

Alma Mater Studiorum Università di Bologna
Archivio istituzionale della ricerca

Anticancer activity of an Artemisia annua L. hydroalcoholic extract on canine osteosarcoma cell lines

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:

Salaroli, R., Andreani, G., Bernardini, C., Zannoni, A., La Mantia, D., Protti, M., et al. (2022). Anticancer activity of an Artemisia annua L. hydroalcoholic extract on canine osteosarcoma cell lines. RESEARCH IN VETERINARY SCIENCE, 152, 476-484 [10.1016/j.rvsc.2022.09.012].

Availability:

This version is available at: <https://hdl.handle.net/11585/899294> since: 2022-11-03

Published:

DOI: <http://doi.org/10.1016/j.rvsc.2022.09.012>

Terms of use:

Some rights reserved. The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>).
When citing, please refer to the published version.

(Article begins on next page)

1 ***Anticancer activity of an Artemisia annua***
2 ***L. hydroalcoholic extract on canine***
3 ***osteosarcoma cell lines***

4 Roberta Salaroli¹, Giulia Andreani^{1*}, Chiara Bernardini¹,
5 Augusta Zannoni¹, Debora La Mantia¹, Michele Protti², Monica
6 Forni¹, Laura Mercolini², Gloria Isani¹

7 ¹Department of Veterinary Medical Sciences (DIMEVET), Alma
8 Mater Studiorum - University of Bologna, 40064 Ozzano Emilia,
9 Bologna, Italy.

10 ²Pharmaco-Toxicological Analysis Laboratory (PTA Lab),
11 Department of Pharmacy and Biotechnology (FaBiT), Alma
12 Mater Studiorum - University of Bologna, 40126 Bologna, Italy*

13 Corresponding author* at: via Tolara di Sopra 50, 40064
14 Ozzano Emilia, Bologna, Italy.

15 E-mail addresses:

16 roberta.salaroli@unibo.it (R. Salaroli)

17 *giulia.andreani2@unibo.it (G. Andreani)

18 chiara.bernardini5@unibo.it (C. Bernardini)

19 debora.lamantia2@unibo.it (D. La Mantia)

20 michele.protti2@unibo.it (M. Protti)

21 monica.forni@unibo.it (M. Forni)

22 laura.mercolini@unibo.it (L. Mercolini)

23 gloria.isani@unibo.it (G. Isani)

24 **Abstract**

25 Since ancient times, *Artemisia annua* (*A. annua*) has
26 been used as a medicinal plant in Traditional Chinese
27 Medicine. In addition, recent studies have
28 investigated the cytotoxic effects of *A. annua* extracts
29 towards cancer cells. The leading aim of the present
30 research is to evaluate the cytotoxic effects of an
31 hydro alcoholic extract of *A. annua* on two canine
32 osteosarcoma (OSA) cell lines, OSCA-8 and OSCA-
33 40, focusing on the possible involvement of
34 ferroptosis.

35 The quantitative determination of Artemisinin
36 concentration in the extract, culture medium and
37 OSA cells was carried out through the use of an
38 instrumental analytical method based on liquid
39 chromatography coupled with spectrophotometric
40 detection and tandem mass spectrometry (HPLC-
41 DAD-MS/MS). OSCA-8 and OSCA-40 were exposed
42 to different dilutions of the extract for the EC₅₀
43 calculation then the uptake of Artemisinin by the cells,
44 the effects on the cell cycle, the intracellular iron
45 level, the cellular morphology and the lipid oxidation
46 state were evaluated. A concentration of Artemisinin
47 of 63.8 ± 3.4 µg/mL was detected in the extract. A
48 dose-dependent cytotoxic effect was evidenced. In

49 OSCA-40 alterations of the cell cycle and a
50 significantly higher intracellular iron content were
51 observed. In both cell lines the treatment with the
52 extract was associated with lipid peroxidation and
53 with the appearance of a “ballooning” phenotype
54 suggesting the activation of ferroptosis. In conclusion
55 the *A. annua* hydroalcoholic extract utilized in this study
56 showed anticancer activity on canine OSA cell lines
57 that could be useful in treating drug resistant canine
58 OSAs.

59 **Keywords:** *Artemisia annua*, canine osteosarcoma
60 cell lines, iron, lipid peroxidation, ballooning
61 phenotype, ferroptosis.

62

63 **1. Introduction**

64 Extracts of *Artemisia annua* L. are well-known
65 remedies in Chinese Traditional Medicine and have
66 been used to treat malaria and fever in Asia and
67 Africa [1]. *A. annua* is characterized by the unique
68 presence of artemisinin, a sesquiterpene trioxane
69 lactone, which contains an endoperoxide bridge
70 essential for its bioactivity. Artemisinin and its
71 derivatives demonstrated also anticancer activity in

72 different human and animal cancer cell lines [2],
73 targeting different pathways, including inhibition of
74 cell proliferation, induction of apoptosis, and
75 inhibition of angiogenesis and metastasis [3]. In
76 addition, artemisinin reveals an additional anticancer
77 mechanism through induction of ferroptotic cell death
78 [4]. To sustain increased proliferation, tumour cells
79 have high iron requirement, a phenomenon also
80 known as “iron addiction” and are characterized by
81 high intracellular iron content [5]. The endoperoxide
82 bridge of artemisinin is strategic for its
83 pharmacological activity, in fact its cleavage leads to
84 the formation of radical species and induces
85 oxidative stress [6]. In addition, in the presence of
86 reduced ferrous ions or heme iron, artemisinin can
87 become a potent alkylating agent, capable of
88 inducing direct oxidative damage. Consequently, an
89 iron-mediated lethal lipid peroxidation called
90 ferroptosis can occur in cancer cells leading to cell
91 death [7, 8]. Thus, iron plays an important role in the
92 selective toxicity of artemisinin towards cancer cells.
93 Osteosarcoma (OSA) is the most common primary
94 bone tumour in dogs and humans [9-11]. In veterinary
95 medicine, OSA accounts for 2-5% of all canine
96 neoplasms [7] and 80-85% of all bone tumours [12].

97 A study on 162 dogs with appendicular
98 osteosarcoma reported a median survival of 19.2
99 weeks. The one-year and two-year survival rates are
100 11.5% and 2%, respectively. Many dogs die or are
101 suppressed due to the presence of pulmonary
102 metastases [13]. Current treatment for canine OSA
103 (cOSA) involves surgery to remove primary tumours;
104 however, dogs treated with surgery alone have a
105 short survival time. Surgery combined with
106 chemotherapy can increase the survival of dogs with
107 OSA, and protocols include doxorubicin, cisplatin,
108 and carboplatin used alone or in combination [12].
109 However, drug resistance is a critical issue
110 determining the failure of therapy in many cases.
111 Therefore, it would be of paramount importance
112 implement the choice of possible drugs to be used in
113 chemotherapy and also to provide low-cost treatment
114 for those animals that do not have access to
115 chemotherapy for economic reasons. Two previous
116 *in vitro* studies have demonstrated the cytotoxicity of
117 dihydroartemisinin on different cOSA cell lines [14]
118 and of an hydroalcoholic extract and pure artemisinin
119 on cOSA D-17 cell line [15, 16].
120 The aim of this research is to deepen the knowledge
121 on the cytotoxic and anti-proliferative effects of an

122 hydroalcoholic commercial extract of *A. annua* on two
123 additional canine osteosarcoma cell lines, OSCA-8
124 and OSCA-40, focusing on the possible involvement
125 of ferroptosis. In detail, to provide more specific
126 therapeutical indications, the aims of the work were
127 to determine: i) the concentration of Artemisinin in the
128 phytoextract and in the culture media and cells after
129 the treatment; ii) the cytotoxicity and the anti-
130 proliferative effects of the extract; iii) the intracellular
131 iron content alteration following the treatment. All
132 tests have been performed for comparison also with
133 the primary compound Artemisinin.

