearly ($P \leq 0.009$) during
48 pellet eating and lying and the root mean square of successive differences of interbeat

intervals (RMSSD) increased linearly ($P = 0.037$) when lying with increasing level of hazel leaves in the diet. In conclusion, our findings indicate that hazel leaves are a promising supplement to improve oxidative status with no effect on cellular immune response and cardiac stress level of sheep.

Key words: heart rate variability, oxygen consumption, peripheral blood mononuclear cells, proliferation, tannins, total antioxidant capacity.

INTRODUCTION

Ruminants undergo oxidative stress when the amount of reactive oxygen species in the animal organism exceeds the capacity of antioxidant defenses (Sies, 1997), which increases the susceptibility of animals to health problems, such as mastitis and metritis (Sordillo and Aitken, 2009; Poławska et al., 2012). Plants rich in secondary compounds can enhance the oxidative and immune status when supplemented to ruminant diets as reviewed by Oh et al. (2017). Tannins can act as natural antioxidants due to the presence of several aromatic rings with one or more hydroxyl groups, which is associated with strong antioxidant capacity by reacting with free radicals to form resonance-stabilized phenoxyl radicals (Rice-Evans et al., 1996). Besides, tannins can enhance directly or indirectly the immune system in ruminants through activating T cells (Holderness et al., 2008), favorably modifying the populations of gastrointestinal bacteria and increasing the availability of feed proteins (Provenza and Villalba, 2010). However, tannin-rich feeds can negatively affect palatability, feed intake and could thus be a stressor for animals. Changes in heart rate (HR) and HR variability (HRV) could be suitable indicators for acute and chronic stress of animals subjected to different environmental challenges (von Borell et al., 2007). Up to now, studies with ruminants reporting the effect of tannins on cardiac activity are rare (Puchala et al., 2005) and the study reporting the effect of hazel tannins on cardiac activity are not available.

74 Also, no information is available on the effects of hazel leaves on *in vivo* antioxidant
75 activity and the immune response. Therefore, we hypothesized that 1) supplementing hazel
76 leaves to the diet improves the antioxidant status and immune response in sheep without
77 negative effects on cardiac activity, and 2) that the active compounds responsible are mainly
78 the tannins.
79

80 **MATERIALS AND METHODS**

81 ***Animals, experimental design and diets***

82 The experimental protocol complied with the Swiss legislation for Animal Welfare and
83 was approved by the Committee on Animal Experimentation of the Cantonal Veterinary
84 office Zurich (license no. ZH 25/16). The experimental design and diets are described in
85 detail by Wang et al. (2018b) with contents of secondary plant compounds and feed intake
86 reprinted in Supplementary Table S1 and S2, respectively. Briefly, six female non-pregnant
87 and non-lactating Swiss Black-Brown Mountain sheep (71 ± 5.7 kg of body weight) at the age
88 of 18 ± 1.7 months were housed in a naturally ventilated and illuminated building at the
89 experimental station AgroVet-Strickhof (Eschikon, Lindau, Canton of Zurich, Switzerland).
90 In addition, there was also diurnal artificial lighting (lights on in the morning, lights off in the
91 evening). The size of individual pens was 1.25 m × 2.5 m, and the floor was covered with
92 sawdust. All sheep were free from worms determined by fecal egg count. The experiment was
93 conducted as a 6 × 4 crossover design with different sequences of the four experimental diets
94 in four 18 d periods where the six animals were kept individually, with 2 d of feeding alfalfa-
95 only (*Medicago sativa*) pellets and hay between the periods where the animals were kept
96 together in a group and no measurements were performed. Thus, each sheep received the four
97 dietary treatments once, and each dietary treatment was replicated six times. The animals
98 were fed 1.6 the maintenance requirements of adult non-performing sheep (Arrigo and Frioud,
99 2016). The diets consisted of three forage ingredients, i.e. ryegrass-dominated (late cut) hay,

