

1 ***Artemisia annua* L. extracts prevent replication of SARS-CoV-2**

2 Nair<sup>1</sup>, M.S., Huang<sup>1</sup>, Y., Fidock<sup>2,3</sup>, D.A., Polyak<sup>4</sup>, S.J., Wagoner<sup>4</sup>, J., Towler<sup>5</sup>, M.J., Weathers<sup>5#</sup>, P.J.

3 <sup>1</sup>Aaron Diamond AIDS Research Center, Columbia University Vagelos College of Physicians and  
4 Surgeons, New York, NY, USA.

5 <sup>2</sup>Department of Microbiology and Immunology, Department of Medicine, Columbia University  
6 Irving Medical Center, New York, NY 10032, USA.

7 <sup>3</sup>Division of Infectious Diseases, Department of Medicine, Columbia University Irving Medical  
8 Center, New York, NY 10032, USA.

9 <sup>4</sup>Department of Laboratory Medicine and Pathology, University of Washington, Seattle, WA, 98104

10 <sup>5</sup>Department of Biology and Biotechnology, Worcester Polytechnic Institute, Worcester, MA  
11 01609, USA.

12  
13 **# Corresponding author:**

14 Pamela Weathers

15 Department of Biology and Biotechnology

16 Worcester Polytechnic Institute

17 100 Institute Rd

18 Worcester, MA 01609 USA

19 Email: [weathers@wpi.edu](mailto:weathers@wpi.edu)

20 Phone: 508-831-5196

21 FAX: 508-831-6362

22

23

24 **ABSTRACT:**

25 SARS-CoV-2 (Covid-19) globally has infected and killed millions of people. Besides remdesivir, there  
26 are no approved small molecule-based therapeutics. Here we show that extracts of the medicinal  
27 plant, *Artemisia annua* L., which produces the antimalarial drug artemisinin, prevents SARS-CoV-2  
28 replication in vitro. We measured antiviral activity of dried leaf extracts of seven cultivars of *A.*  
29 *annua* sourced from four continents. Hot-water leaf extracts based on artemisinin, total  
30 flavonoids, or dry leaf mass showed antiviral activity with IC<sub>50</sub> values of 0.1-8.7 μM, 0.01-0.14 μg,  
31 and 23.4-57.4 μg, respectively. One sample was >12 years old, but still active. While all hot water  
32 extracts were effective, concentrations of artemisinin and total flavonoids varied by nearly 100-  
33 fold in the extracts and antiviral efficacy was inversely correlated to artemisinin and total flavonoid  
34 contents. Artemisinin alone showed an estimated IC<sub>50</sub> of about 70 μM, and antimalarial  
35 artemisinin derivatives artesunate, artemether, and dihydroartemisinin were ineffective or  
36 cytotoxic at elevated micromolar concentrations. In contrast, the antimalarial drug amodiaquine  
37 had an IC<sub>50</sub> = 5.8 μM. The extracts had minimal effects on infection of Vero E6 or Calu-3 cells by a  
38 reporter virus pseudotyped by the SARS-CoV-2 spike protein. There was no cytotoxicity within an  
39 order of magnitude of the antiviral IC<sub>90</sub> values. Results suggest the active component in the  
40 extracts is likely something besides artemisinin or is a combination of components acting  
41 synergistically to block post-entry viral infection. Further studies will determine in vivo efficacy to  
42 assess whether *A. annua* might provide a cost-effective therapeutic to treat SARS-CoV-2 infections.

43  
44 **KEY WORDS:** *Artemisia annua*, artemisinin, SARS-Cov-2, Covid-19, artesunate, artemether,  
45 amodiaquine, dihydroartemisinin

46

47 **INTRODUCTION:**

48 The global pandemic of SARS-CoV-2 (the etiologic agent of COVID-19) has infected over 80 million  
49 people and killed nearly 1.8 million as of December 29, 2020 (<https://coronavirus.jhu.edu/>). There  
50 is an intense effort to distribute the registered Pfizer/BioNTech and Moderna vaccines, but to our  
51 knowledge, there is no approved therapeutic and global infections keep rising with the sporadic  
52 advent of new variants.

53

54 The medicinal plant *Artemisia annua* L. produces the antimalarial therapeutic artemisinin, a  
55 sesquiterpene lactone produced and stored in the glandular trichomes located on the shoots and  
56 especially the leaves and flowers of the plant. Both the plant and artemisinin have been used  
57 safely for over 2,000 years to treat a variety of ailments, especially malaria. Artemisinin derivatives  
58 (Figure 1) are front-line therapeutics for treating malaria and are delivered with a second  
59 antimalarial drug, such as lumefantrine or amodiaquine, which are formulated as artemisinin-  
60 based combination therapies (Blasco et al. 2017). Artemisinins also have some antiviral activity  
61 (Efferth 2018). Extracts of *A. annua* showed anti-SARS-CoV-1 activity, suggesting that they may be  
62 active against SARS-CoV-2 (Li et al. 2005).

63

64 Artemisinin delivered *per os* from *A. annua* consumption is highly bioavailable and distributes  
65 through peripheral blood and into a plethora of organs including lungs, liver, heart, and brain  
66 (Desrosiers et al. 2020). Furthermore, both artemisinins and the plant *A. annua* reduce levels of  
67 inflammatory cytokines including IL-6 and TNF- $\alpha$  *in vivo* (Desrosiers et al. 2020; Hunt et al. 2015;  
68 Shi et al. 2015). These effector molecules can be problematic during the “cytokine storm” suffered  
69 by many SARS-CoV-2 patients (Schett et al. 2020). Artemisinin also blunts fibrosis (Larson et al.