134

135 **2. Materials and Methods**

136 **Cells, chemicals and reagents**

137 Canine osteosarcoma cell lines OSCA-8 and OSCA-
138 40 were purchased from Kerfast, Inc. (Boston, MA,
139 USA). Minimum Essential Medium (MEM) with
140 GlutaMAX, Foetal Bovine Serum (FBS), Antibiotic-
141 Antimycotic solution, Dulbecco Phosphate Buffered
142 Saline (DPBS), DPBS without calcium and
143 magnesium (PBS w/o Ca²⁺ and Mg²⁺), RNaseA/T1
144 were purchased from Thermo Fisher Scientific

145 (Waltham, MA, USA). Dimethyl Sulfoxide (DMSO),
146 Fluoroshield™ histology mounting medium and
147 erastin were purchased from Merck (Darmstadt,
148 Germany). Propidium iodide (PI) and Hoechst 33342
149 staining solution were purchased from Miltenyi Biotec
150 (Bergisch Gladbach, Germany). Lipid Peroxidation
151 Assay Kit was purchased from Abcam (Cambridge,
152 UK). All plastic supports for cell culture and 8-well
153 slide chambers were purchased from Corning-
154 Beckton-Dickinson (Franklin Lakes, NJ, USA).
155 Artemisinin (CAS number: 63968-64-9), acetonitrile,
156 methanol, formic acid (all mass spectrometry-grade)
157 were obtained from Sigma Aldrich (St. Louis, MO,
158 USA). Artemisinin-D3 pure powder, used as the
159 internal standard (IS), was provided by Biosynth (St.
160 Gallen, Switzerland). All solutions used for LC-DAD-
161 MS/MS analysis were stored protected from light in
162 amber glass vials certified for mass spectrometry
163 from Waters Corporation (Milford, MA, USA). A
164 commercial hydroalcoholic extract obtained from
165 aerial parts of *A. annua* and composed by 65%
166 ethanol, 20% of aerial parts and water was used
167 (*Artemisia annua* hydroalcoholic solution, Sarandrea
168 Marco C. srl, Fr, Italy).

169 **MEPS-LC-DAD-MS/MS determination of**
170 **artemisinin**

171 Quali-quantitative analytical determinations were
172 carried out exploiting a previously developed and
173 fully validated methodology based on microextraction
174 by packed sorbent (MEPS) coupled to liquid
175 chromatography with diode array detection and
176 tandem mass spectrometry (LC-DAD-MS/MS) for the
177 determination of Artemisinin in extracts and
178 commercial products [17]. Briefly, LC-DAD-MS/MS
179 analysis was performed using a Waters (Milford, MA,
180 USA) Alliance e2695 chromatographic system
181 equipped with autosampler coupled to a Waters 2998
182 photodiode array detector and a Waters Micromass
183 Quattro Micro triple-quadrupole mass spectrometer,
184 interfaced with an electrospray ion source working in
185 positive ionisation mode (ESI+). Chromatography
186 was obtained a Restek (Bellefonte, PA, US) Ultra AQ
187 reverse-phase C18 column (50 × 2.1mm I.D., 3µm),
188 kept at room temperature and equipped with a C18
189 guard column (10 × 2.1mm I.D., 3µm), while injection
190 volume was 10 µL. An automated composition
191 gradient program managed a 2-component mobile
192 phase composed of 0.25% formic acid in water
193 (component A) and 0.25% formic acid in acetonitrile

194 (component B), flowing at a constant rate of 0.2
195 mL/min: T=0 min, A:B 70:30; T=2 min, A:B 10:90;
196 T=5 min, A:B 10:90; T=6 min, A:B 70:30; T=8, A:B
197 70:30. To detect Artemisinin, DAD was set at 232 nm,
198 while for MS/MS analysis, multiple reaction
199 monitoring (MRM) was used exploiting two different
200 exclusive *m/z* transitions (one for quantitative
201 purposes, one for qualitative confirmation) for both
202 Artemisinin (283.24 → 209.45; 283.24 → 265.36)
203 and Artemisinin-D3, used as internal standard (IS,
204 286.31 → 212.38; 286.31 → 268.34). For sample
205 pretreatment, all the samples involved in this study
206 (hydroalcoholic extract, cell pellets and cell culture
207 supernatant) were subjected to MEPS pretreatment
208 before LC analysis. Cell pellets from 1×10^6 cells were
209 preliminarily homogenized in 0.1 M, pH 5.5 sodium
210 phosphate buffer (1 mL/sample). The mixtures were
211 centrifuged at 4000 rpm for 10 min (4 °C) and the
212 supernatants were collected. 100- μ L aliquots of the
213 hydroalcoholic extract/cell pellet extract/cell culture
214 supernatant were then subjected to a MEPS
215 following a protocol developed ad-hoc for Artemisinin
216 analysis and involving a miniaturised apparatus
217 based on C8 sorbent [17].

218 **Cell culture and treatments**

219 OSCA-8 and OSCA-40 were cultured in MEM with
220 GlutaMAX, 5% foetal bovine serum (FBS) and 1%
221 antibiotic/antimycotic solution and expanded in T-25
222 or T-75 culture flasks at 2.5×10^4 cells/cm² seeding
223 density, at 37°C and 5% CO₂. The commercial
224 extract was directly diluted in the culture medium to
225 obtain the required artemisinin concentrations, based
226 on the artemisinin concentration determined in the
227 phytoextract as previously described. Artemisinin
228 powder was firstly dissolved in DMSO and then
229 diluted in the culture medium. Control cells were
230 treated with equivalent amount of ethanol (ranging
231 dilution 0.3-10%) or DMSO (0.05-3%) used as
232 specific vehicles.

233 **Cytotoxicity and EC₅₀ determination**

234 The two cell lines were seeded in 96-well plates
235 (1×10^4 cells/well) and exposed, for 24 h, to increasing
236 doses of *A. annua* hydroalcoholic extract
237 corresponding to Artemisinin concentrations of 0,
238 0.22, 0.44, 1.1, 2.2, 4.4, and 35.2 µM, calculated on
239 the measured concentration of Artemisinin in the
240 hydroalcoholic extract or with increasing
241 concentrations of pure Artemisinin (0, 50, 100, 500,
242 1000, 2000, 3000 µM). Cytotoxicity was measured

243 using tetrazolium salt (In Vitro Toxicology Assay Kit,
244 MTT-based). Briefly, the MTT substrate was added
245 to the culture medium and incubated for 3 h, then the
246 MTT solubilization solution was added to the cells to
247 dissolve the formazan crystals. The formazan
248 absorbance was measured at a wavelength of 570
249 nm, using Infinite[®] F50/Robotic Absorbance
250 microplate readers (TECAN, LifeScience). The
251 background absorbance of multiwell plates at 690 nm
252 was also measured and subtracted from the 570 nm
253 measurements. EC₅₀ values were calculated from
254 dose-response curves using nonlinear regression
255 analysis tool in GraphPad Prism 7 software
256 [log(agonist) vs. normalized response - Variable
257 slope] (GraphPad San Diego, CA, USA). Each assay
258 was performed thrice independently, with seven
259 replicates each.