100 alfalfa and hazel leaves. The alfalfa and the hazel leaf material were purchased from Landi
101 Sense-Düdingen (Heitenried, Switzerland) and Alfred Galke GmbH (Bad Grund, Germany),
102 respectively. Four types of experimental pellets were produced by thoroughly mixing alfalfa
103 and hazel leaves in different proportions, including alfalfa alone as a control, 30% and 60% of
104 hazel leaves. The diet with the highest hazel leaf proportion was also tested with the addition
105 of 3.8% polyethylene glycol (PEG; molecular weight of 6000; Sigma, St. Louis, MO, USA)
106 on a dry matter (DM) basis by replacing the respective proportion of alfalfa in the pellets. The
107 corresponding total tannins content in each experimental pellet was 0.76, 2.82, 4.80 and 4.36%
108 of DM, respectively (Wang et al. 2018b, Supplemental Table S1, see the online version of the
109 article). The complete diets consisted of experimental pellets and hay at a ratio of 80%:20% in
110 DM, resulting in hazel leaf proportions of approximately 0, 25 and 50% in the total diet
111 (realized: 0, 23.4 and 46.8%). The pellets were offered in equal amounts twice daily at 0800 h
112 and 1500 h, and 30 min later the corresponding proportion of hay was offered. Animals did
113 not differ in intake of pellets and hay (Wang et al. 2018b, Supplemental Table S2). The
114 animals had free access to water.
115
116 ***Heart rate and heart rate variability measurements***
117 The continuous measurement of the heart beat parameters of each sheep started before
118 morning feeding and stopped after afternoon feeding on d 10 and 11 of each period, resulting
119 in 7 to 10 h of recordings per day, by using Polar Team2 (Polar® Electro Oy, Kempele,
120 Finland). In order to increase the electrode-skin contact, the electrodes were positioned on
121 shaved skin. Ultrasound gel (Henry Schein, NY, USA) was used to improve conductivity
122 between electrodes and the sheep body. The device was set to record every heartbeat of the
123 animals. The data recorded in the transmitter was sent to a laptop computer by using an
124 interface (base station) and Polar Team2 software (version 1.4.5).

Using the program Polar ProTrainer 5 Equine Edition (version 5.42.007), the HR (beat per minute), the time domain-related parameters of RMSSD (root mean square of successive differences of inter-beat intervals; ms) and SDNN (standard deviation of all inter-beat intervals; ms) were extracted. The RMSSD/SDNN ratio was calculated in Microsoft Excel (Microsoft Office Professional Plus 2016) based on the extracted RMSSD and SDNN values.

The exact start and end time for three focal activities of the sheep during the measurement period, i.e. the consumption of pellets, of hay and resting while lying was recorded by a camera recorder (HDR-CX240E and HDR-PJ240E, Sony, Shanghai, China) positioned in a way that all six sheep and the respective activities could be fully recorded. The first and last minute of each activity period (i.e., eating pellets, eating hay and lying) of the cardiac dataset were excluded in order to avoid a potential bias by previous and subsequent activities. In each dataset, the first two segments with 3 min and less than 5% errors were taken into account, and then the correction of the tachograms within the Polar software was carried out by using the correction routines to correct for any artefacts prior to analysis. If the segment (e.g. minute 2 to 4) could not be used, it was moved one minute forward (e.g. minute 3 to 5) and examined as described above. For the pellet and hay eating activities, the values from the first two segments recorded in the respective morning (2 × 3 min) and afternoon feeding (2 × 3 min) were averaged per animal and per day. For lying activity, the first lying period that lasted at least 30 min after the morning feeding was included in the analysis, which resulted in 24 samples including three lasting only 20, 26 and 26 min. From the beginning, middle and end of each lying period, one segment fitting to the aforementioned criteria was extracted respectively, and the resulting three segments (3 × 3 min) of each lying bout were averaged per animal and per day. Finally, the average of two days measurement was used for data analysis. This resulted in n = 6 for each dietary treatment.