70 2019; Dolivo et al. 2020), another problem experienced by SARS-CoV-2 survivors that causes more  
71 lasting damage to organs (Lechowicz et al. 2020; Liu et al. 2020a). A recent report showed that a  
72 number of artemisinin-related compounds have some anti-SARS-CoV-2 activity, with  
73 dihydroartemisinin, artesunate, and arteannuin B having IC<sub>50</sub> values <30 μM (Cao et al. 2020), and  
74 dihydroartemisinin ACTs with 1-10 μM IC<sub>50</sub>s (Bae et al. 2020). Artesunate was reported to have  
75 IC<sub>50</sub> values against SARS-CoV-2 of 7-12 μg/mL (0.7-1.2 μM; Gilmore et al. 2020) and 2.6 μM (Bae et  
76 al. 2020). Knowing that artemisinin is much more bioavailable *per os* when delivered via *A. annua*  
77 (Weathers et al. 2011; Weathers et al. 2014; Desrosiers et al. 2020), we posited that encapsulated  
78 powdered dried leaves of *A. annua* may be a safe, cost-effective therapeutic to combat SARS-CoV-  
79 2 infections. Here we report *in vitro* results from testing extracts of a diversity of *A. annua* cultivars  
80 against SARS-CoV-2 propagated in Vero E6 cells, with correlation analyses of antiviral efficacy to  
81 artemisinin and total flavonoid contents.

82

### 83 **METHODS:**

84 **Plant material, extract preparations, and artemisinin and total flavonoid analyses:** Batches of  
85 dried leaves of various cultivars of *Artemisia annua* L. with source, age, and voucher identity when  
86 known are shown in Table 1. Hot-water extracts (tea infusions) were prepared as follows: dried  
87 leaves at 10 g/L were added to boiling water on a stir plate and boiled for 10 min, then poured  
88 through a 2 mm stainless steel sieve to retain most solids. Extracts were then cooled and sterile-  
89 filtered (0.22 μm) prior to being stored at -20°C. Dichloromethane (DCM) extracts of dried leaves  
90 were also prepared by extraction of 25 mg in 4 mL DCM for 30 min in a sonicating water bath  
91 (Fischer Scientific FS60, 130 W), separating solvent from solids with Pasteur pipets, drying under  
92 nitrogen flow, and storing at -20°C until analyzing for artemisinin using gas chromatography / mass

93 spectrometry, as detailed in Martini et al. (2020). For artemisinin analysis of tea infusions, two-  
94 phase overnight aliquots extracted in DCM in a 1:1 ratio were separated by using Pasteur pipets,  
95 dried under nitrogen flow, and stored at -20°C until analysis as previously noted (Martini et al.  
96 2020). Total flavonoids were analyzed in DCM extracts via the aluminum chloride method of  
97 Arvouet-Grand et al. (1994) and were quantified as quercetin equivalents. Artemisinin and total  
98 flavonoid contents of tea infusions are shown in Table 2. The DCM extract of *A. annua* (cv SAM)  
99 contained a total of 34 mg of artemisinin. After solubilizing in PEG400 containing 5% DMSO the  
100 concentration was 8.95 mg/mL.

101  
102 **Viral culture and analyses:** Vero E6 cells, obtained from the American Type Culture collection  
103 (ATCC CRL-1586), were cultured in Minimal Essential Eagle Medium (EMEM) containing penicillin-  
104 streptomycin (1x 100 U/mL) and 10% fetal calf serum. SARS-CoV-2 isolate USA/WA1 was from BEI  
105 Resources ([www.beiresources.org](http://www.beiresources.org)). We infected Vero E6 cells with the USA/WA1 isolate according  
106 to Liu et al. (2020b). Briefly, infected cells were incubated in flasks until a viral cytopathic effect  
107 was observed. The supernatant was then harvested and titered for its tissue culture infective dose  
108 (TCID) using an end point dilution method. TCID was calculated using the Reed Muench  
109 proportional distance method (Reed and Muench 1938). Viral aliquots were frozen, then later  
110 thawed and used for infection experiments at their desired infectivity (multiplicity of infection  
111 (MOI).

112  
113 **Assays for determining drug inhibition of SARS-CoV-2:** Except for tea infusions that were diluted  
114 in water and used directly, amodiaquine, artesunate, artemether, artemisinin, deoxyartemisinin,  
115 and dihydroartemisinin compounds were solubilized and diluted in 5% DMSO in PEG400 or 5%

116 DMSO in EMEM enriched with fetal calf serum at a final concentration of 7.5%, prior to testing for  
117 efficacy against SARS-CoV-2. Indicated dilutions of the drug were incubated for 1 h in wells of 96  
118 well tissue culture plates containing a monolayer of Vero E6 cells seeded the day before at 20,000  
119 cells/well. Post incubation of the drug with the cells, SARS-CoV-2 USA/WA1 virus was added to  
120 each well at a multiplicity of infection of 0.1. Cells were cultured for 3 days at 37°C in 5% CO<sub>2</sub> and  
121 scored for cytopathic effects as detailed in Liu et al. (2020b). Vesicular Stomatitis Virus (VSV)-spike  
122 pseudoviruses were generated as described (Hoffmann et al. 2020; Whitt 2010), using the spike  
123 gene from SARS-CoV-2 containing the D614G mutation (Korber et al. 2020). The construct also  
124 contains a deletion of 18 amino acids from the C-terminus, which facilitates loading onto  
125 pseudovirus particles. The construct ( $\Delta$ 18 D614G) was kindly provided by Markus Hoffmann and  
126 Stefan Pöhlmann (Leibniz-Institut für Primatenforschung, Germany). The day prior to infection,  
127 Vero E6 and Calu-3 cells (ATCC HTB-55) were plated in black, clear-bottomed plates at 10,000 and  
128 30,000 cells/well, respectively, in a final volume of 90  $\mu$ l. Cells were then treated with 10  $\mu$ l of  
129 serially diluted *Artemisia* extract in water and incubated for 1 h prior to infection with 100  $\mu$ l of  
130 VSV-spike  $\Delta$ 18 D614G pseudovirus. At 22 h post-infection, PrestoBlue was added 2 h before the  
131 end of assay, so that cell viability in parallel non-infected, drug-treated wells could be measured.  
132 Virus-produced Renilla luciferase activity was measured by Renilla-Glo assay at 24 h post-infection.  
133 Results were converted into percent of control. Drug concentrations were log transformed and the  
134 concentration of drug(s) that inhibited virus by 50% (*i.e.*, IC<sub>50</sub>), and the concentration of drug(s)  
135 that killed 50% of cells (*i.e.*, CC<sub>50</sub>), were determined via nonlinear logistic regressions of  
136 log(inhibitor) versus response-variable dose-response functions (four parameters) constrained to a  
137 zero-bottom asymptote by statistical analysis using GraphPad Prism 9 (GraphPad Software, Inc.) as  
138 described by Hulseberg et al. (2019).