260 **Cell cycle analysis**

261 OSCA-8 and OSCA-40 cells were seeded (2.5×10^5)
262 in 6-wells plates in complete medium and, when
263 confluence reached round about 70%, cells were
264 treated with *A. annua* hydroalcoholic extract and with
265 Artemisinin EC₅₀ doses for 24 h in a humidified CO₂
266 incubator. EtOH and DMSO exposed cells were

267 considered as controls as described above. After 24
268 h of treatment, cells were harvested, counted,
269 washed twice in 5 mL of DPBS w/o Ca²⁺ and Mg²⁺
270 then fixed overnight in 70% ice-cold EtOH
271 (1mL/1x10⁶ cells) added drop-by-drop under
272 continuous vortex mixing. After fixation, the cells
273 were washed with 10 mL DPBS w/o Ca²⁺ and Mg²⁺
274 and cellular pellet was incubated with 1mL/10⁶ cells
275 of staining solution [50 µg/mL PI + 100 µg/mL
276 RNaseA/T1 in DPBS w/o Ca²⁺ and Mg²⁺] for 30 min
277 in the dark at room temperature (RT). The DNA
278 contents 2N (G0/G1 phase), 2– 4N (S phase), and
279 4N (G2/M phase) were evaluated by MACSQuant®
280 Analyzer10 (Miltenyi Biotec, Bergisch Gladbach,
281 Germany) and Flow Logic software (Inivai
282 Technologies, Australia) as previously described
283 [18]. Dean-Jett-Fox Univariate Model was used to
284 determine the percentage of the cell population in the
285 distinct phases of the cell cycle [19]. The experiment
286 was repeated three times.

287 **Iron quantification in OSCA-8 and OSCA-40**

288 For the quantification of intracellular iron, cells were
289 seeded and grown in wells as previously described.
290 Then, cells were treated with *A. annua* hydroalcoholic

291 extract or Artemisinin at the respective EC_{50} doses,
292 for 24 h. After the treatment cells were harvested and
293 centrifuged at 800 x g for 10 min. The pellet was
294 washed twice with DPBS, and then 1×10^6 cells were
295 resuspended in 1 ml of a solution of 1 M HNO_3 ,
296 digested at room temperature until completely
297 dissolved, and finally used for iron quantification
298 using a Spectra AA-20 atomic absorption
299 spectrometer (Varian) equipped with a GTA-96
300 graphite tube atomizer and a sample dispenser. Final
301 data were expressed as pg Fe/cell.

302 The optimization of the analytical method was
303 obtained following Tüzen [20] with minor changes.
304 The graphite tubes employed were coated GTA
305 tubes (Agilent Technologies, Germany), the hollow
306 cathode lamp current was 7 mA and measurements
307 were performed at 248.3 nm resonance lines using a
308 spectral slit width of 0.2 nm. During
309 spectrophotometer readings, internal argon flow rate
310 in the partition graphite tubes was maintained at 300
311 mL/min and was interrupted in the atomization
312 phase. Ramp and hold times for drying, pyrolysis,
313 atomization and cleaning temperatures were
314 optimized to obtain maximum absorbance without

315 significant background absorption, therefore,
316 background correction was not necessary.
317 The calibration curve was obtained by diluting 1
318 mg/mL standard stock solution of iron (Iron Standard
319 for AAS, Sigma-Aldrich, St Louis, Missouri, USA) with
320 Suprapur water (Supelco, St Louis, Missouri, USA) to
321 obtain working standards containing 0, 20, 40 and 60
322 ng/mL of iron and by plotting the absorbance at 248.3
323 nm against iron concentrations. The equation of the
324 curve was $y = 0.0121x$ and the calculated regression
325 coefficient (r) was 0.996. The method was validated
326 with standard reference material (ERM[®] - BB422)
327 and the accuracy of the method, calculated as the
328 percentage of the certified value, resulted of 106 %.
329 The detection limit (LOD), defined as the
330 concentration corresponding to 3 times the standard
331 deviation of 6 blanks, was 0.8 ng/mL

332 **Light microscopic evaluation**

333 OSCA-8 and OSCA-40 cell lines were treated for 24
334 h with *A. annua* hydroalcoholic extract, Artemisinin at
335 the EC₅₀ dose or with erastin (10 μM) that triggers
336 ferroptosis [21], an iron-dependent form of non-
337 apoptotic cell death. The cell death morphology was
338 observed and acquired using an inverted microscope

339 (Eclipse TS100, Nikon, Tokyo, Japan) equipped with
340 a digital camera (Digital C-Mount Camera TP3100,
341 Kowa, Aichi, Japan).

342 **Lipid Peroxidation Assay**

343 Lipid peroxidation in OSCA-8 and OSCA-40 treated
344 with *A. annua* hydroalcoholic extract or with
345 Artemisinin was evaluated by the Lipid Peroxidation
346 Assay Kit (Abcam, Cambridge, UK) following
347 manufacturer's instructions. The day before the
348 experiment 1×10^5 cells/well were seeded in 8 well
349 chamber slides and the cells were incubated for 24 h
350 with *A. annua* hydroalcoholic extract, with Artemisinin
351 at the respective EC₅₀ doses or with vehicle controls.
352 To have positive controls OSCA-8 and OSCA-40
353 were treated with erastin (10 μ M) for 24 h. Lipid
354 Peroxidation Assay Kit uses a sensitive sensor that
355 changes its fluorescence from red to green upon
356 peroxidation by ROS in cells. The cells were also
357 stained with Hoechst 33342 during the last 10
358 minutes of incubation with lipid peroxidation sensor.
359 Fluorescence of the cells was monitored with a
360 fluorescence microscope (Eclipse E600, Nikon)
361 equipped with a digital camera (RETIGA-2000RV,
362 Surrey, Canada) through FITC/TRITC channels.

363 **Statistical analysis**

364 Data for MTT were analysed with one-way analysis
365 of variance (ANOVA) followed by post hoc Dunnett's
366 multiple comparison test. Data of the cell cycle and
367 iron content were analysed by paired Student's t-test.
368 $p < 0.05$ was considered significant.

369 **3. Results and discussion**

370 **Quantification of artemisinin in *A. annua* extract**
371 **and artemisinin cOSA uptake**

372 For this purpose, a very sensitive method was
373 developed, based on high performance liquid
374 chromatography coupled to diode array detection
375 and tandem mass spectrometry (HPLC-DAD-
376 MS/MS). This method was previously validated with
377 satisfactory results in terms of sensitivity (LOQ=5
378 ng/mL and LOD=1.5 ng/mL), linearity ($r^2 > 0.9995$
379 over the 5-1000 ng/mL artemisinin concentration
380 range), extraction yield (>85 %), precision
381 (RSD%<3.5) and accuracy (88-93% range), allowing
382 an accurate determination of artemisinin
383 concentrations in different matrices.

384 In the hydroalcoholic extract of *A. annua* considered
385 in this study, a concentration of artemisinin of $63.8 \pm$

386 3.4 µg/mL, corresponding to 0.23 mM, was detected.
387 The value is in accordance with those reported by
388 Protti et al. (2019) [17]. In that research, extracts
389 prepared ad hoc from herbal material by the authors
390 were analysed (Artemisinin concentration was 21.40
391 µg/mL for the hydroalcoholic extract and 109.40
392 µg/mL for the artemisinin-enriched extract prepared
393 following Chinese Pharmacopeia), as well as a
394 commercial extract sold as food supplement (94.79
395 µg/mL). The results obtained in this study are also
396 consistent with previously reported data, even if
397 Artemisinin concentration shows a pronounced
398 variability depending on the source, ranging from 60
399 µg/mL [22] to 200-500 µg/mL [23].

400 To verify the uptake of artemisinin by the cells,
401 Artemisinin content was determined either in the
402 incubation media or in the OSCA-8 and OSCA-40 cell
403 lines after 24 hours of exposure to *A. annua* extract
404 or Artemisinin at the EC₅₀ doses (Table 1). Cells
405 actively took up Artemisinin, which reached a
406 concentration of 1.66 pg/cell in OSCA-40 cell line. In
407 both cell lines, the intracellular concentration of
408 Artemisinin is higher in the case of exposure to pure
409 Artemisinin than to the phytoextract, in agreement

410 with the higher concentrations in the medium and
411 higher EC₅₀ values.