Blood sampling

151 Blood samples were collected from the jugular vein with lithium- and sodium-heparinized
152 and EDTA vacutainers (BD, Polymouth, UK) 1 h after morning feeding on d 19 of each
153 period. The lithium-heparinized and EDTA blood samples were centrifuged at 1300 × g for
154 20 min. The plasma was collected and stored at -80°C until analysis of phenol concentration
155 and antioxidant status. The sodium-heparinized blood samples were transferred on ice to the
156 laboratory for peripheral blood mononuclear cells (PBMC) isolation. The blood of one sheep
157 was hemolytic in all periods and was thus excluded from analysis, resulting in n = 5.
158

159 ***PBMC activation and proliferation***

160 The PBMC were isolated by density-gradient centrifugation. Briefly, the ice-cold blood was
161 diluted (1:1 with RPMI 1640 medium) and transferred gently on top of the separating Biocoll
162 (1.077 g/mL; Biochrom GmbH, Berlin, Germany). After centrifugation, the middle layer
163 containing PBMC was collected and suspended in RPMI 1640 medium supplemented with 10%
164 fetal bovine serum superior and 2 mmol/L L-glutamine (Biochrom) and 10 mmol/L HEPES
165 (PAN Biotech, Aidenbach, Germany). The remaining erythrocytes were hypotonically lysed
166 by sterile pure water (PAN Biotech) and isotonicity was restored with sodium chloride
167 solution. Finally, PBMC were resuspended in complete RPMI 1640 medium, and the cell
168 number and viability were determined using an automatic cell counter (Eve, NanoEnTek,
169 Secol, Korea). The cell number of each sample was adjusted to 1×10^6/mL. Cells were seeded
170 in quadruplicate with and without phytohaemagglutinin (PHA) at the concentration of 4
171 µg/mL (Bioswisstec AG, Schaffhausen, Switzerland) each for the activation and proliferation
172 assay.

173 Cell activation was assessed using the oxygen consumption rate (OCR) of PBMC at 24 h
174 of incubation (Schwarm et al., 2013; Wang et al., 2018a). The PHA was added to cell
175 suspensions after equilibration for 1 h in an atmosphere of humidified air-5% CO_2 at 39°C in
176 fluorophore-coated 96-well round-bottom OxoPlates (PreSens Precision Sensing GmbH,

177 Regensburg, Germany). After incubation for 24 h, the fluorescence was measured from the
178 bottom with a plate reader (BioTek, Luzern, Switzerland) in the dual kinetic mode using two
179 different filter pairs (540/650 nm and 540/590 nm). Fluorescence units were converted to
180 oxygen consumption rate following the manufacturer's instructions and Schwarm et al. (2013)
181 using 0.35 cm^2 surface area and 0.71 cm diffusion path length. Counting of cells from parallel
182 plates incubated for 24 h in the presence and absence of PHA enabled the scaling of oxygen
183 consumed per number of cells. The activation index was calculated as the ratio of oxygen
184 consumption rate [nmol/min/(10^7 cells)] of PBMC in the presence and absence of PHA.

185 Cell proliferation was measured using the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl
186 tetrazolium bromide (MTT) assay at 72 h of incubation (Tuchscherer et al., 1998). Incubation
187 was performed in standard 96-well round-bottom microplates in an atmosphere of humidified
188 air-5% CO_2 at 39℃. After 72 h, the plates were centrifuged at 220 × g and 20°C for 10 min
189 and then 100 µL of supernatant per well were removed. Incubation was resumed for 4 h after
190 quick application of 10 µL MTT solution (5 mg/mL of phosphate-buffered saline) and
191 accomplished overnight after addition of 100 µL of preheated 10% sodium dodecyl sulphate.
192 The optical densities at 550 and 690 nm (test and reference wavelength, respectively) were
193 measured from the top with a plate reader (BioTek). The proliferation index was calculated as
194 the ratio of optical density of PBMC in the presence and absence of PHA.

