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### 1 Anticancer activity of an Artemisia annua

# L. hydroalcoholic extract on canine osteosarcoma cell lines

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#### 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  $EC_{50}$ 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 state were evaluated. A concentration of Artemisinin 46 47 of 63.8  $\pm$  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 idroalcoholic 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, balloning
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 artemisinin bridge of 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 lipid 89 iron-mediated lethal peroxidation called 90 ferroptosis can occurs 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 with Α study on 162 dogs 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 knowledge121 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 Kerafast, 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 magnesium (PBS w/o Ca<sup>2+</sup> and Mg<sup>2+</sup>), RNaseA/T1 143 144 were purchased from Thermo Fisher Scientific 145 (Waltham, MA, USA). Dimethyl Sulfoxide (DMSO), 146 Fluoroshield<sup>TM</sup> 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 aerial parts of A. annua and composed by 65% 165 166 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  $\times$  2.1mm I.D., 3µm), while injection volume was 10 µL. An automated composition 190 191 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 Artemisinin (283.24  $\rightarrow$  209.45; 283.24  $\rightarrow$  265.36) 202 203 and Artemisinin-D3, used as internal standard (IS, 204  $286.31 \rightarrow 212.38$ ;  $286.31 \rightarrow 268.34$ ). 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 1x10<sup>6</sup> 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-µL 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 or T-75 culture flasks at 2.5 x 10<sup>4</sup> cells/cm<sup>2</sup> seeding 222 density, at 37°C and 5% CO2. The commercial 223 224 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 diluited 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 EC<sub>50</sub> determination

234 The two cell lines were seeded in 96-well plates (1x10<sup>4</sup> cells/well) and exposed, for 24 h, to increasing 235 236 doses of Α. 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 with extract or 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 using Infinite® F50/Robotic Absorbance nm, 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. EC<sub>50</sub> 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 ( $2.5 \times 10^5$ ) 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 EC<sub>50</sub> doses for 24 h in a humidified CO<sub>2</sub> 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, washed twice in 5 mL of DPBS w/o Ca<sup>2+</sup> and Mg<sup>2+</sup> 269 270 then fixed overnight in 70% ice-cold EtOH 271 (1mL/1x10<sup>6</sup> cells) added drop-by-drop under 272 continuous vortex mixing. After fixation, the cells were washed with 10 mL DPBS w/o Ca2+ and Mg2+ 273 274 and cellular pellet was incubated with 1mL/10<sup>6</sup> cells 275 of staining solution [50 µg/mL PI + 100 µg/mL RNaseA/T1 in DPBS w/o Ca<sup>2+</sup> and Mg<sup>2+</sup>] for 30 min 276 277 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<sup>®</sup> 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

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

291 extract or Artemisinin at the respective EC<sub>50</sub> 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 1x10<sup>6</sup> cells were 295 resuspended in 1 ml of a solution of 1 M HNO<sub>3</sub>, 296 digested at room temperature until completely 297 dissolved, and finally used for iron quantification 298 Spectra AA-20 atomic using а 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 slit width of 0.2 spectral 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<sup>®</sup> - BB422) 327 and the accuracy of the method, calculated as the 328 percentage of the certified value, resulted of 106 %. 329 limit (LOD), The detection 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 EC<sub>50</sub> dose or with erastin (10  $\mu$ M) 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 1x10<sup>5</sup> 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 EC<sub>50</sub> doses or with vehicle controls. 352 To have positive controls OSCA-8 and OSCA-40 353 were treated with erastin (10 µM) 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
- 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

#### 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 ng/mL and LOD=1.5 ng/mL), linearity ( $r^2 > 0.9995$ 378 379 over the 5-1000 ng/mL artemisinin concentration 380 extraction yield (>85 %), range), precision 381 (RSD%<3.5) and accuracy (88-93% range), allowing 382 determination an accurate of artemisinin concentrations in different matrices. 383

In the hydroalcoholic extract of *A. annua* considered 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  $EC_{50}$  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 and411 higher EC<sub>50</sub> values.

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

417

Table 1. Artemisinin concentration determined in culture
medium and in OSCA-8 and OSCA-40 cell lines after 24 hours
of exposure to pure Artemisinin (A) and *A. annua* extract (E) at
the EC<sub>50</sub> doses.

	Medium	Intacellular Artemisinin
	µg/mL	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

## 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 EC<sub>50</sub> of 3.3 and 2.6 µM for OSCA-8 and OSCA-40 435 respectively, while Artemisinin showed an EC<sub>50</sub> 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 EC<sub>50</sub> values than 445 Artemisinin (Fig. 1). The EC<sub>50</sub> 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 anti-tumour candidate for further promising 465 development.

466



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 ± 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<sub>50</sub> 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.



Figure 2\_ *A. annua* hydroalcoholic extract and Artemisinin
impair cell cycle of the canine OSA cell lines. The cells were
treated with *A. annua* extract and Artemisinin at the EC<sub>50</sub> doses

- 505 or vehicle (EtOH or DMSO) for 24 h and fluorescence of the PI506 stained cells was measured using MACSQuant® Analyzer10
  507 and analysed by Flow Logic software (Inivai Technologies,
  508 Australia). 5 × 10<sup>5</sup> 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



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 EC<sub>50</sub> 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 ± 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 Artemisinin, of 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 by shedding apoptotic bodies, cellular fragments 542 543 holding pieces of chromatin, broken nuclei, 544 chromosomes, and cellular debris [30]. It could be 545 hypothesised that Artemisinin in Α. 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 and unable to lead to a significant cell cycle 564 565 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 a sensitive and accurate method. This is a 582 583 challenging task, due to the very small amount of the 584 biological samples; consequently, а 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/1x10<sup>6</sup> cells. OSCA-40 cells treated with A. 599 annua extract at the EC<sub>50</sub> 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



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 EC<sub>50</sub> 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 ХС -), 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



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 EC<sub>50</sub> dose (b, f), erastin  $10\mu M$  (c, g) or EtOH (a, 661 e). Following the treatment with A. annua hydroalcoholic extract 662 or erastin 10µM bothcell 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- 40, pure Artemisinin treatment induced a "ballooning"phenotype. Scale bar: 100 µm.

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 EC<sub>50</sub> 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 D-17 cOSA cell [14] in 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 EC<sub>50</sub> 703 dose (b, e), with pure Artemisinin at the EC<sub>50</sub> 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 EC<sub>50</sub> 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 OSCA-8. Alteration of iron metabolism is in 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 EC<sub>50</sub> 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

intracellular content of iron as well as a different
kinetics in DNA damage response mechanisms in the
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 idroa	alcoholic extract of <i>A. annua</i> showed
786 cytotoxicit	y on two canine OSA cell lines with
787 increase o	of total iron, accumulation of lipid peroxides
788 and a "ba	llooning" death phenotype, suggesting the
789 activation	of ferroptosis. However, it should be
790 emphasize	ed that any conclusions from this study
791 must nece	essarily 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
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