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2 Effect of simulated foregut digestion on the antioxidant capacity of
3 plants naturally consumed by horses

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12
13 **Abstract**

14 Herbal medicine has impacted veterinary medicine including equine practice. Many of
15 the postulated health-promoting effects of medicinal plants are discussed in the context of
16 their antioxidative properties, but different factors such as digestion can have an effect on the
17 activities of compounds. The effect of simulated foregut digestion conditions on the
18 antioxidant capacity of seven different plants commonly consumed by horses and/or present
19 in their complementary diet was investigated. Plants included willow tree (*Salix cinerea*),
20 purple willow (*Salix purpurea*), willow regrowth (new sprouts growing at the foot of willows
21 (*Salix cinerea*), oak tree (*Quercus* spp.), meadowsweet (*Filipendula ulmaria* spp.), hawthorn

22 (*Crataegus*), and blackthorn (*Prunus spinosa*). Aqueous extracts obtained from the bark and
23 from the leaves were compared. Two methods, Oxygen Radical Absorbance Capacity
24 (ORAC) and Trolox equivalent antioxidant capacity (TEAC) using 2,2-azinobis-(3-
25 ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assays, were used to
26 measure the total antioxidant capacity (TAC) of the extracts before and after the digestion
27 process. The results showed that the TAC of all studied plant extracts decreased ($P < 0.05$)
28 following the digestion process indicating that the active compounds present in the extracts
29 were degraded. In fact, a decrease in TAC values of up to 80 % and 83 % was observed using
30 TEAC and ORAC assays respectively. Such a drop in the TAC must be taken into account
31 when considering the antioxidative efficiency of plants consumed by animals.

32

33 **Keywords:** TEAC Trolox equivalent antioxidant capacity using 2,2-azinobis-(3-
34 ethylbenzothiazoline-6- sulfonic acid) (ABTS), ORAC oxygen radical absorbance capacity,
35 herbal extract, equine.

36 **Abbreviations:**

37 AAPH, 2,2'-azobis-(2-methylpropionamidine) dihydrochloride

38 ABTS, 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)

39 ASE, accelerated solvent extraction system

40 DMSO, dimethyl sulfoxide

41 HCl, chloridic acid

42 KCl, potassium chloride

43 $K_2S_2O_8$, potassium persulfate

44 KH_2PO_4 , potassium phosphate

45 $Na_2HPO_4 \cdot 2H_2O$, di-sodium hydrogen phosphate dihydrate

- 46 NaCl, sodium chloride
- 47 NaOH, sodium hydroxide
- 48 ORAC, oxygen radical absorbance capacity
- 49 PBS, phosphate buffer saline
- 50 TAC, total antioxidant capacity
- 51 TEAC, trolox equivalent antioxidant capacity

52

53 **1. Introduction**

54 Plant-based recipes have been traditionally used for their potential health promoting
55 properties since ancient times, but they have gained increased interest over the past two
56 decades. As a consequence, the global phytotherapy and homeopathy market value has
57 increased from \$19.4 billion in 1999 to \$83 billion in 2008 (Robinson and Zhang, 2011). The
58 use of medicinal plants is impacting not only human but also veterinary medicine, with herbal
59 treatments being increasingly used for animal performance improvement or as alternative
60 therapies for injuries or diseases. It is a traditional practice for horse owners, breeders or
61 trainers to use various plant extracts for therapeutic applications or feed additives (Elghandour
62 et al., 2018). With regard to equine diet, willow (*Salix*) bark is used as feed supplement to
63 treat joint and metabolic problems, and ginseng, which is one of the most commonly used
64 supplements, is used to reduce stress, improve performance and stimulate the immune system
65 (Williams and Lamprecht, 2008). However some plant species such as St. John's wort
66 (*Hypericum perforatum*), mistle-toe (*Phoradendron flavescens*), black cherry (*Prunus*
67 *serotina*), and castor bean (*Ricinus communis*) are considered to be toxic for equines (Lans et
68 al., 2006).