195

196 ***Chemical analysis of plasma***

197 The phenol concentration in EDTA plasma was determined based on Serafini et al. (1998).
198 Briefly, a modified Folin-Ciocalteu method was applied for total phenols and calculations
199 were done as gallic acid equivalents (Sigma, St. Louis, MO, USA). Commercial kits
200 (OxiSelectTM, Cell Biolabs, San Diego, CA, USA) were used to determine total antioxidant
201 capacity (TAC, STA-360), which represents the non-enzymatic antioxidant substances, and
202 antioxidant enzyme activity including superoxide dismutase (SOD, STA-340), catalase (CAT,

203 STA-341) and glutathione reductase (GR, STA-812), in lithium-heparinized plasma according
204 to the corresponding manufacturer's instructions.
205
206 ***Statistical analysis***
207 All data were subjected to ANOVA with the Mixed procedure of SAS (version 9.4, SAS
208 Institute, Cary, NC) with treatment and period as fixed effects and animal as random effect.
209 Multiple comparisons among means were performed by Tukey's method. Linear and
210 quadratic effects of the level of hazel leaves (0%, 25% and 50%) without the treatment with
211 50%+PEG were evaluated by orthogonal polynomial contrasts. Effects were declared as
212 statistically significant at $P < 0.05$ and as trends at $0.05 \leq P < 0.10$.
213
214 **RESULTS**
215 There was no effect ($P = 0.23$) of dietary hazel leaves on the concentration of total
216 phenols in the plasma (Table 1). Feeding the high level of hazel leaves resulted in an increase
217 ($P = 0.006$) in the plasma TAC, which was linearly ($P = 0.016$) increased along with
218 increasing the proportion of hazel leaves. No effect ($P \geq 0.72$) of dietary hazel leaves on the
219 activities of antioxidant enzymes, namely SOD and CAT among the treatments was observed.
220 The GR activity was slightly linearly enhanced ($P = 0.047$) with increasing hazel leaf
221 proportions.
222 The viability of isolated PBMC before incubation was 85 ± 1% (mean ± SE, not shown in
223 Figure). The *in vitro* activation and proliferation index of PBMC was not affected ($P \geq 0.42$)
224 by the partial replacement of alfalfa by hazel leaves in sheep (Figure 1).
225 The sheep fed with 50% hazel leaves with or without PEG had a lower HR while eating
226 pellets and lying than the sheep fed without hazel leaves ($P \leq 0.005$; $P = 0.076$ for eating hay,
227 Table 2). In addition, the decrease was in a linear ($P \leq 0.009$) manner for eating pellets and

228 lying, and at linear tendency ($P = 0.055$) while eating hay. When lying down, the RMSSD and
229 the ratio of RMSSD to SDNN of sheep consuming the diet with hazel leaves linearly ($P \leq$
230 0.011) increased. This effect was alleviated by adding PEG. The RMSSD was higher ($P =$
231 0.049) during the lying period than during the time spent feeding (both on pellets and hay),
232 and no differences regarding the HR and HR variability were observed between the sheep
233 when ingesting pure alfalfa pellets or when ingesting hay (data not shown).
234

235 **DISCUSSION**

236 To our knowledge, the present study is the first to investigate the effect of
237 supplementation of hazel leaves on the plasma phenol concentration, antioxidant status,
238 cellular immune function and heart beat parameters in the sheep. So far, antioxidant effects of
239 hazel leaves extracts have been demonstrated *in vitro*, by showing a great reducing power,
240 scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals and bleaching β-carotene
241 (Oliveira et al., 2007). These effects may be attributed to the hazel leaves' richness of phenolic
242 compounds, thus making them a potential candidate for a natural antioxidant (Oliveira et al.,
243 2007). In line with this, the present *in vivo* study revealed that the consumption of hazel
244 leaves by sheep had an enhancing effect on TAC and GR activity in blood, although the
245 enzyme activities of SOD and CAT were not affected. The antioxidants in the animal can be
246 classified into two categories, i.e. enzymatic antioxidants such as SOD, CAT and GR, and
247 non-enzymatic antioxidants represented by e.g. tocopherols, ascorbic acid, glutathione and
248 lipoic acid (Sordillo and Aitken, 2009). The overall antioxidant capacity of the non-enzymatic
249 antioxidant substances in the present study determined by TAC analysis was better than the
250 enzymatic ones, indicating different responses towards dietary hazel leaf supplementation.
251 Although the metabolic fate of tannins in ruminants is not entirely understood, the following
252 two explanations for the observed improvement in antioxidant defense caused by tannins are
253 likely. First, ingested tannins might be degraded and absorbed from ruminant gastrointestinal