139

140 **Cell viability assay:** To determine the viability of Vero E6 cells post drug treatment, cells were  
141 exposed to indicated doses of tea infusions diluted in EMEM containing fetal calf serum at a final  
142 concentration of 7.5%, and incubated at 37°C in 5% CO<sub>2</sub> for 24 h. Cells were then washed and  
143 treated with 100 µL XTT reagent premixed with activation agent, followed by incubation for  
144 another 2 h at 37°C in 5% CO<sub>2</sub>. Culture medium was removed, and absorbance measured at 450  
145 nm. The absorbance ratio of treated to untreated cells was plotted as percent viability. Imatinib,  
146 an FDA-approved apoptosis inducer and tyrosine kinase inhibitor, was used as a positive control.  
147

148 **Chemicals and reagents:** Unless otherwise stated all reagents were from Sigma-Aldrich (St. Louis,  
149 MO) DCM was from ThermoFisher (Waltham, MA, USA); artemisinin was from Cayman Chemical  
150 (Ann Arbor, MI, USA); artemether, artesunate, and dihydroartemisinin were gifts from Prof. J.  
151 Plaizier-Vercammen (Brussels, Belgium); deoxyartemisinin was from Toronto Research Chemicals  
152 (North York, ON, Canada), amodiaquine HCl hydrate (Cat #: 562290) and imatinib (Cat # 100956)  
153 were from Medkoo Biosciences Inc. (Morrisville, NC, USA); EMEM (Cat # 30-2003) and XTT reagent  
154 (Cat # 30-1011k) were from ATCC; PrestoBlue was from Life Technologies (Cat #P50201); Renilla-  
155 Glo was from Promega (E2720).

156

157 **Statistical analyses:** All *in vitro* anti-SARS-CoV-2 analyses were done at least in triplicate. Plant  
158 extract analyses had n≥6 independent assays. IC<sub>50</sub> and IC<sub>90</sub> values were calculated using GraphPad  
159 Prism V8.0. Correlations between antiviral activity and artemisinin or total flavonoids used  
160 Spearman's Rho analysis (Spearman 1904).

161

162 **RESULTS:**

163 **Artemisia annua hot water extracts have anti-SARS-CoV-2 activity.** Hot water extracts of the *A.*  
164 *annua* cultivars used in the study had considerably different artemisinin contents ranging from  
165  $20.1 \pm 0.8$  to  $149.4 \pm 4.4$   $\mu\text{g}/\text{mL}$  (Table 2). Total flavonoid content of leaf material ranged from  $7.3$   
166  $\pm 0.2$  to  $37.2 \pm 0.7$   $\mu\text{g}/\text{mL}$  (Table 2). All cultivars showed anti-SARS-CoV-2 activity (Figure 2; Table  
167 2), and  $\text{IC}_{50}$  values calculated on the basis of artemisinin or total flavonoid content ranged from  
168  $0.1$ - $8.7$   $\mu\text{M}$ , or  $0.01$ - $0.14$   $\mu\text{g}/\text{mL}$ , respectively (Table 2). On the basis of leaf dry mass,  $\text{IC}_{50}$  values  
169 ranged from  $13.5$ - $57.4$   $\mu\text{g}$  dry weight (DW). On a  $\mu\text{g}$  artemisinin/mL tea basis, the  $\text{IC}_{50}$  of the  
170 samples ranged from  $0.03$  to  $2.5$   $\mu\text{g}/\text{mL}$ . Analysis of frozen (SAM -20C) extracts remained potent  
171 upon thawing and reanalysis (Table 2, Figure 2). Leaf samples that were 12 years old were also  
172 active with an  $\text{IC}_{50}$  of  $32.9$   $\mu\text{g}$  DW. Infection of Vero E6 or Calu-3 human lung cells by VSV-spike  
173 pseudoviruses was minimally inhibited by the extract, except perhaps at the highest dose tested of  
174  $500$   $\mu\text{g}/\text{mL}$  (Figure 3). Indeed, GraphPad Prism-calculated  $\text{IC}_{50}/\text{CC}_{50}$  values were  $545/3564$   $\mu\text{g}/\text{mL}$   
175 for Calu-3 and  $410/810$   $\mu\text{g}/\text{mL}$  for Vero E6 cells.