412 The innovative analytical method gave the
413 opportunity to accurately determine the concentration
414 of Artemisinin taken up by cells and allowed an
415 evidence-based discussion of the cytotoxic effects of
416 the extracts.

417

418 Table 1. Artemisinin concentration determined in culture
419 medium and in OSCA-8 and OSCA-40 cell lines after 24 hours
420 of exposure to pure Artemisinin (A) and *A. annua* extract (E) at
421 the EC₅₀ doses.

422

	Medium µg/mL	Intacellular Artemisinin pg/cell
OSCA-8 (A)	4.05	2.54
OSCA-8 (E)	0.41	1.36
OSCA-40 (A)	3.76	2.42
OSCA-40 (E)	0.38	1.66

423

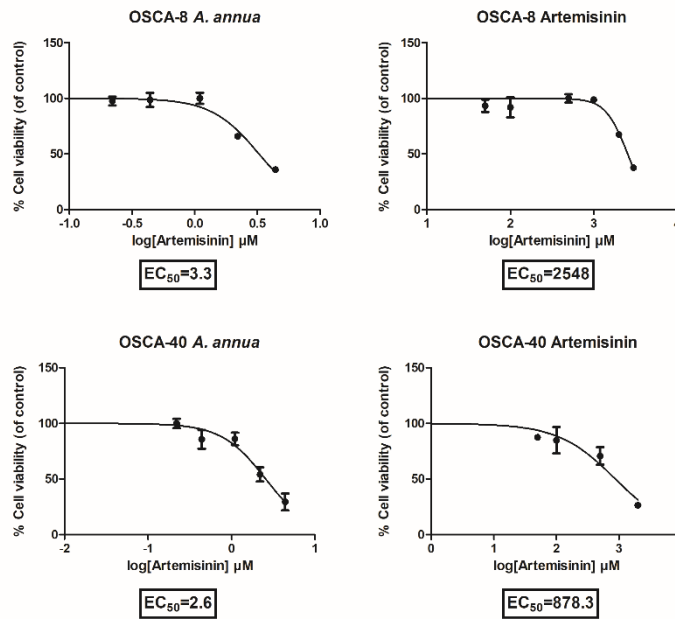
424

425 ***Artemisia annua* hydroalcoholic extract is**
426 **cytotoxic for canine OSA cell lines**

427 MTT assay was used to determine the effect of an *A.*
428 *annua* hydroalcoholic extract containing 63.8 µg
429 artemisinin/mL or primary compound Artemisinin on
430 the growth of 2 different canine OS cell lines: OSCA-
431 8 e OSCA-40. The *A. annua* hydroalcoholic extract
432 showed a dose-dependent cytotoxic effect inhibiting
433 the proliferation of the two canine OSA cell lines with
434 EC₅₀ of 3.3 and 2.6 µM for OSCA-8 and OSCA-40
435 respectively, while Artemisinin showed an EC₅₀ of
436 2548 µM for OSCA-8 and of 878.3 µM for OSCA-40.
437 (Figure 1). Accordingly, a similar toxic effect was
438 previously reported for D-17 canine OSA cell line by
439 Isani et al., (2019) [15] and a marked dose-
440 dependent toxic effect of an extract of *A. annua*,
441 obtained by pressurized cyclic solid–liquid extraction,
442 was reported by Culurciello et al. (2021) [16] on a
443 different canine OS cell line (CRL2130). The extract
444 presented significantly lower EC₅₀ values than
445 Artemisinin (Fig. 1). The EC₅₀ values for Artemisinin
446 determined in this study are one-order magnitude

447 lower than those reported for pure Artemisinin in two
448 other canine tumour cell lines, DH82 and D-GBM, by
449 Saeed et al. (2020) [24], suggesting a more potent
450 cytotoxic effect of the phytoextract. Indeed, the
451 extract contains many other cytotoxic compounds in
452 addition to Artemisinin, including polyphenols,
453 flavonoids, coumarins, and phytosterols. Important
454 constituents are camphene, camphor, beta-
455 caryophyllene, pinene, 1,8-cineole, and scopoletin
456 [25]. Volatile essential oils are also present at
457 concentrations of 0.20-0.25%. All these secondary
458 metabolites acting in a multi-specific manner against
459 tumours can contribute to the toxic effect of the
460 phytoextract [26]. The data reported in the present
461 research add more evidence on the potency of *A.*
462 *annua* extracts, which inhibit the growth of canine
463 osteosarcoma cells, and might be considered
464 promising anti-tumour candidate for further
465 development.

466

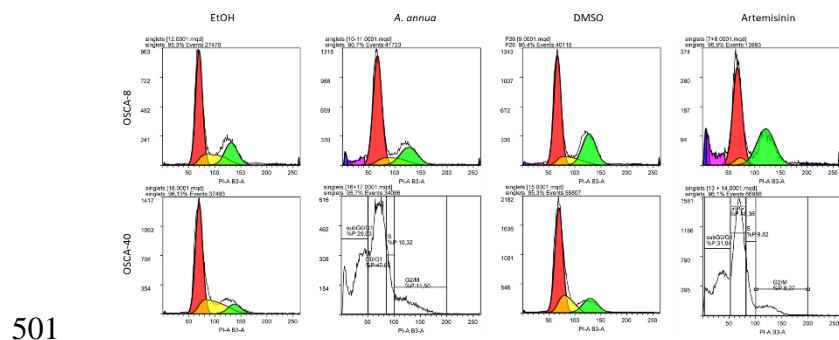


467

468 **Figure 1_ *A. annua* hydroalcoholic extract and Artemisinin**
 469 **impair cell viability of the canine OSA cell lines OSCA-8 and**
 470 **OSCA-40.** The cells were treated with increasing
 471 concentrations of *A. annua* hydroalcoholic extract, Artemisinin
 472 or vehicles for 24 h and the cell viability measured by MTT
 473 assay. Dose-response curves represent mean \pm SD from three
 474 independent experiments with seven replicates each($n=3$).

475 The cytotoxic effects of *A. annua* could be related to
 476 DNA damage, oxidative stress, and alteration of
 477 tumour-related signal transduction pathways [2, 24].
 478 The effect of the extract on cell cycle was evaluated
 479 by flow cytometry and data were analysed with Flow
 480 Logic software. The cells grew as asynchronous
 481 populations represented by cells in all stages of the
 482 cell cycle. For OSCA40 cell line treated with *A. annua*
 483 extract and with Artemisinin at the EC_{50} doses, the

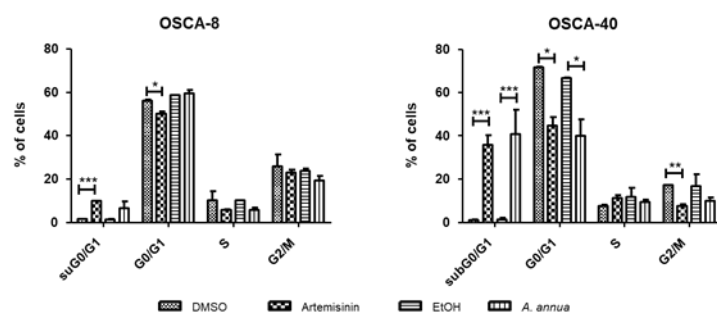
484 gates were inserted manually because Dean-Jett-
 485 Fox Model failed to distinguish the different phases of
 486 the cell cycle. Considering the cells treated with the
 487 vehicles (EtOH or DMSO) as control, it can be
 488 observed that *A. annua* extract and Artemisinin
 489 treatments impair the cellular distribution in the
 490 different cell cycle phases. Particularly, in OSCA-8
 491 cell line pure Artemisinin, but not *A. annua* extract,
 492 determined a significant decrease ($P<0.05$) of the
 493 cells in G0/G1 accompanied by a significant increase
 494 ($P<0.001$) of the cells in sub-G0/G1 phase. In OSCA-
 495 40, both treatments strongly influenced the cell
 496 distribution with significant decrease ($p<0.05$) of the
 497 cells in G0/G1 accompanied by a significant increase
 498 ($p<0.001$) of the cells in sub G0/G1 phase. (Fig. 2,3).
 499 These data add further evidence to the effects of
 500 Artemisinin on the cell cycle.