254 tract into the blood stream and serve as exogenous antioxidants. Second, tannins serve as
255 antioxidants in the lumen of the gastrointestinal tract by removing or chelating pro-oxidant
256 compounds and thereby decreasing their uptake into the blood stream (López-Andrés et al.,
257 2013). The latter potential mechanism seems to be plausible for the observed increased
258 plasma TAC, as no change of total phenol concentrations in the blood was observed. The
259 greater plasma TAC in sheep fed the high level of hazel leaves compared to those fed only
260 alfalfa or lower levels of hazel leaves indicates that the hazel leaves improved the antioxidant
261 status of sheep in a dose-dependent manner. The plasma TAC results for the treatments with
262 or without PEG were not different, suggesting that the improved antioxidant status caused by
263 hazel leaves was not solely due to tannins, which is in line with an earlier study showing
264 comparable TAC levels in the serum of lambs fed with purple prairie clover (*Dalea purpurea*
265 Vent.) hay with and without polyethylene glycol (Peng et al., 2016). Thus, it is assumed that
266 other bioactive ingredients, such as non-tannin phenols that made up 28% of dietary total
267 phenols in our study (published previously by Wang et al. 2018b, Supplemental Table S1) are
268 likely to contribute to the increasing effect on the antioxidant defense in sheep. However, the
269 PEG-to-total tannin ratio was only 0.8:1, what might not have been high enough to
270 completely inhibit the bioactivity of the tannins. Moreover, the complexation of tannins with
271 PEG or protein may have affected but not eliminated their antioxidant activities (Riedl and
272 Hagerman, 2001). Glutathione reductase is a homodimeric enzyme that indirectly prevents
273 oxidative damage in cells by supporting the maintenance of the intracellular reduced
274 glutathione, which is another non-enzymatic antioxidant. The present study revealed that the
275 consumption of hazel leaves by sheep could linearly increase GR activity, possibly leading to
276 an increased glutathione level as an explanation for the enhanced TAC in the plasma, which
277 was also reported in a study with humans (Ahmadpoor et al., 2009). However, there was no
278 effect observed on enzyme activities of SOD and CAT, although inclusion of tannins from
279 chestnut and purple prairie clover has been reported to enhance SOD and CAT activities in

280 serum of lambs (Liu et al., 2016; Peng et al., 2016). The difference between the present and
281 previous studies may be explained by the source, dosage and structure of the tannins. In the
282 study of Peng et al. (2016), the lambs were provided with two times the amount of condensed
283 tannins than in the present study (3.8 vs. 1.9% of DM). Although low concentrations of
284 condensed tannins (0.5 and 1.0% of DM) were supplied in the study of Liu et al. (2016), the
285 lambs used were subject to heat-stress, which may have enhanced the antioxidant effect of
286 tannins.