176

177 **Activity of antimalarials.** In a separate analysis, DCM and hot water extracts of *A. annua* were  
178 compared, yielding  $\text{IC}_{50}$  values of  $12.0$  and  $11.8$   $\mu\text{M}$ , respectively (Figure 4). However, due to  
179 solvent toxicity at higher concentrations of the drug on Vero E6 cells, the  $\text{IC}_{50}$  of the DCM extract  
180 had to be estimated. Similar solvent toxicity was encountered with artemisinin that subsequently  
181 was estimated to have an  $\text{IC}_{50}$  of  $70$   $\mu\text{M}$  (Figure 4). Artemether efficacy was estimated at  $1.23$   $\mu\text{M}$   
182 but was cytotoxic at concentrations slightly above that level (Figure 4). Artesunate and  
183 dihydroartemisinin were inactive at  $<100$   $\mu\text{M}$ . In contrast, amodiaquine showed efficacy at  $5.8$   $\mu\text{M}$   
184 (Figure 4).

185

186 *Anti-SARS-CoV-2 activity of hot water extracts inversely correlated to artemisinin or total flavonoid*  
187 *content.* A Spearman's Rho analysis showed that neither IC<sub>50</sub> nor IC<sub>90</sub> values of the hot water  
188 extracts correlated to either artemisinin or total flavonoid content (Figure 5). Results of IC<sub>50</sub> and  
189 IC<sub>90</sub> calculations based on dry leaf mass used to prepare the tea were tightly grouped (Figure 2).  
190 Although cultivar IC<sub>50</sub> ranking from most to least effective on dry weight basis was BUR, MED, A3,  
191 #15, PEG01, SAM1, SAM2, and FLV5 (Table 2), the maximum differential was less than 44 µg DW of  
192 dried leaves, or ~4.4 µL of tea infusion, an inconsequential difference.

193

194 **Hot water extracts are not cytotoxic.** When cytotoxicity of the hot water extracts to the Vero E6  
195 cells was measured, cell viability did not substantially decrease (Figure 6A) at 24 h post treatment.  
196 In comparison, the apoptotic inducer imatinib showed a dose-dependent decrease in viability of  
197 the cells by 90% (Figure 6B). At the higher concentrations of hot water extracts, there appeared to  
198 be proliferation of Vero E6 cells (Figure 6A).

199

200 *Human bioavailability.* To query the potential of using dried leaf *A. annua* (DLA) as a potential  
201 therapeutic, we tracked artemisinin as a marker molecule post consumption of *per os* delivered  
202 DLA in a human. One of us (PJW) consumed 3 g of encapsulated DLA of the SAM cultivar, had her  
203 blood drawn at 2 and 5 h post consumption, and 7.04 and 0.16 µg artemisinin/mL serum,  
204 respectively, were measured (See Supplemental Data). Thus, at 2 h post ingestion, 36% of the  
205 original DLA-delivered artemisinin was detected in the serum, dropping to 0.8% at 5 h post  
206 ingestion (See Supplemental data Table S1). This corresponded at 2 h to 2.35 µg artemisinin/mL  
207 serum of DLA-delivered artemisinin per gram of DLA consumed.

208

209 **DISCUSSION:**

210 This is the first report of anti-SARS-CoV-2 efficacy of hot water extracts of a wide variety of  
211 cultivars of *A. annua* sourced from four continents. These extracts had an IC<sub>50</sub> corresponding to  
212 <12 μM artemisinin, with DCM extracts of *A. annua* showing similar efficacy. In contrast,  
213 artemisinin alone had an estimated IC<sub>50</sub> about sixfold greater (~70 μM), suggesting the plant  
214 extracts were more potent against SARS-CoV-2. Furthermore, the anti-SARS-CoV-2 effect was  
215 inversely correlated to the artemisinin content of the extracts that varied by one to nearly two  
216 orders of magnitude for IC<sub>50</sub> and IC<sub>90</sub> values. Total flavonoid content also was inversely correlated  
217 to antiviral activity. One of the cultivar samples was obtained in 2008 and was still active at a level  
218 comparable to the most recently harvested cultivar samples, suggesting that the active principle is  
219 ubiquitous to different *A. annua* cultivars and chemically stable during long-term room  
220 temperature dry storage. None of the plant extracts were cytotoxic to Vero 6 or Calu-3 cells at  
221 concentrations approaching the IC<sub>50</sub> or IC<sub>90</sub> values. Finally, the minimal antiviral effects against VSV  
222 pseudoviruses containing the SARS-CoV-2 spike protein suggests that *Artemisia* inhibits SARS-CoV-  
223 2 infection primarily by targeting a post-entry step.

224

225 Although Cao et al. (2020) reported an EC<sub>50</sub> of 10.28 μM for arteannuin B, a metabolite that is  
226 formed in a side branch of the artemisinin biosynthetic pathway and that is often present in *A.*  
227 *annua* extracts, only three of the tested tea extracts had any detectable arteannuin B with SAM  
228 having 3.2 μg/mL. Arteannuin B in BUR and MED was barely detectable. Thus, arteannuin B was  
229 eliminated as the principle active component, although if present in an extract, arteannuin B may  
230 be providing some antiviral effect as part of the more complex plant extract mixture. Although

231 they can be present in substantial amounts in *A. annua* (Weathers and Towler 2014; Towler and  
232 Weathers 2015; see supplemental Table S2), neither artemisinic acid nor deoxyartemisinin, also  
233 metabolites in the artemisinin biosynthetic pathway, showed anti-SARS-CoV-2 activity in this  
234 study.