502 **Figure 2_ *A. annua* hydroalcoholic extract and Artemisinin**
 503 **impair cell cycle of the canine OSA cell lines.** The cells were
 504 treated with *A. annua* extract and Artemisinin at the EC₅₀ doses

505 or vehicle (EtOH or DMSO) for 24 h and fluorescence of the PI-
 506 stained cells was measured using MACSQuant® Analyzer10
 507 and analysed by Flow Logic software (Inivai Technologies,
 508 Australia). 5×10^5 cells were examined for each sample and
 509 experiment was repeated three times. Representative DNA
 510 content frequency histograms in OSCA-8 and OSCA-40. Sub
 511 G0/G1 blue/purple, G0/G1 red, S yellow, G2/M green.

512



513

514 **Figure 3_ Grouped histograms graphs of cell cycle**
 515 **distribution in OSCA-8 and OSCA-40.** The cell lines were
 516 treated with *A. annua* hydroalcoholic extract and Artemisinin at
 517 the EC₅₀ doses for 24 h or vehicles (EtOH or DMSO). Cell
 518 percentages were averaged over triplicate samples, and the
 519 data are expressed as the mean \pm SD. Paired Student's t-test,
 520 (*p<0.05, **p<0.01; ***p<0.001) was performed between
 521 controls and treated cells (n=3).

522 As a matter of fact, Artemisinin and its derivatives
 523 (dihydroartemisinin, artesunate, artemether,
 524 arteether) are known to affect the cell cycle of several
 525 types of tumour cells in different ways, depending on
 526 specific defects of the machinery regulating the cell

527 cycle of tumour cell lines [27]. In OSCA-8 the
528 exposure to pure Artemisinin but, in OSCA-40, also
529 the exposure to *A. annua* extract induced a significant
530 decrease of the cells in G0/G1 accompanied by a
531 significant increase of the cells in sub G0/G1 phase.
532 The same profile was also reported for other canine
533 and human OSA cell lines treated with
534 dihydroartemisinin [14, 28]. A dose-dependent
535 accumulation of MDA-MB-468 and SK-BR-3 breast
536 cancer cells in the sub-G1 fraction following the
537 exposure to artesunate, a semi-synthetic derivative
538 of Artemisinin, has been reported also by
539 Greenshields et al. (2019) [29]. Sub-G0/G1 peak is
540 composed by dead cells (apoptosis, necrosis,
541 oncosis) and by cells that had already lost their DNA
542 by shedding apoptotic bodies, cellular fragments
543 holding pieces of chromatin, broken nuclei,
544 chromosomes, and cellular debris [30]. It could be
545 hypothesised that Artemisinin in *A. annua*
546 hydroalcoholic extract extensively impairs DNA
547 integrity in OSCA-40 cells and an efficient G1
548 checkpoint machinery hosted by this canine OSA cell
549 line leads cells to die before replicating their
550 damaged DNA. The DNA damage response (DDR)
551 is a complex system, a network of biochemical

552 pathways that detects DNA damage and decides the
553 cell fate. These pathways include the repair
554 throughout different phases of proliferation, the delay
555 of cell cycle, and the arrest of cell cycle to allow for
556 more comprehensive DNA repair [31]. If the level of
557 DNA damage exceeds the cells repairing ability, cell
558 death is stimulated. DNA damage is caused by
559 various internal and extrinsic factors including
560 reactive oxygen species (ROS) and environmental
561 mutagens [32]. In OSCA-8 cell line, where only pure
562 Artemisinin is able to impair the cell cycle, the DNA
563 damage induced by *A. annua* might be less extensive
564 and unable to lead to a significant cell cycle
565 impairment or the cellular repair machinery could be
566 so efficient to allow a complete repair. This
567 hypothesis is supported by the lower intracellular
568 concentration of Artemisinin measured in OSCA-8
569 (Table 1).

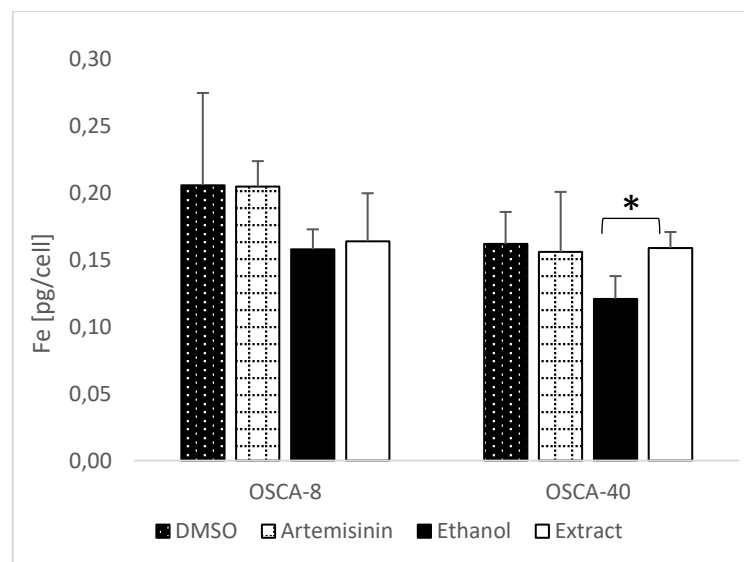
570 ***Artemisia annua* hydroalcoholic extract modifies**
571 **intracellular iron content in canine OSA cell line**

572 Tumours are characterized by high iron content, to
573 satisfy their increased metabolic demand [5]. This is
574 achieved through some crucial changes in iron
575 metabolism, including the increased expression of

576 transferrin receptor-1 (TfR1) in many tumours [33],
577 including cOSA [34]. The intracellular iron content
578 influences the sensitivity of cells to ferroptosis. As a
579 result, to study the iron involvement in the cytotoxicity
580 of *A. annua* extract the need arose to measure the
581 iron content in OSCA-8 and OSCA-40 cell lines with
582 a sensitive and accurate method. This is a
583 challenging task, due to the very small amount of the
584 biological samples; consequently, a specific
585 elemental detector with low detection limit is needed.
586 Complex analytical methods with different degree of
587 accuracy and sensitivity are currently available to
588 measure iron in cells, including FAAS, ICP-MS and
589 TXRF [35]. The analytical FAAS method used in this
590 research with a detection limit of 0.8 ng/mL was able
591 to detect iron in all the samples analysed. Iron
592 content in cells is reported in Figure 4. The variety of
593 analytical methods and the related different units of
594 measurement to express the intracellular iron content
595 hamper the comparison with the data in the literature.
596 Iron content in control untreated cells is like the value
597 reported in canine D-17 OSA cells [15], if expressed
598 as ng/1x10⁶ cells. OSCA-40 cells treated with *A.*
599 *annua* extract at the EC₅₀ dose had a significantly
600 (p<0.05) higher iron content than those treated with

601 the vehicle, while no significant difference was
602 detected for OSCA-8. An increase of intracellular iron
603 content, though measured with a less accurate and
604 specific colorimetric method, was also reported in
605 Saos-2 and U2os human OSA cell lines treated with
606 EF24, a synthetic analogue of curcumin [36]. In both
607 cell lines, no significant effect of Artemisinin was
608 detected in comparison with the vehicle (Figure 4).