287 With regard to the immune system, tannins can have protective, health-promoting effects
288 with an improved immune response (Provenza and Villalba, 2010). The mechanism of this
289 immune modulation by tannins or their metabolites may involve direct stimulating effects on
290 immune cells (Holderness et al., 2008) and indirect effects such as changes in populations of
291 commensal bacteria by the bactericidal action and the improvement of protein degradation in
292 ruminants (Provenza and Villalba, 2010). In the latter case, the immune response may be
293 supported by the high-quality protein bypass from the rumen to the small intestine attributed
294 to the presence of tannins, as the availability of specific amino acids like arginine, glutamine
295 and cysteine can enhance lymphocyte activity (Li et al., 2007). However, those effects vary
296 depending on the source, structure and supplemented levels of tannins. In fact, the observation
297 that apparent N digestibility in sheep was not increasing with increasing proportions of hazel
298 leaves in the diet (Wang et al., 2018b), is in line with the unchanged PBMC response in the
299 present study. In addition, tannins, especially hydrolysable tannins could be degraded in the
300 lumen of the gastrointestinal tract (Goel et al., 2005) and the resulting metabolites may exert
301 their function by passing the intestinal barrier and entering the blood system. Thus, systemic
302 effects on immune cells may occur, affecting the potential of immune cells to be activated and
303 to proliferate. Both activation and proliferation of PBMC were comparable when feeding
304 different amounts of hazel leaves to sheep. This is in line with the lack in changes of total
305 phenol concentrations in the blood. Thus, the consumption of hazel leaves by sheep did not

306 enhance the immune response of PBMC. However, hazel leaves had also no inhibiting effect
307 on PBMC response. Besides the source, structure and dosage of the tannins, other factors such
308 as the physiological status of the animal and the animal species can influence the potential of
309 tannins to modulate the immune response. For example, Tibe et al. (2012) reported that
310 condensed tannins could *in vitro* activate gamma-delta T lymphocytes from young goats, but
311 not from lambs and calves, which suggested that the response of lymphocytes to tannins
312 varies among animal species. In addition, the observed high variation in immune response
313 among individuals, especially in PBMC proliferation in the present study makes it difficult to
314 demonstrate an effect of dietary tannins on immune cells of animals. Indeed, it is quite
315 difficult to specify the reason for this variation in immune response due to the quite limited
316 research regarding the effect of feeding supplements high in phenols to ruminants on their
317 cellular immune function. More research is therefore needed in this area.

318 The measurements of HR and HR variability (i.e. RMSSD, SDNN and RMSSD/SDNN)
319 that have been introduced from human to farm animals over the past decades can be realized
320 with a non-invasive approach to investigate the dynamic functioning of the autonomic
321 nervous system (ANS), especially the sympathovagal balance (von Borell et al., 2007). When
322 animals suffer from stress, the RMSSD (reflecting only short-term heart variability), and the
323 SDNN (reflecting short-term and long-term heart variability) decrease, reflecting alterations
324 in the sympathovagal balance that is sympathetically mediated. In the present study, when the
325 sheep were fed with hazel leaves, the decrease in HR and the increased RMSSD and
326 RMSSD/SDNN ratio when animals were lying indicated a shift towards more dominant vagal
327 activity and less stress for the sheep. Since this is the first paper studying differences in ANS
328 function of sheep fed with different proportions of hazel leaves and phenols thereof, it is
329 impossible to compare our results to earlier findings in this field. However, it is known that
330 the HR is correlated with energy expenditure or heat production as was shown in cattle (Brosh,
331 2007) and yaks (Han et al., 2002). The decreased HR observed in the current study may partly