235

236 There is some discrepancy among IC<sub>50</sub> molar values in this and other studies for anti-SARS-CoV-2  
237 efficacy (Table 3). In contrast to Bae, Cao, and Gilmore, we did not observe any anti-SARS-CoV-2  
238 activity for artesunate or dihydroartemisinin. Artemether in our study had an IC<sub>50</sub> of 1.23 μM,  
239 while Cao et al. (2020) reported an EC<sub>50</sub> of 73.8 μM but with less toxicity than we observed. In  
240 particular, we noted cytotoxicity of artemether. The contrasts are likely the result of differences in  
241 how we conducted our viral challenge experiments or solvents used to challenge the virus in Vero  
242 E6 cells. For example, our study solubilized our pure artemisinin and other antimalarial  
243 compounds in 5% DMSO in PEG400, while the other two studies solubilized compounds in DMSO.  
244 Our preliminary experiments indicated that solubilizing in pure DMSO was too toxic to Vero cells  
245 to achieve dosing of drug concentrations needed to obtain an IC<sub>50</sub> value. In addition, Cao et al. also  
246 had a different viral assay system. We used an endpoint assay to measure the cytopathic effect of  
247 the replicating virus at 72 h and estimate the IC<sub>50</sub> values while they collected supernatants to assay  
248 the total RNA levels at 24 h post infection using RT-PCR. We recognize that such inherent  
249 variations in the biological assays would offset the calculated values. Gilmore et al. (2020) also  
250 tested a hot water extract of *A. annua* and observed EC<sub>50</sub> values ranging from 260-390 μg/mL.  
251 However, our hot water extracts are not directly comparable to those of Gilmore et al. because we  
252 did not dry, concentrate, and then weigh our extracts. Furthermore, we extracted for 10 min in  
253 boiling water, while they extracted for 200 min in boiling water. At present it is not possible to

254 compare our hot water extracts directly. In addition, different viruses were used in our study  
255 versus that of Gilmore et al., which could affect the inherent replication kinetics of the assay and in  
256 turn affect the specific IC<sub>50</sub> numbers.

257

258 We and others noted there was anti-SARS-Cov-2 activity by other non-artemisinin antimalarial  
259 drugs including amodiaquine at an IC<sub>50</sub> = 5.8 μM (this study), tafenoquine at an IC<sub>50</sub> of 2.6 μM  
260 (Dow et al. 2020), and lumefantrine at a reported IC<sub>50</sub> = 23.2 μM (Cao et al. 2020). Gendrot et al.  
261 (2020) also reported anti-SARS-CoV-2 activity of various ACTs drugs at doses used for treating  
262 malaria with mefloquine-artesunate (550 mg + 250 mg, respectively) providing the maximum  
263 inhibition, namely 72% of viral replication at serum C<sub>max</sub>. Other combinations were less effective.

264

265 The high bioavailability of artemisinin after oral consumption of dried-leaf *A. annua* (DLA) was not  
266 surprising considering that a series of earlier studies in rodents showed the drug is >40 fold more  
267 bioavailable when delivered via the plant than in purified form (Weathers et al. 2011; Weathers et  
268 al. 2014). The increased bioavailability is mainly the result of three mechanisms: essential oils in  
269 the plant material improving the solubility of artemisinin, improved passage across the intestinal  
270 wall, and especially the inhibition of liver cytochrome P450s, 2B6, and 3A4 that are critical in first-  
271 pass metabolism (Desrosiers and Weathers 2016, 2018; Desrosiers et al. 2020). The anti-SARS-CoV-  
272 2 IC<sub>90</sub> of the SAM1 and SAM2 cultivar samples ranged from 12.3-18.8 μM, equal to 1.7-2.6 μg/mL,  
273 so 1 g of the SAM cultivar delivered *per os* yielded 2.6 μg/mL in a patient's serum. Thus, 1 g of DLA  
274 could deliver enough artemisinin/DLA to achieve the IC<sub>90</sub> of the hot water extract. While clearly  
275 human trials are required, these hypothetical estimations suggest that reasonable amounts of DLA  
276 consumed *per os* may be able to provide a cost-effective anti-SARS-CoV-2 treatment. Indeed, the

277 broad scale use of both artemisinin and non-artemisinin compound antimalarials including *A.*  
278 *annua* tea infusions across Africa may help in part explain why despite having anti-SARS-CoV-2  
279 antibodies, Africans have not to date suffered the clinical scourge of SARS-CoV-2 like the rest of  
280 the world (Uyoga et al. 2020).

281

## 282 **CONCLUSIONS:**

283 This is a first report of the *in vitro* anti-SARS-CoV-2 activity of hot water extracts of *A. annua*  
284 wherein there was no cytotoxicity and where we showed reasonable levels of orally consumed  
285 plant material. If subsequent clinical trials are successful, *A. annua* could potentially serve as a safe  
286 therapeutic that could be provided globally at reasonable cost and offer an alternative to vaccines.

287

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299

300 **CONFLICT OF INTEREST STATEMENT:**

301 Authors declare they have no competing conflicts of interest in the study.

302

303 **AUTHOR CONTRIBUTIONS:**

304 MSN – conducted SARS-CoV-2 experiments, helped analyze the data, and contributed to the  
305 manuscript.

306 YH – conducted SARS-CoV-2 experiments, helped analyze the data, and contributed to the  
307 manuscript.

308 DAF – provided reagents, helped analyze the data, and edited the manuscript.

309 SJP – helped plan and analyze pseudovirus data, and contributed to manuscript

310 JW – conducted pseudovirus experiments, helped analyze data, and contributed to manuscript

311 MJT – prepared and analyzed plant extracts and human samples, helped analyze the data, and  
312 contributed to the manuscript.