69 The health-promoting effects of plant extracts are often attributed to their antioxidative
70 properties. These extracts are in fact known to possess potent antioxidant properties mainly

71 due to the presence of phenolic compounds. Such antioxidant compounds have been identified
72 in plant extracts used as feed additives in horse nutrition (Elghandour et al., 2018; Williams
73 and Lamprecht, 2008). Diet supplementation with such compounds can reduce the risk of
74 developing diseases related to oxidative stress. Siard et al. suggested that polyphenols can be
75 used to combat chronic inflammation in horses (Siard et al., 2016). In fact, they are known to
76 quench the proliferation of radical oxygen and reactive nitrogen species which are implicated
77 in etiology of different diseases such as cancer, obesity, coronary heart diseases, diabetes,
78 asthma and rhinitis in humans (Barnham et al., 2004; Eberhardt et al., 2000; Finkel and
79 Holbrook, 2000).

80 Chemical and biochemical assays exist that can be used to quantify the Total Antioxidant
81 Capacity (TAC) of plant extracts. These assays involve reactions with either a single electron
82 transfer or a hydrogen atom transfer (Huang et al., 2005). Yet, a major drawback of these
83 assays is the lack of information on the bioavailability of the bioactive molecules following
84 the digestion process for instance (Stahl et al., 2002). *In vitro* studies have shown that some
85 individual antioxidants may remain stable and active throughout the human digestion (Ryan
86 et al., 2008; Wootton-Beard et al., 2011). Such data is lacking when it comes to the equine
87 digestion system which is quite different from the human one.

88 The aim of this study was to evaluate the stability of the antioxidant capacity of different
89 plants commonly consumed by horses and/or used in their complementary diet after a
90 simulated *in vitro* horse digestion. The TAC was evaluated using the oxygen radical
91 absorbance capacity (ORAC) and Trolox equivalent antioxidant capacity (TEAC) using 2,2-
92 azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assays.

93

94 **2. Materials and methods**

95 **2.1 Reagents**

96 All solvents used in this study were of analytical grade. Ethanol, dimethyl sulfoxide (DMSO),
97 2,2'-azino-bis-(3-ethylbenthioazoline-6-sulfonic acid) (ABTS), 3H-xanthene-3-one
98 (fluorescein), 2,2'-azobis-(2-methylpropionamidine) dihydrochloride (AAPH), and 6-
99 hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) were purchased from
100 Sigma-Aldrich (Steinheim, Germany). Chloridic acid (HCl), sodium hydroxide (NaOH) and
101 sodium chloride (NaCl) were obtained from Thermo Fisher Scientific (Illkirch-Graffenstaden,
102 France). Porcine pepsin (500 U/g USP) was purchased from VWR (Fontenay-sous-bois,
103 France). Porcine pancreatin (25 000 U/g USP), potassium persulfate (K₂S₂O₈) and potassium
104 chloride (KCl) were obtained from Alfa Aesar (Kandel, Germany). Di-sodium hydrogen
105 phosphate dihydrate (Na₂HPO₄·2H₂O) and potassium phosphate (KH₂PO₄) were purchased
106 from Merck (Molsheim, France). Milli-Q water (18.2 MΩ) was generated by Millipore
107 synergy system (Molsheim, France).

108

109 **2.2 Plants**

110 Plants were selected based on their spontaneous consumption by semi-free ranging domestic
111 horses (group 1, n = 13, pasture = 17ha, Louhans, France; group 2, n = 7, pasture = 10ha,
112 Hauteville-Lompnes, France). Plant consumption was based on behavioral observation
113 conducted 6 hours per day, 6 days a week over a period of 3 months. Behavioral observations
114 were made by two observers on foot that maintained a minimum distance of 50 meters with
115 horses. Observations were recorded using a videocamera and/or a voice recorder. Observers
116 recorded any feeding event that was not grass grazing by stating the start and end time as well
117 as the number of bites. In the case of tree browsing, observers counted the number of
118 branches that were eaten while also indicating their approximate length and whether leaves