332 be attributed to the lower heat production as the energy used for heat production in the
333 animals was numerically decreased from 10.6 to 9.9 MJ/d along with the increase of hazel
334 leaves in the diet from 0 to approximately 50% (Wang et al., 2018b). In addition, the
335 increased concentration of dietary phenols may exert an influence on the ANS. It has been
336 reported that the tannin-containing extracts from *Terminalia arjuna* could decrease the blood
337 pressure and HR in rat (Takahashi et al., 1997). Based on the present results, the lowering
338 effect of hazel leaves on HR was alleviated to some extent by adding PEG that can counteract
339 the biological function caused by tannins. No differences were found with the other
340 parameters reflecting HR variability across concentrations of hazel leaves applied in the
341 present study. Overall, the supplementation of sheep diets with hazel leaves caused no cardiac
342 stress to animals but enhanced the cardiovascular functions to some extent. The HR, RMSSD
343 and SDNN did not differ between eating alfalfa pellets and eating hay, which again suggested
344 that the ingestion of hazel leaves did not cause cardiac stress for the sheep.
345
346 The present study showed that hazel leaf supplementation to sheep resulted in an
347 enhancement in plasma TAC and GR activity, indicating a significant potential of hazel
348 leaves as forage for ruminants to mitigate oxidative stress. Tannins in hazel leaves were not
349 the sole active ingredients. In addition, feeding hazel leaves with the current dosages
350 maintained the response of immune cells and did not cause any cardiac stress to the sheep.
351 The underlying mechanism of hazel leaves to improve oxidative status in animals needs to be
352 elucidated in further studies.
353
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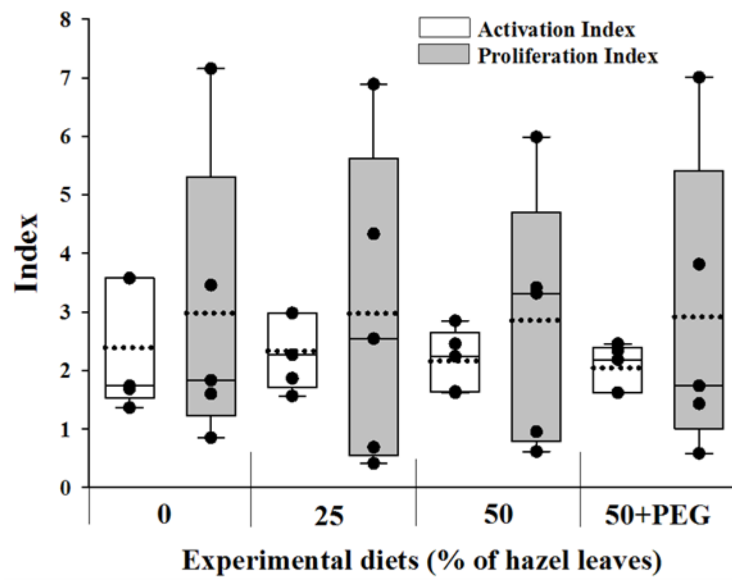
448

449 **Figure caption**

450 **Figure 1.** Effect of hazel leaf supplementation on the *in vitro* activation ($P > 0.10$) and
451 proliferation index ($P > 0.10$) of peripheral blood mononuclear cells (PBMC) from sheep (n =
452 5) after 24 and 72 h of incubation, respectively. The indices were calculated as the ratio of
453 phytohaemagglutinin-stimulated and untreated PBMC. Dotted lines indicate arithmetic means.
454 PEG, polyethylene glycol.

455

456



457

458 **Figure 1**

Table 1

Effect of hazel leaves on phenols concentration and oxidative status in plasma of sheep (n = 5).

	Experimental diets				SEM	*P*-value		
Hazel leaves (% of diet)	0	25	50	50+PEG		Diet	L[1]	Q[1]
Total phenols, µg/mL	229	217	225	230	3.9	0.23	0.67	0.12
Total antioxidant capacity (TAC), µmol/L	180[b]	193[ab]	217[a]	213[a]	5.6	0.006	0.016	0.44
Superoxide dismutase (SOD), inhibition %	38.6	37.0	38.8	39.4	0.96	0.75	0.87	0.43
Catalase (CAT), U/mL	30.0	25.7	24.2	27.4	3.03	0.72	0.48	0.77
Glutathione reductase (GR), mU/mL	26.5	27.0	29.1	28.7	1.37	0.83	0.047	0.39

PEG, polyethylene glycol; L, linear effect of hazel leaf proportion; Q, quadratic effect of hazel leaf proportion; SEM, standard error of mean.

Means carrying no common superscript are different at $P < 0.05$.

[1]For this analysis, only diets 0, 25 and 50 were compared.

Table 2
Effect of hazel leaves on heart rate and heart rate variability of sheep with different activities (n = 6)[1].