313 PJW – wrote manuscript, conducted the single human PK test, provided reagents, and helped  
314 analyze the data.

315

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| <b>Table 1.</b> <i>Artemisia annua</i> L. cultivars used in the SARS-CoV-2 antiviral analyses. |   |                    |                |   |
|--|---|--------------------|----------------|---|
| Cultivar code/ID   | Voucher   | Yr leaves obtained | Country source | Donor   |
| SAM  | MASS 00317314   | 2020               | USA            | WPI, originated from F2 generation of PEG01; clonally propagated and grown by Atelier Temenos, Miami , FL |
| A3 (Anamed A-3)  | None <i>per se</i> ;<br><a href="http://www.anamed-edition.com">http://www.anamed-edition.com</a> | 2016               | Ethiopia       | Mary Vanderkooi, Soddo Christian Hospital, Soddo Walaita  |
| PEG01 (PEG01, F2 generation)   | None <i>per se</i> ; Process Engineering Group 01   | 2008               | China          | Chunzhao Liu, Chinese Acad Science, Beijing   |
| BUR  | LG0019527   | 2016               | Burundi        | Ingo Vincens Burow, Savanor, Mutambara, Burundi   |
| MED (Apollon Mediplant)  | KL/015/6407   | 2019               | Kenya          | Jean Jacques Shul, IDAY, Belgium  |
| FLV5   | Artemisia 5' CPMA-UNICAMP 1246  | 2011               | Brazil         | Pedro Melillo de Magalhães, CPQBA-UNICAMP, Paulínia-SP  |

|     |               |                         |     |  |
|-----|---------------|-------------------------|-----|--|
| #15 | MASS 00317313 | Pooled<br>2013-<br>2015 | USA | WPI, originated from F2<br>generation of PEG01 |
|-----|---------------|-------------------------|-----|--|

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**Table 2.** Calculated IC<sub>50</sub> and IC<sub>90</sub> values for the 7 *A. annua* cultivars against Vero E6 cells infected with SARS-CoV-2 USA/WA1 (MOI 0.1) based on their measured artemisinin (ART), total flavonoids (tFLV), and dry leaf mass content (DW) in the hot water extracts.

| Sample ID       | Artemisinin                                      |   |   | Total Flavonoids                             |                                       |                                       | Dry <i>A. annua</i> Leaf Mass |  |  |
|-----------------|--|---|---|--|---------------------------------------|---------------------------------------|-------------------------------|--|--|
|                 | ART in tea<br>( $\mu\text{g/mL} \pm \text{SE}$ ) | IC <sub>50</sub><br>( $\mu\text{M ART}$ ) | IC <sub>90</sub><br>( $\mu\text{M ART}$ ) | tFLV**<br>( $\mu\text{g/mL} \pm \text{SE}$ ) | IC <sub>50</sub><br>( $\mu\text{g}$ ) | IC <sub>90</sub><br>( $\mu\text{g}$ ) | Leaves extracted<br>(g/L)     | IC <sub>50</sub><br>( $\mu\text{g DW}$ ) | IC <sub>90</sub><br>( $\mu\text{g DW}$ ) |
| SAM1*<br>(-20C) | 149.4<br>$\pm 4.4$                               | 8.7                                       | 18.8                                      | 35.4 $\pm$ 0.2                               | 0.13                                  | 0.28                                  | 10                            | 34.9                                     | 75.2                                     |
| SAM2*<br>(4C)   | 131.6<br>$\pm 3.4$                               | 5.9                                       | 12.3                                      | 37.2 $\pm$ 0.7                               | 0.14                                  | 0.29                                  | 10                            | 38.4                                     | 79.0                                     |
| A3              | 42.5<br>$\pm 1.8$                                | 1.4                                       | 2.7                                       | 10.5 $\pm$ 0.3                               | 0.03                                  | 0.06                                  | 10                            | 28.9                                     | 54.9                                     |
| PEG01           | 82.7<br>$\pm 2.8$                                | 3.2                                       | 13.6                                      | 17.6 $\pm$ 0.6                               | 0.06                                  | 0.25                                  | 10                            | 32.9                                     | 139.3                                    |

|      |               |     |      |            |      |      |    |      |       |
|------|---------------|-----|------|------------|------|------|----|------|-------|
| FLV5 | 73.3<br>± 2.5 | 4.9 | 14.5 | 7.9 ± 0.1  | 0.07 | 0.21 | 10 | 57.4 | 167.8 |
| #15  | 47.8<br>± 2.5 | 1.8 | 5.4  | 10.7 ± 0.2 | 0.05 | 0.15 | 10 | 32.3 | 95.7  |
| BUR  | 20.1<br>± 0.8 | 0.1 | 0.2  | 7.3 ± 0.2  | 0.01 | 0.03 | 10 | 13.5 | 37.7  |
| MED  | 59.4<br>± 1.6 | 0.4 | 1.1  | 22.3 ± 0.5 | 0.05 | 0.13 | 10 | 23.4 | 58.7  |

415 \* SAM1 and SAM2 are replicated hot water extracts from the same batch of *A. annua* leaves grown and processed from Atelier

416 Temenos; SAM1 was stored at -20C, thawed and reanalyzed at the same time as SAM2. Data are the average of ≥ 6 independently

417 extracted leaf samples.

418 \*\* Quercetin equivalents.