119 were present. Plant species identification was first made at a distance and was later confirmed
120 by the observer once the feeding event was over and the focal horse had moved away.
121 During the period of observation, horses did not receive any supplemental hay. Nearby rivers
122 provided an *ad libitum* access to fresh water. In total the leaves and barks of 7 different plants
123 were manually collected: willow tree (*Salix cinerea*), purple willow (*Salix purpurea*), willow
124 regrowth (new sprouts growing at the foot of willows), oak tree (*Quercus* spp.), meadowsweet
125 (*Filipendula ulmaria*), hawthorn (*Crataegus* spp.) and blackthorn (*Prunus spinosa*). For each
126 plant species, samples were taken from 7 different specimens that were spread all over the
127 pasture and at least 30 meters apart. A total of 700 g of plant material was collected for each
128 specimen (total 7 x 700 g = 4900 g for each plant species). Plant samples were stored at –
129 20°C within 2 hours of collection and then cryogenically grounded with a grid of 1 mm in
130 diameter in order to obtain a fine powder which was then stored at room temperature in sealed
131 bags protected from the light.

132

133 **2.3 Simulated foregut digestion**

134 The simulated foregut horse digestion was adapted from Abdouli and Attia (2007). The used
135 protocol simulated the digestion of feed in the stomach and small intestine of the horse. First,
136 a pepsin aqueous solution at 10 g/L was prepared in 0.1 mol/L HCl at a final pH of 2.0. Then,
137 1 g of finely ground dry plant was added to 20 mL of the previously prepared pepsin solution.
138 This mixture was incubated at 39 °C in a temperature-controlled oven under agitation for 2
139 hours. This first step simulated the digestion in the horse stomach. After the required
140 incubation time had elapsed, 12 mL of a solution of NaOH at 0.2 mol/L was added to the
141 previous mixture to obtain a pH of 7. Then, 13 mL of an aqueous pancreatin solution at 10
142 g/L were added. The resulting mixture was incubated at 39 °C under agitation for additional 4
143 hours. In those conditions, the digestion in the horse small intestine was simulated as

144 pancreatin was a mixture of amylase and lipase, two necessary enzymes for this digestion.
145 Finally, to neutralize the enzymes activity, the mixture was placed at 100 °C for 3 min. The
146 mixture was then placed at -80 °C overnight and lyophilized (Labconco FreeZone, Fisher
147 Scientific, Illkirch, France) for about 48 hours until a dry powder was obtained.

148

149 **2.4 Extraction**

150 Plant extracts were obtained by solid-liquid extraction under pressure using an ASE-350
151 Accelerated Solvent Extraction system (Dionex, Sunnyvale, CA, USA). For each plant, three
152 extracts were done for the leaves and three extracts for the bark. The extraction solvent was a
153 50/50 % (v/v) mixture of water and ethanol. For the extraction, 1 g of finely grounded plant
154 powder or 1 g of plant which was subjected to the simulated foregut digestion mixed with
155 chemically inert Fontainebleau sand (previously heated at 600 °C for 4 hours) were placed
156 into a 10-mL stainless steel extraction cell. Two 27 mm cellulose filters (Dionex, Sunnyvale,
157 CA, USA) were placed at the bottom and at the top of the extraction cell. The extraction cell
158 was then subjected to three cycles of extraction at 40 °C and 100 bars. The duration of one
159 cycle was 10 min. The whole extraction process took 30 min. After extraction, the solvent
160 was evaporated under a gentle nitrogen flow. The extracts were weighed and kept at 4 °C in
161 amber vials to protect the molecules from light degradation.

162 To evaluate the antioxidant capacity, the extracts were dissolved at a concentration of
163 1 mg/mL in DMSO prior to the different assays carried out.

164

165 **2.5 Antioxidant capacity**

166 For both TEAC and ORAC assays, trolox was used as a standard for quantitative assessment
167 of the antioxidant activity and calibration curves were done using a range of concentration
168 from 50 to 500 $\mu\text{mol/L}$ and were prepared in Milli-Q water (Molsheim, France)..