	Experimental diets				SEM	*P*-value		
Hazel leaves (%)	0	25	50	50+PEG		Diet	L[2]	Q[2]
Eating pellets								
Heart rate (HR), bpm[3]	84.0^{a}	79.1^{ab}	73.3^{b}	76.5^{b}	1.55	0.005	0.009	0.65
RMSSD, ms	91.5	98.7	104.5	100.6	7.01	0.50	0.24	0.81
SDNN, ms	84.8	84.1	94.3	87.7	3.54	0.30	0.20	0.16
RMSSD/SDNN	1.08	1.17	1.09	1.14	0.047	0.34	0.76	0.13
Eating hay								
Heart rate (HR), bpm	$89.7^{(a)}$	$83.0^{(ab)}$	$81.8^{(b)}$	$82.9^{(ab)}$	1.82	0.076	0.055	0.62
RMSSD, ms	92.3	101.3	97.8	103.1	6.04	0.40	0.56	0.52
SDNN, ms	83.1	87.0	86.7	91.5	3.65	0.31	0.53	0.92
RMSSD/SDNN	1.10	1.17	1.14	1.13	0.035	0.55	0.64	0.26
Lying								
Heart rate (HR), bpm	76.4^{a}	72.3^{ab}	65.1^{c}	68.4^{bc}	1.52	0.002	0.005	0.43
RMSSD, ms	117^{b}	125^{ab}	140^{a}	122^{ab}	10.5	0.037	0.008	0.41
SDNN, ms	97.6	98.7	101.0	93.2	5.98	0.74	0.41	0.73
RMSSD/SDNN	1.17^{b}	1.28^{ab}	1.37^{a}	1.32^{ab}	0.043	0.044	0.011	0.78

RMSSD, root mean square of successive differences of interbeat intervals; SDNN, standard deviation of all interbeat intervals; PEG, polyethylene glycol; L, linear effect of hazel leaf proportion; Q, quadratic effect of hazel leaf proportion; SEM, standard error of mean.
Means carrying no common superscript are different at $P < 0.05$; superscripts in brackets indicate a trend of a difference among means, $P < 0.10$.
[1]The ratio of pellet to hay in total dietary DM was 80%:20%.
[2]For this analysis, only diets 0, 25 and 50 were compared.
[3]Beat per min.

Supplemental Material to Wang et al.

Table S1. Phenol composition in hay, pure hazel leaves and experimental pellets (% of DM). Source: Wang et al. (2018b)[1]

Analysed composition (% of dry matter)	Hay[2]	Hazel leaves	Experimental pellets[2]			
			0[3]	30	60	60+PEG
Total phenols	1.43	8.16	1.72	4.14	6.55	5.94
Non-tannin phenols	0.82	1.95	0.96	1.33	1.75	1.58
Total tannins	0.61	6.21	0.76	2.82	4.80	4.36
Condensed tannins	0.02	3.39	0.01	1.11	2.43	1.36
Hydrolysable tannins	0.59	2.82	0.74	1.71	2.37	3.00

[1]Data in this table have been published previously by Wang et al. (2018b)
[2]The ratio of hay to pellet was 20%:80% in total dietary dry matter. Experimental pellets were produced from alfalfa and hazel leaves containing 0%, 30% or 60% hazel leaves on a dry matter basis.
[3]Equivalent to the composition of alfalfa
PEG, polyethylene glycol

Table S2. Effect of hazel leaves on intake of the sheep (n =6). Source: Wang et al. (2018b)[1]

	Experimental diets				SEM	*P* values		
Hazel leaves (% of diet)	0	25	50	50+PEG		Diet	L[2]	Q[2]
Dry matter intake (g/day)								
Total	2182	2147	2174	2170	45.5	0.64	0.71	0.56
Pellets	1794	1762	1780	1792	36.4	0.73	0.52	0.59
Hay	388	385	394	378	14.5	0.50	0.48	0.88

[1]Data in this table have been published previously by Wang et al. (2018b)
[2]For this analysis, only diets 0, 25 and 50 were compared.
PEG, polyethylene glycol; L, linear effect of hazel leaf proportion; Q, quadratic effect of hazel leaf proportion; SEM, standard error of mean.

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