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**Table 3.** Comparative IC/EC50s for artemisinin derivatives and partner drug antimalarials.

| Compound           | Cao et al. (2020) | Gilford et al.<br>(2020) | Bae et al.<br>(2020)         | This report |
|--------------------|-------------------|--------------------------|------------------------------|-------------|
|                    | μM                |                          |                              |             |
| Artemisinin        | 64.5              | 534.8                    | NM                           | 70          |
| Arteannuin B       | 10.3              | NM                       | NM                           | NM          |
| Artemisinic acid   | >100              | NM                       | NM                           | NM          |
| Deoxyartemisinin   | NM                | NM                       | NM                           | >100        |
| Dihydroartemisinin | 13.3              | NM                       | NM                           | >100        |
| Artesunate         | 13.0              | 18.2                     | 53, 1.8<br>(Vero E6, Calu-3) | >100        |
| Arteether          | 31.9              | NM                       | NM                           | NM          |
| Artemisone         | 49.6              | NM                       | NM                           | NM          |
| Amodiaquine        | NM                | NM                       | NM                           | 5.8         |
| Lumefantrine       | 23.2              | NM                       | NM                           | NM          |

423 NM = not measured

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425

426 **FIGURE LEGENDS**

427 Figure 1. Compounds used in this study and the plant *Artemisia annua* L.

428 Figure. 2. Inhibition plots of extracts for efficacy against Vero E6 cells infected with SARS-CoV-2

429 USA/WA1 (MOI 0.1) based on: artemisinin (A); total flavonoids (tFLV) (B); or dry mass of *A. annua*

430 leaves (C) used in the experiments. Data are plotted from an average of three replicates with  $\pm$  SE.

431 Figure 3. VSV spike pseudovirus in Calu-3 and Vero E6 cells and their viability in response to

432 increasing hot water *Artemisia* extracts as percent of solvent controls. *Artemisia* concentration

433 refers to dry leaf mass extracted with hot water. Data plotted using nonlinear regression curve

434 fitting using GraphPad Prism. Data are averages of triplicate samples per condition and error bars

435 are  $\pm$  SD. Data are a representative experiment that was repeated twice.

436 Figure 4. Comparison of *A. annua* SAM extracts and other antimalarial and artemisinin compounds

437 against Vero E6 cells infected with SARS-CoV-2 USA/WA1 (MOI 0.1). A full concentration series for

438 all samples except for the *A. annua* tea could not be fully tested due to solvent toxicity, which was

439 also observed for *A. annua* in dichloromethane (DCM) at higher concentrations. Data are plotted

440 from an average of three replicates with  $\pm$  SE.

441 Figure 5. Spearman's correlation scatter plots between artemisinin concentration or total

442 flavonoid levels vs. calculated IC<sub>50</sub> and IC<sub>90</sub> for the hot water extract of each cultivar from data in

443 Table 2.

444 Figure 6. Cytotoxicity of Vero 6 cells in response to imatinib (A) and *A. annua* hot water extracts

445 (B). Data are plotted from an average of three replicates with  $\pm$  SE

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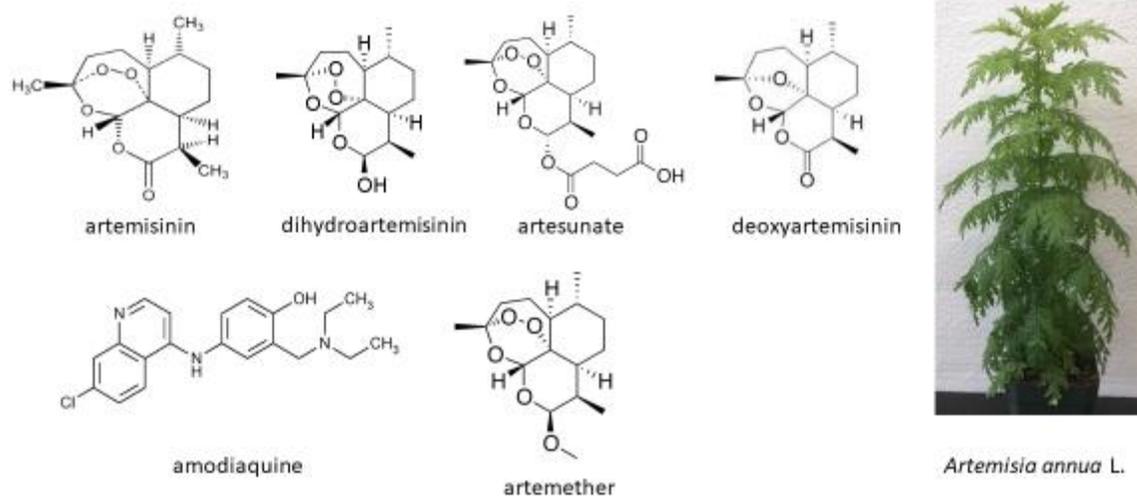
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**Figure 1.** Compounds used in this study and the plant *Artemisia annua* L.