169 **2.5.1 TEAC**

170 The principle of the antioxidant capacity evaluation by the TEAC assay is based on the
171 electron loss of the cationic radical $\text{ABTS}^{\cdot+}$. Indeed, during this reduction by an antioxidant,
172 $\text{ABTS}^{\cdot+}$, initially colored blue-green, becomes colorless. The measured absorbance can then
173 be directly and quantitatively linked to the total antioxidant capacity of the extracts. The
174 radical cation $\text{ABTS}^{\cdot+}$ was formed by adding a solution of potassium persulfate at 2.5 mmol/L
175 to a solution of ABTS at 7.5 mmol/L. The solution was left overnight at 4 °C protected from
176 light exposure. From this $\text{ABTS}^{\cdot+}$ stock solution, a 2 % dilution in a phosphate buffer saline
177 (PBS) was obtained. The PBS was prepared by dissolving 8.2 g of NaCl, 1.4 g of Na_2HPO_4 ,
178 0.27 g of KH_2PO_4 and 0.15 g of KCl in 1 L of Milli-Q water. The TEAC assay was conducted
179 on a 96-wells microplate (Thermo Fisher scientific, Newport, United Kingdom) in which 10
180 μL of each extract and 200 μL of the 2 % $\text{ABTS}^{\cdot+}$ solution were added in each well. To
181 promote redox reactions between the $\text{ABTS}^{\cdot+}$ and the molecules in the extracts, the microplate
182 was placed at 37 °C for 10 min. Finally, the absorbance at 734 nm was read in each well by a
183 Varioskan spectrophotometer (Thermo Fisher Scientific, Illkirch-Graffenstaden, France).
184 Prior to the absorbance measurement, the plate was agitated for 10 seconds. The extracts with
185 an antioxidant capacity were then identified by a decrease in intensity as the radical cation
186 $\text{ABTS}^{\cdot+}$ would have lost its blue-green color. The TAC values of each extract were measured
187 in triplicate.

188 The loss of TAC in plant extracts after simulated foregut digestion was calculated as follows:
189 $(\text{TAC value of non-digested plant extract} - \text{TAC value of digested plant extract})/(\text{TAC value}$
190 $\text{of non-digested plant extract})$ and multiplied with 100 for reporting the loss in percent.

191

192 **2.5.2 ORAC**

193 The ORAC assay is based on a competition between a fluorescent probe and the antioxidant
194 compounds present in the extract. The fluorescent probe used in this study was the
195 3H-xanthen-3-one, also known as fluorescein. This compound is degradable by oxidation
196 reactions by a transfer of protons, leading to the extinction of its fluorescence. AAPH (2,2'-
197 azobis-(2-methylpropionamide)) was used as the initiator of the oxidation reactions as it
198 dissociates at 27 °C by releasing peroxylic radicals. Antioxidant molecules present in the
199 analyzed extract will absorb the free radicals generated by AAPH, the fluorescein will be
200 protected and there will be a delay in the decrease of the fluorescence. The TAC of the extract
201 is thus directly linked to the decreasing curve of the measured fluorescence delay. To quantify
202 the TAC, the area under the fluorescence curve of the studied extracts is calculated.

203 The ORAC assay was conducted on a 96-wells dark microplate (Thermo Fisher scientific,
204 Newport, United Kingdom), to prevent the degradation of the fluorescein by light. In each
205 well, 10 µL of the extract and 150 µL of a solution of fluorescein at 2.8×10^{-5} g/L in Milli-
206 Q water were added. The microplate was then placed in a fluorescence microplate reader
207 (Varioskan, Thermo Fisher Scientific, Illkirch-Graffenstaden, France), shaken for 20 sec and
208 incubated at 37 °C for 15 min. Then, using an automatic dispenser integrated into the
209 fluorescence reader, 30 µL of an AAPH solution in PBS at 41.5 g/L were added to each well.
210 The microplate was agitated again for 5 sec. The fluorescence kinetics was then measured
211 every 5 min for 2 hours with excitation and emission wavelengths of 485 nm and 530 nm
212 respectively. The experiments for each extract were conducted in triplicate.