609



610

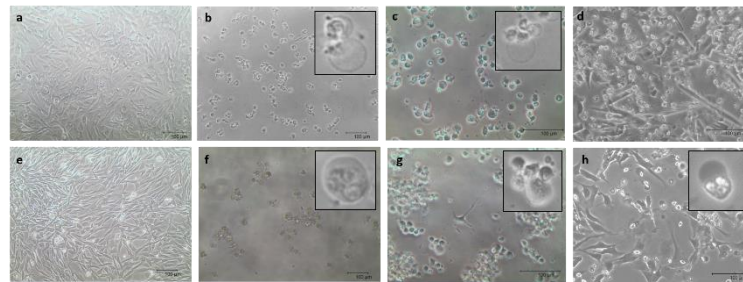
611 **Figure 4_ Intracellular iron content in OSCA-8 and OSCA-**
612 **40.** The vehicles (DMSO for Artemisinin and 65% EtOH for
613 extract) were used as control. Data are expressed as pg Fe/cell
614 and are reported as mean \pm SD from three independent
615 experiments (n=3), each performed in duplicate. Paired
616 Student's t-test, (*p<0.05) was performed between control and
617 treated cells.

618 ***Artemisia annua* hydroalcoholic extract induces**
619 **“ballooning” phenotype in canine OSA cell lines**

620 Cells were exposed for 24 h to *A. annua*
621 hydroalcoholic extract or to Artemisinin at the EC₅₀
622 doses and to 10 μM erastin to investigate the possible
623 involvement of ferroptosis. Erastin, a well-known
624 inducer of ferroptosis, inhibits cystine uptake by the
625 cystine/glutamate antiporter (system xc⁻),
626 decreasing the antioxidant defences of the cell, and
627 ultimately leading to oxidative cell death [21].
628 Ferroptosis is dependent upon intracellular iron and
629 is morphologically, biochemically, and genetically
630 distinct from apoptosis, necrosis, and autophagy [21].
631 It is known that, following the treatment with a pro-
632 ferroptotic agent such as erastin, an initial cell
633 shrinking is followed by condensation of cytoplasmic
634 constituents and a “ballooning” phenotype, which
635 involves the formation of a clear, rounded
636 morphology consisting mainly of empty cytosol. The
637 exact mechanisms underlying the phenotypic
638 changes that occur during ferroptosis remain unclear
639 [37]. In both cell lines treated with *A. annua*
640 hydroalcoholic extract or with 10 μM erastin, and in
641 OSCA-40 cells treated with pure Artemisinin, the
642 microscopic examination revealed loss of attachment

643 to the culture plate and dead cells showed a clear
644 “ballooning” phenotype suggesting that not only
645 erastin but also *A. annua* could trigger ferroptosis in
646 canine OS cell lines. (Fig. 5 b, c, f, g and h) In
647 contrast, the cells treated with the vehicle (EtOH) had
648 no evidence of cytotoxicity nor of such specific
649 phenotype. (Fig. 5 a, e). On the other hand, the
650 OSCA-8 cells treated with pure Artemisinin showed
651 evidence of cytotoxicity, but not a clear “ballooning”
652 phenotype. (Fig. 5d)

653



654

655

656 **Figure 5_ *A. annua* hydroalcoholic extract induces**
657 **“ballooning” phenotype in canine OSA cell lines OSCA-8**
658 **and OSCA-40.** Representative images of OSCA-8 (a, b, c, d)
659 and OSCA-40 (e, f, g, h) treated with *A. annua* hydroalcoholic
660 extract at the EC₅₀ dose (b, f), erastin 10µM (c, g) or EtOH (a,
661 e). Following the treatment with *A. annua* hydroalcoholic extract
662 or erastin 10µM both cell lines showed a “ballooning” phenotype
663 which involves the formation of a clear, rounded morphology
664 consisting mainly of empty cytosol (see in the boxes). In OSCA-

665 40, pure Artemisinin treatment induced a “ballooning”
666 phenotype. Scale bar: 100 μ m.

667

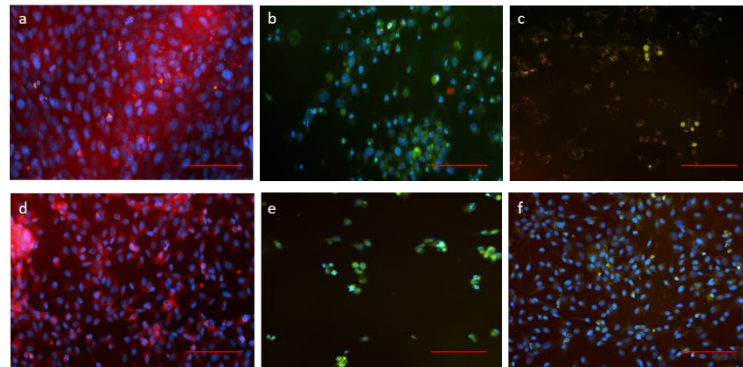
668 ***Artemisia annua* hydroalcoholic extract induces**
669 **Lipid Peroxidation in canine OSA cell lines**

670 In OSCA-8 and OSCA-40 canine OSA cell lines, the
671 treatment with *A. annua* hydroalcoholic extract and
672 with pure Artemisinin at the EC₅₀ doses for 24 h leads
673 to extensive lipid peroxidation as indicated by a clear
674 shift from red to green of the Lipid Peroxidation
675 Sensor (Fig. 6). Lipid peroxidation is an oxidative
676 degradation and ROS play a dual role, beneficial
677 and/or deleterious. Indeed, a growing body of
678 evidence shows that within cells ROS act as
679 secondary messengers in intracellular signalling
680 cascades, inducing and maintaining the oncogenic
681 phenotype of cancer cells both in humans and dogs
682 [38, 39]. However, ROS can also induce cellular
683 senescence, apoptosis, ferroptosis and can therefore
684 function as anti-tumourigenic species [38].
685 Artemisinin and its derivatives induce ROS
686 overproduction, triggering peroxidation of membrane
687 lipids and cell death in a wide range of cellular types,
688 including plants, and mammalian cancer cells [8, 40].
689 The increase of ROS production in a dose-dependent

690 manner was also reported by Hosoya et al. (2008)
691 [14] in D-17 cOSA cell line treated with
692 dihydroartemisinin. Since ferroptosis is associated
693 with accumulation of lipid peroxides [21, 37] it could
694 be further speculated that the cytotoxicity of *A. annua*
695 involves ferroptotic cell death.

696

697



698

699 **Figure 6_***A. annua* hydroalcoholic extract induces Lipid
700 Peroxidation in canine OSA cell lines OSCA-8 and OSCA-
701 40. Representative images of OSC-8 (a, b, c) and OSCA-40
702 (d,e,f) treated with *A. annua* hydroalcoholic extract at the EC₅₀
703 dose (b, e), with pure Artemisinin at the EC₅₀ dose (c,f) and
704 controls (untreated cells, a, d). The cells were stained with 1X
705 Lipid Peroxidation Sensor for 30 minutes in complete growth
706 medium at 37°C and stained with Hoechst 33342 during the last
707 10 minutes of incubation. In b, c, e and f a clear shift from red to
708 green was observed. Scale bar: 100 µm.