213 The percentage loss of TAC of extract plant non digested and digested was calculated as
214 TEAC assay.

215

216 **2.6. Statistical analysis**

217 The means of triplicate TAC values (experimental unit) for each plant extract obtained with
218 TEAC or ORAC assays were compared before and after simulated digestion, the paired, two-
219 tailed *t* test was used using the software Minitab (2017) v.17. Means with P value < 0.05 were
220 considered statistically different.

221

222 **3. Results**

223

224 In this study, two methods, TEAC and ORAC, were used to evaluate the TAC of seven
225 selected plant extracts before and after a simulated foregut digestion. The concerned plants
226 were willow tree, oak tree, purple willow, willow regrowth, meadowsweet, hawthorn, and
227 blackthorn. Aqueous extracts from the leaves and from the bark of willow tree, oak tree,
228 purple willow and willow regrowth were used. For the three remaining plants, only aqueous
229 leaf extracts were used, either because they don't have a bark (meadowsweet) or because their
230 bark is not consumed by horses due to the presence of large thorns (hawthorn and blackthorn).

231 Figure 1 shows the TAC expressed in μmol Trolox equivalent per gram of plant (μmol
232 TE/g of plant) of each plant extract before and after simulated foregut digestion for TEAC
233 (figure 1.A) and ORAC (figure 1.B) methods.

234

235 **3.1. TEAC assay**

236 Prior to simulated foregut digestion, the TAC of all plant extracts assessed using
237 TEAC assay showed a level of antioxidant activity with values ranging from 1 538 to
238 10 119 μmol TE/g of plant extract. The lowest TAC value was obtained with the extract of
239 purple willow leaves and the highest with the extract of oak tree bark.

240 Among bark extracts, the lowest TAC value was observed with the extract of purple
241 willow ($3\,778 \pm 387 \mu\text{mol TE/g}$ of plant) while the highest value was obtained with the oak
242 tree extract ($10\,119 \pm 1748 \mu\text{mol TE/g}$ of plant). Among leaf extracts, purple willow extract
243 ($1\,538 \pm 65 \mu\text{mol TE/g}$ of plant) and oak tree extract ($7\,083 \pm 1\,256 \mu\text{mol TE/g}$ of plant)
244 showed the lowest and the highest TAC value using TEAC assay, respectively. The bark and
245 leaf extracts of oak tree showed the highest TAC values compared to the other plant extracts.
246 The bark extracts of willow tree, purple willow, willow regrowth and oak tree showed higher
247 TAC values compared to their respective leaf extracts ($P < 0.05$). This suggests that the bark
248 may contain more antioxidants than the leaves.

249 After the simulated foregut digestion, the TAC values of all plant extracts decreased
250 ($P < 0.05$) (figure 1). The highest TAC value was observed with the bark extract of the willow
251 tree ($2\,421 \pm 322 \mu\text{mol TE/g}$ of plant) and the lowest value with blackthorn leaf extract ($1\,008$
252 $\pm 87 \mu\text{mol TE/g}$ of plant). Table 1 shows the percentage loss of TAC before and after
253 digestion for each plant extract for both TEAC and ORAC assays. The loss ranged from 80 %
254 to 16 % with the TEAC assay. The TAC values of the bark and leaf extracts of oak tree,
255 which were the highest before digestion, decreased by 80 % after being subjected to the
256 simulated foregut digestion process (table 1). Moreover, the observed pattern with non-
257 digested extracts of same plant, where the TAC values of bark were higher than those of
258 leaves, was not observed with the digested extracts. In fact, the TAC values of bark extracts
259 were similar or lower than those obtained with leaf extracts.

260

261 **3.2. ORAC assay**

262 For the ORAC assay, the TAC values obtained prior to simulated foregut digestion
263 ranged from 2 216 to 16 915 $\mu\text{mol TE/g}$ of plant.

264 Among the studied extracts, the bark extract of the willow tree showed the highest
265 TAC value ($16\,915 \pm 1\,384 \mu\text{mol TE/g}$ of plant) while its leaf extract showed the lowest value
266 ($2\,216 \pm 311 \mu\text{mol TE/g}$ of plant). Among bark extracts, the lowest TAC value was observed
267 with the extract of purple willow ($3\,790 \pm 74 \mu\text{mol TE/g}$ of plant) while the highest value was
268 obtained with the willow tree extract ($16\,915 \pm 1\,384 \mu\text{mol TE/g}$ of plant). Among leaf
269 extracts, the lowest TAC value was observed with the willow tree extract ($2\,216 \pm 311 \mu\text{mol}$
270 TE/g of plant) and the highest value with the oak tree extract ($12\,426 \pm 418 \mu\text{mol TE/g}$ of
271 plant). As with the TEAC assay, the bark extracts of the willow tree, purple willow and
272 willow regrowth showed higher TAC values than the respective leaf extract ($P < 0.05$) (figure
273 1). However, this pattern was not observed with the oak tree extracts with the leaves showing
274 higher ORAC TAC values than the bark ($P < 0.05$) (figure 1).

275 After the simulated foregut digestion, the ORAC TAC values of all plant extracts were
276 lower ($P < 0.05$) and ranged from 932 to $5108 \mu\text{mol TE/g}$ of plant (figure 1). According to
277 table 1, the decrease ranged from 83 % to 20 %. The bark extract of the willow tree which
278 showed the highest TAC value was reduced by 83 % following simulated digestion. As with
279 the TEAC assay, this reduction resulted in TAC values equivalent to those of leaf extracts.

280

281

282 **Discussion**

283 In this study, TAC of seven different plants naturally consumed by horses was measured
284 by two different methods, TEAC and ORAC. The TEAC method measures the capacity of the
285 compound to donate electrons, while the ORAC method measures the capacity of a molecule
286 to transfer hydrogen atoms. The obtained TAC values with the two methods can therefore not
287 be compared as they represent different antioxidant phenomena measured in different
288 conditions (Schaich et al., 2015). The TAC values were between 1 538 and $10\,119 \mu\text{mol}$

289 TE/g of plant using TEAC assays. By comparison, according to Jiménez-Zamora et al. (2016),
290 the aqueous extract of green tea leaves has a TAC of about 2 289 $\mu\text{mol TE/g}$ of plant as
291 measured by the TEAC assay. This shows that the plants studied have a high antioxidant
292 capacity as their TAC are higher or at least close to that of green tea leaves, a plant well-
293 known for its antioxidant activity (Jiménez-Zamora et al., 2016). Furthermore, the TAC
294 values measured for leaves were lower than those of the bark ($P < 0.05$) (figure 1). This result
295 shows the importance of studying different parts of the plants as differences may exist in the
296 contents of active compounds.

297 The effect of simulated foregut digestion on the TAC of the seven studied plants was
298 also investigated. The results of both TEAC and ORAC assays showed a dramatic decrease in
299 TAC following digestion (-16 % to -80 % with TEAC and -20 % to -83 % with ORAC)
300 (table1). To the best of our knowledge, this study is the first to investigate the influence of our
301 model of simulated foregut horse digestion on the TAC of plants and to demonstrate the
302 decrease of TAC values following simulated foregut digestion. As far as comparison can be
303 made, our results are in agreement with those of other studies using simulated *in vitro* human
304 digestion which showed a similar trend (Bermúdez-Soto et al., 2007; Chen et al., 2013;
305 McDougall et al., 2005). For example, Chen et al. studied the influence of *in vitro* human
306 digestion on the TAC of tea juices measured by the TEAC assay. They showed that the
307 simulated digestion process led to a decrease of the TAC values of six tea juices out of nine
308 (Chen et al., 2013). Bermúdez-Soto et al. (2007) showed a decrease in TAC after an *in vitro*
309 pancreatic digestion of different chemical compounds found in chokeberry juice such as
310 anthocyanins (-43 %), flavonols (-26 %) and flavan-3-ols (-19 %). In other studies based on
311 the TEAC assay, it was observed on the other hand that some extracts can stay stable
312 throughout the digestive process (gastric and pancreatic) such as extract Perennial chamomile

313 (*Chamaemelum nobile*) or Japanese plum (*Prunus salicina*) seeds extract (Chen et al., 2015,
314 2016; Wootton-Beard et al., 2011).