709

710 **Involvement of ferroptosis**

711 Three main traits define ferroptotic cell death, namely
712 the increase of free iron, the accumulation of lipid
713 peroxides, and a “ballooning” death phenotype that is
714 morphologically distinct from autophagic, apoptotic,
715 or necrotic cell death phenotypes [21]. In both cell
716 lines, *A. annua* hydroalcoholic extract at the EC₅₀
717 doses triggered the appearance of a “ballooning”
718 phenotype as well as extensive lipid peroxidation,
719 while the iron content increased in OSCA-40, but not
720 in OSCA-8. Alteration of iron metabolism is
721 recognized as central mediator of ferroptosis. Ferric
722 ions bound to transferrin are imported into cells using
723 the transferrin receptor 1 (TFR1) and then included in
724 the endosome. In the endosome, ferric ions are
725 reduced to ferrous ions and finally transported into
726 the cytoplasm through the divalent metal transporter
727 1 (DMT1). In the cell cytoplasm a dynamic and
728 controlled labile iron pool (LIP) is present and serves
729 as a crossroad of intracellular iron metabolism [41].
730 In normal cells, this pool is maintained within a
731 narrow range of concentration, while in cancer cells,
732 a reduction of ferritin iron storage can increase the
733 LIP and the risk of oxidative stress, which in turn is
734 able to determine a massive lipid peroxidation. In
735 different human tumour cell lines, including OSA,

736 exposed to dihydroartemisinin an increase of LIP has
737 been reported, due to the increased lysosome-
738 mediated ferritin degradation [8]. However, despite
739 an increasing number of studies, the role of iron in
740 ferroptotic cell death is still to be completely
741 understood, due to the complexity of iron metabolism
742 and homeostasis. OSCA-40 cell line is more
743 sensitive to the cytotoxic effect of the extract, has a
744 lower EC₅₀ value for *A. annua* extract and or pure
745 Artemisinin, a higher intracellular Artemisinin and iron
746 content, and extensive lipid peroxidation associated
747 with a “ballooning” phenotype appeared following the
748 exposure to *A. annua* extract and to pure Artemisinin
749 for 24 hours. This experimental evidence argues in
750 favour of the activation of ferroptosis. Although the
751 method for iron analysis used in this research allows
752 the quantification of total intracellular iron, an
753 imbalance of its metabolism can be hypothesised,
754 leading to an increased ferritin degradation and
755 finally to increased LIP. In OSCA-8 treated with *A.*
756 *annua* extract, even in the presence of the
757 “ballooning” phenotype and lipid peroxidation, no
758 increase in total iron content and no impairment of
759 cell cycle were observed. However, it cannot be
760 excluded an increase of LIP without modifying the

761 intracellular content of iron as well as a different
762 kinetics in DNA damage response mechanisms in the
763 two cell lines.

764 The high chemoresistance is a negative trait of most
765 OS [42] and ferroptosis is considered as an
766 interesting therapeutic strategy to overcome
767 multidrug resistance. Recently, it has been reported
768 that ferroptosis makes OSA cells more susceptible to
769 doxorubicin, collaboratively strengthening the
770 apoptosis-based doxorubicin chemotherapy [43].
771 Therefore *A. annua* may be especially effective in
772 treating drug resistant osteosarcomas. Considering
773 the similarities between many human and canine
774 tumours, advances in deepening knowledge and
775 improving therapeutic protocols may be relevant for
776 both species, in a model of mutual translational
777 medicine. The relevance of *A. annua* as anticancer
778 compound is enhanced by the fact that it is cheap, as
779 compared to other pharmacological interventions
780 available on the market. This could be an advantage
781 for low-income countries [44] or contexts such as for
782 the dog owners' reluctance to choose chemotherapy
783 treatments.

784 **4. Conclusions**

785 The hydroalcoholic extract of *A. annua* showed
786 cytotoxicity on two canine OSA cell lines with
787 increase of total iron, accumulation of lipid peroxides
788 and a “ballooning” death phenotype, suggesting the
789 activation of ferroptosis. However, it should be
790 emphasized that any conclusions from this study
791 must necessarily be confirmed on more cell lines.

792

793 **Author Contributions:**

794 Conceptualization: GI, GA, MF

795 Methodology: RS, CB, DLM, AZ, MF, GA, LM, MP

796 Validation, formal analysis: RS, CB, DLM, AZ, MF, GA,
797 GI, LM, MP

798 Data curation: RS, CB, DLM, AZ, MF, GA, LM

799 Writing—original draft preparation: GI, RS, LM

800 Writing— review and editing: GI, GA, LM, MP

801 Project administration and funding acquisition: GI.

802 All authors have read and agreed to the published version
803 of the manuscript.

804 **Funding:** This research was supported by a grant from
805 “Fondazione Carisbo” (Bologna) (2019.0535, project title:

806 Attività antitumorale di estratti di *Artemisia* in colture
807 cellulari di osteosarcoma di cane: un progetto
808 multidisciplinare di medicina traslazionale).

809 **Conflicts of Interest:** The authors declare no conflict of
810 interest.

811 **References**

812 1. Tu Y. The discovery of artemisinin (qinghaosu) and gifts from
813 Chinese medicine. *Nat Med.* 2011;17:1217–20.

814 2. Efferth T. From ancient herb to modern drug: *Artemisia annua* and
815 artemisinin for cancer therapy. *Semin Cancer Biol.* 2017;46:65–83.

816 3. Ho WE, Peh HY, Chan TK, Wong WSF. Artemisinins:
817 pharmacological actions beyond anti-malarial. *Pharmacol Ther.*
818 2014;142:126–39.

819 4. Zhu S, Yu Q, Huo C, Li Y, He L, Ran B, et al. Ferroptosis: A Novel
820 Mechanism of Artemisinin and its Derivatives in Cancer Therapy.
821 *Curr Med Chem.* 2021;28:329–45.

822 5. Torti SV, Torti FM. Iron and cancer: more ore to be mined. *Nat Rev*
823 *Cancer.* 2013;13:342–55.

824 6. Krishna S, Uhlemann A-C, Haynes RK. Artemisinins: mechanisms
825 of action and potential for resistance. *Drug Resist Updat.* 2004;7:233–
826 44.

827 7. Farcas N, Arzi B, Verstraete FJM. Oral and maxillofacial
828 osteosarcoma in dogs: a review: Canine oral osteosarcoma. *Vet Comp*

- 829 Oncol. 2014;12:169–80.
- 830 8. Chen G-Q, Benthani FA, Wu J, Liang D, Bian Z-X, Jiang X.
831 Artemisinin compounds sensitize cancer cells to ferroptosis by
832 regulating iron homeostasis. *Cell Death Differ.* 2020;27:242–54.
- 833 9. Gola C, Giannuzzi D, Rinaldi A, Iussich S, Modesto P, Morello E,
834 et al. Genomic and Transcriptomic Characterization of Canine
835 Osteosarcoma Cell Lines: A Valuable Resource in Translational
836 Medicine. *Front Vet Sci.* 2021;8:666838.
- 837 10. Massimini M, Romanucci M, De Maria R, Della Salda L. An
838 Update on Molecular Pathways Regulating Vasculogenic Mimicry in
839 Human Osteosarcoma and Their Role in Canine Oncology. *Front Vet*
840 *Sci.* 2021;8:722432.
- 841 11. Sánchez-Céspedes R, Accornero P, Miretti S, Martignani E,
842 Gattino F, Maniscalco L, et al. In vitro and in vivo effects of toceranib
843 phosphate on canine osteosarcoma cell lines and xenograft orthotopic
844 models. *Veterinary and Comparative Oncology.* 2020;18:117–27.
- 845 12. Morello E, Martano M, Buracco P. Biology, diagnosis and
846 treatment of canine appendicular osteosarcoma: Similarities and
847 differences with human osteosarcoma. *The Veterinary Journal.*
848 2011;189:268–77.
- 849 13. Meuten DJ, editor. *Tumors in Domestic Animals.* Hoboken, NJ,
850 USA: John Wiley & Sons, Inc.; 2016.
- 851 14. Hosoya K, Murahari S, Laio A, London CA, Couto CG, Kisseberth
852 WC. Biological activity of dihydroartemisinin in canine osteosarcoma
853 cell lines. *Am J Vet Res.* 2008;69:519–26.