315 The decrease of the TAC of the plant extracts may be due to the degradation of
316 antioxidant compounds present in the extracts. In fact, some compounds known for their
317 antioxidant activity, such as polyphenols – a major family of natural products found in plants-
318 may be decomposed into different structural forms with different chemical and biological
319 properties during the pancreatic digestion due to their sensitivity to pH variations (Bermúdez-
320 Soto et al., 2007). Indeed it has been shown *in vitro* that the pH influences oxidoreductions of
321 phenolic compounds. Some phenolic compounds have for instance displayed prooxidant
322 activities at pH 7.4, while at lower pH (5.8) they were reported to possess antioxidant
323 activities (Decker, 2009; Moran et al., 1997). Other factors such as the interactions of
324 phenolic compounds with other food components released during digestion (iron, other
325 minerals, dietary fiber, proteins) may influence the antioxidant properties of phenolic
326 compounds. In fact, it has been shown that the antioxidant activity of free phenols is higher
327 compared to iron-phenol chelates (Argyri et al., 2006; Hajji et al., 2006; Seraglio et al., 2017).
328 In addition, the antioxidant activity of phenolic compounds also depends on their chemical
329 structure, mainly the number and position of the hydrogen-donating hydroxyl groups on the
330 aromatic rings (Rice-Evans et al., 1996). Previous works have also showed the degradation of
331 anthocyanins, known antioxidant compounds, after being subjected to the alkaline conditions
332 of the pancreatic digestion process (McDougall et al., 2005; Pérez-Vicente et al., 2002).

333 In this study, a simulated foregut digestion with porcine enzymes was conducted.
334 However, the results obtained with our mimetic horse foregut digestion have to be nuanced.
335 Firstly, the conditions of the simulated foregut horse digestion, based on the work of Abdouli
336 and Attia (2007), used a temperature lightly higher (39°C) than equine body temperature
337 which is in range of 37.5°C and 38°C. Secondly incubation time used in our model was

338 different than what happens during horse's digestion. Indeed food passes the stomach quickly
339 (15 min) in case of it is roughage, and more slowly if it is concentrates and ingested feed
340 reaches the caecum within 2 hours (Jones, 1993). And finally, compared to eight other
341 species, it appear than horses pancreatic secretions into the duodenum have low enzyme
342 activity (Frape, 2004; Lorenzo-Figueras et al., 2007). It was showed in the study of Lorenzo-
343 Figueras et al. (2007) than the specific enzyme activity of five pancreatic enzymes of swine
344 (amylase, lipase, elastase, trypsin, chymotrypsin) was higher than for horses. The activity
345 measurement of five pancreatic enzymes from the adult equine and porcine pancreas was
346 reported by Sweeney (2012), expressed as mean units per milligram of DNA: for amylase, the
347 activity was 2.3 and 107 for equine and porcine respectively, for lipase 41.5 and 49, elastase
348 0.07 and 0.22, trypsin 0.13 and 0.44 and for chymotrypsin 0.36 and 2.26. Thus, considering
349 these different points, in our study the impact of the enzymes on the TAC values is higher
350 than expected *in vivo*. *In vivo* animal studies will have to come in support of the obtained *in*
351 *vitro* data.