- 854 15. Isani G, Bertocchi M, Andreani G, Farruggia G, Cappadone C,
855 Salaroli R, et al. Cytotoxic Effects of *Artemisia annua* L. and Pure
856 Artemisinin on the D-17 Canine Osteosarcoma Cell Line. *Oxid Med*
857 *Cell Longev.* 2019;2019:1615758.
- 858 16. Culurciello R, Bosso A, Di Fabio G, Zarrelli A, Arciello A, Carella
859 F, et al. Cytotoxicity of an Innovative Pressurised Cyclic Solid-Liquid
860 (PCSL) Extract from *Artemisia annua*. *Toxins (Basel)*. 2021;13:886.
- 861 17. Protti M, Mandrioli R, Mandrone M, Cappadone C, Farruggia G,
862 Chiocchio I, et al. Analysis of *Artemisia annua* extracts and related
863 products by high performance liquid chromatography-tandem mass
864 spectrometry coupled to sample treatment miniaturisation. *J Pharm*
865 *Biomed Anal.* 2019;174:81–8.
- 866 18. Levi M, Salaroli R, Parenti F, De Maria R, Zannoni A, Bernardini
867 C, et al. Doxorubicin treatment modulates chemoresistance and affects
868 the cell cycle in two canine mammary tumour cell lines. *BMC Vet*
869 *Res.* 2021;17:30.
- 870 19. Fox MH. A model for the computer analysis of synchronous DNA
871 distributions obtained by flow cytometry. *Cytometry.* 1980;1:71–7.
- 872 20. Tüzen M. Determination of heavy metals in soil, mushroom and
873 plant samples by atomic absorption spectrometry. *Microchemical*
874 *Journal.* 2003;74:289–97.
- 875 21. Dixon SJ, Lemberg KM, Lamprecht MR, Skouta R, Zaitsev EM,
876 Gleason CE, et al. Ferroptosis: an iron-dependent form of
877 nonapoptotic cell death. *Cell.* 2012;149:1060–72.
- 878 22. Bai H, Wang C, Chen J, Peng J, Cao Q. A novel sensitive
879 electrochemical sensor based on in-situ polymerized molecularly

880 imprinted membranes at graphene modified electrode for artemisinin
881 determination. *Biosens Bioelectron.* 2015;64:352–8.

882 23. Soktoeva TE, Ryzhova GL, Dychko KA, Khasanov VV,
883 Zhigzhitzhapova SV, Radnaeva LD. Artemisinin content in *Artemisia*
884 *annua* L. extracts obtained by different methods. *Russ J Bioorg Chem.*
885 2013;39:761–4.

886 24. Saeed MEM, Breuer E, Hegazy M-EF, Efferth T. Retrospective
887 study of small pet tumors treated with *Artemisia annua* and iron. *Int J*
888 *Oncol.* 2020;56:123–38.

889 25. Ferreira JFS, Luthria DL, Sasaki T, Heyerick A. Flavonoids from
890 *Artemisia annua* L. as antioxidants and their potential synergism with
891 artemisinin against malaria and cancer. *Molecules.* 2010;15:3135–70.

892 26. Efferth T, Herrmann F, Tahrani A, Wink M. Cytotoxic activity of
893 secondary metabolites derived from *Artemisia annua* L. towards
894 cancer cells in comparison to its designated active constituent
895 artemisinin. *Phytomedicine.* 2011;18:959–69.

896 27. Fenger JM, London CA, Kisseberth WC. Canine osteosarcoma: a
897 naturally occurring disease to inform pediatric oncology. *ILAR J.*
898 2014;55:69–85.

899 28. Ji Y, Zhang Y-C, Pei L-B, Shi L-L, Yan J-L, Ma X-H. Anti-tumor
900 effects of dihydroartemisinin on human osteosarcoma. *Mol Cell*
901 *Biochem.* 2011;351:99–108.

902 29. Greenshields AL, Fernando W, Hoskin DW. The anti-malarial
903 drug artesunate causes cell cycle arrest and apoptosis of triple-negative
904 MDA-MB-468 and HER2-enriched SK-BR-3 breast cancer cells. *Exp*
905 *Mol Pathol.* 2019;107:10–22.

- 906 30. Simpson S, Dunning MD, de Brot S, Grau-Roma L, Mongan NP,
907 Rutland CS. Comparative review of human and canine osteosarcoma:
908 morphology, epidemiology, prognosis, treatment and genetics. *Acta*
909 *Vet Scand.* 2017;59:71.
- 910 31. Kuczler MD, Olseen AM, Pienta KJ, Amend SR. ROS-induced
911 cell cycle arrest as a mechanism of resistance in polyan euploid cancer
912 cells (PACCs). *Prog Biophys Mol Biol.* 2021;165:3–7.
- 913 32. Matsui A, Hashiguchi K, Suzuki M, Zhang-Akiyama Q-M.
914 Oxidation resistance 1 functions in the maintenance of cellular
915 survival and genome stability in response to oxidative stress-
916 independent DNA damage. *Genes Environ.* 2020;42:29.
- 917 33. Brown RAM, Richardson KL, Kabir TD, Trinder D, Ganss R,
918 Leedman PJ. Altered Iron Metabolism and Impact in Cancer Biology,
919 Metastasis, and Immunology. *Front Oncol.* 2020;10:476.
- 920 34. De Vico G, Martano M, Maiolino P, Carella F, Leonardi L.
921 Expression of transferrin receptor-1 (TFR-1) in canine osteosarcomas.
922 *Vet Med Sci.* 2020;6:272–6.
- 923 35. Cerchiaro G, Manieri TM, Bertuchi FR. Analytical methods for
924 copper, zinc and iron quantification in mammalian cells. *Metallomics.*
925 2013;5:1336–45.
- 926 36. Lin H, Chen X, Zhang C, Yang T, Deng Z, Song Y, et al. EF24
927 induces ferroptosis in osteosarcoma cells through HMOX1.
928 *Biomedicine & Pharmacotherapy.* 2021;136:111202.
- 929 37. Dodson M, Castro-Portuguez R, Zhang DD. NRF2 plays a critical
930 role in mitigating lipid peroxidation and ferroptosis. *Redox Biol.*
931 2019;23:101107.

- 932 38. Valko M, Leibfritz D, Moncol J, Cronin MTD, Mazur M, Telser J.
933 Free radicals and antioxidants in normal physiological functions and
934 human disease. *Int J Biochem Cell Biol.* 2007;39:44–84.
- 935 39. Macotpet A, Suksawat F, Sukon P, Pimpakdee K,
936 Pattarapanwichien E, Tangrassameeprasert R, et al. Oxidative stress in
937 cancer-bearing dogs assessed by measuring serum malondialdehyde.
938 *BMC Vet Res.* 2013;9:101.
- 939 40. Yan Z-Q, Wang D-D, Ding L, Cui H-Y, Jin H, Yang X-Y, et al.
940 Mechanism of artemisinin phytotoxicity action: induction of reactive
941 oxygen species and cell death in lettuce seedlings. *Plant Physiol*
942 *Biochem.* 2015;88:53–9.
- 943 41. Kakhlon O, Cabantchik ZI. The labile iron pool: characterization,
944 measurement, and participation in cellular processes(1). *Free Radic*
945 *Biol Med.* 2002;33:1037–46.
- 946 42. Hattinger CM, Patrizio MP, Fantoni L, Casotti C, Riganti C, Serra
947 M. Drug Resistance in Osteosarcoma: Emerging Biomarkers,
948 Therapeutic Targets and Treatment Strategies. *Cancers (Basel).*
949 2021;13:2878.
- 950 43. Fu J, Li T, Yang Y, Jiang L, Wang W, Fu L, et al. Activatable
951 nanomedicine for overcoming hypoxia-induced resistance to
952 chemotherapy and inhibiting tumor growth by inducing collaborative
953 apoptosis and ferroptosis in solid tumors. *Biomaterials.*
954 2021;268:120537.
- 955 44. Waseem Y, Hasan CA, Ahmed F. Artemisinin: A Promising
956 Adjunct for Cancer Therapy. *Cureus.* 2018;10:e3628.