352 **Conclusion**

353 This study shows that despite the negative impact of the digestion on their antioxidant
354 activities, the studied plants remain a source of antioxidant compounds. Despite the
355 acknowledged limitations of *in vitro* models that try to mimic *in vivo* conditions, they remain
356 a useful tool to investigate enzymatic activity and its effect on biological activities of
357 antioxidants. It would be important to investigate in detail the outcome of the bioactive
358 compounds after the digestion by identifying their degradation products and the subsequent
359 impact on their bioavailability and biological activity.

360

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364

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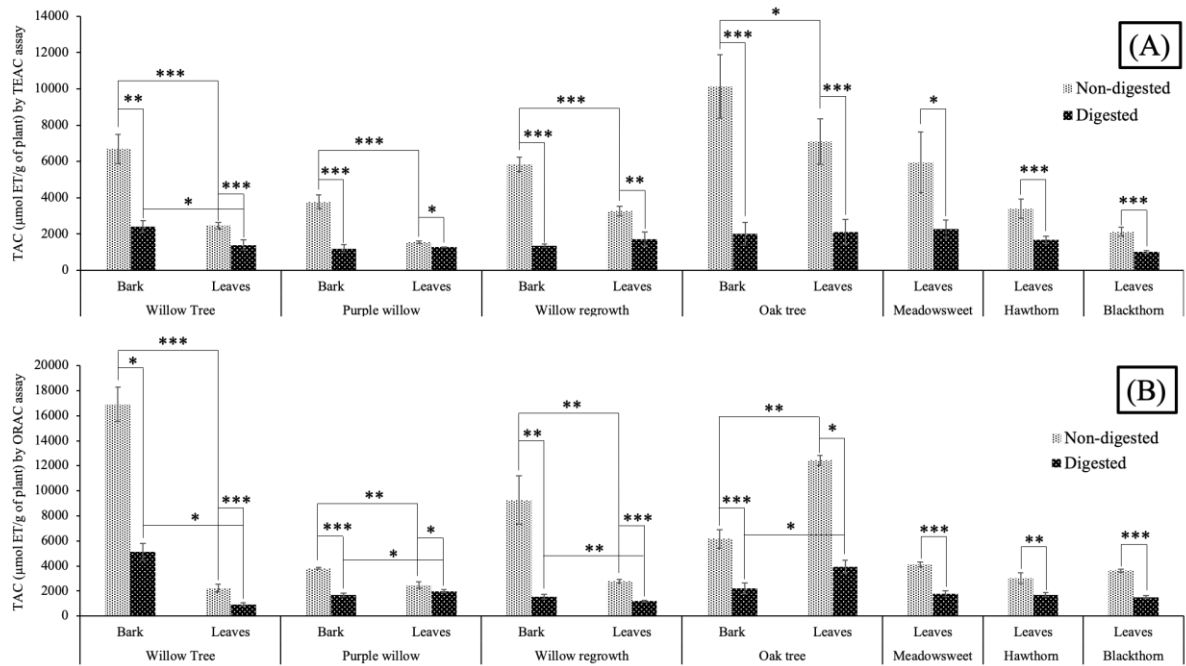
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Figure 1. Total antioxidant capacity (TAC) of plant aqueous extracts before and after simulated foregut digestion obtained with TEAC (A) and ORAC (B) assays (n=3, *t*-test, $\alpha=0.05$, * $0.01 < P < 0.05$; ** $0.001 < P < 0.01$; *** $P < 0.001$).

471 **Table 1.** Loss of total antioxidant capacity (TAC) in bark, leaf and whole plant extracts after
 472 simulated foregut digestion, in percent.

Plants		TAC loss (%)	TAC loss (%)
		TEAC assay	ORAC assay
Willow Tree	Bark	64 %	70 %
	Leaves	43 %	58 %
Oak tree	Bark	80 %	64 %
	Leaves	70 %	68 %
Purple willow	Bark	68 %	55 %
	Leaves	16 %	20 %
Willow regrowth	Bark	77 %	83 %
	Leaves	47 %	58 %
Meadowsweet	Whole plant	62 %	57 %
Hawthorn	Whole plant	51 %	44 %
Blackthorn	Whole plant	52 %	58 %

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