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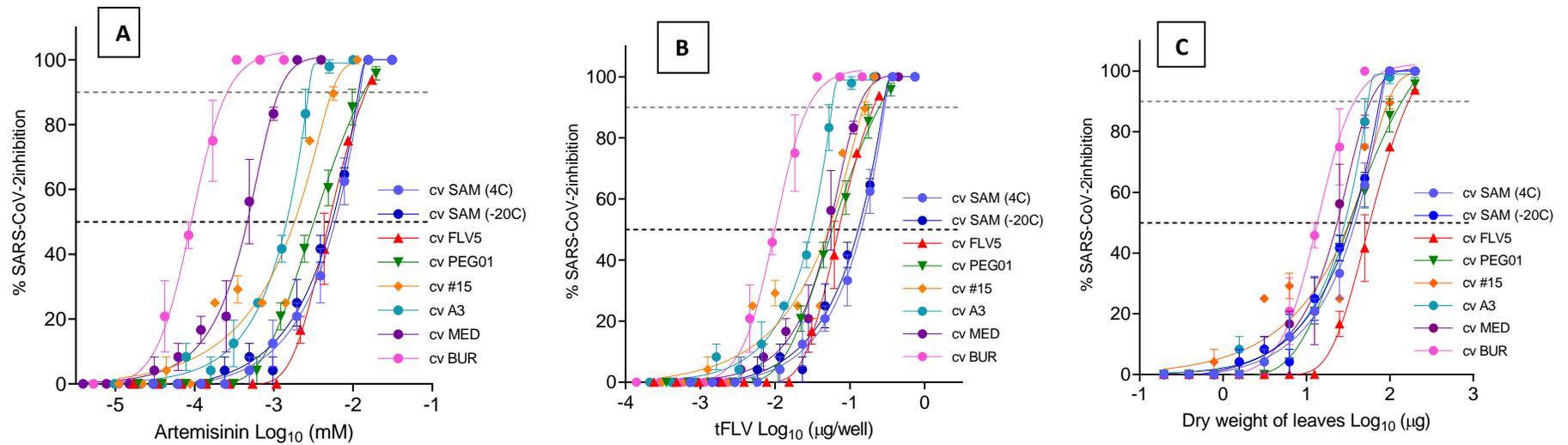
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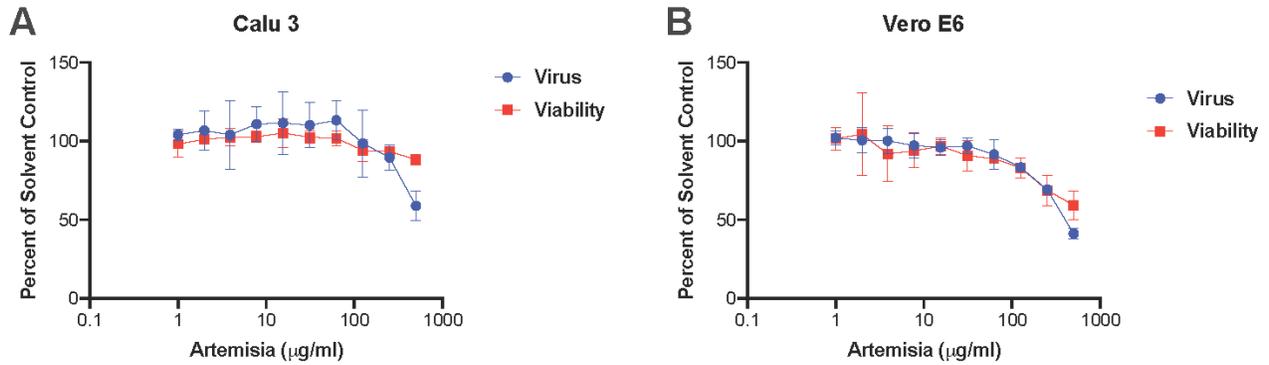
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**Figure. 2.** Inhibition plots of extracts for efficacy against Vero E6 cells infected with SARS-CoV-2 USA/WA1 (MOI 0.1) based on: artemisinin (A); total flavonoids (tFLV) (B); or dry mass of *A. annua* leaves (C) used in the experiments.

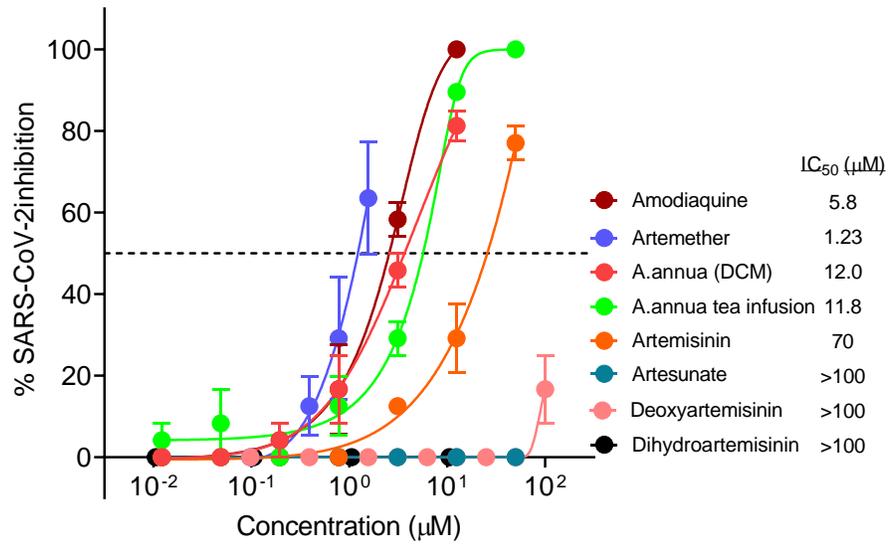
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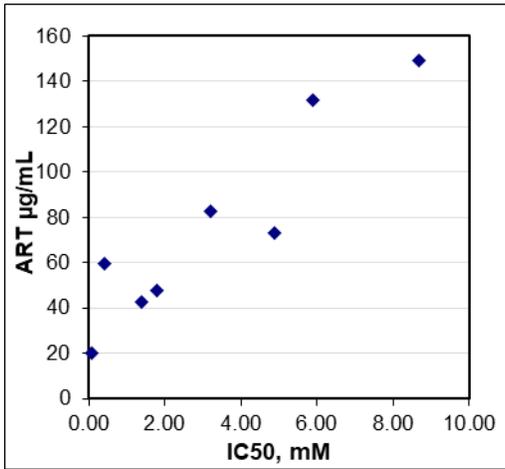


**Figure 3.** VSV spike pseudovirus in Calu-3 and Vero E6 cells and their viability in response to increasing hot water *Artemisia* extracts as percent of solvent controls. *Artemisia* concentration refers to dry leaf mass extracted with hot water. Data plotted using nonlinear regression curve fitting using GraphPad Prism. Data are averages of triplicate samples per condition and error bars are  $\pm$  SD. Data are a representative experiment that was repeated twice.

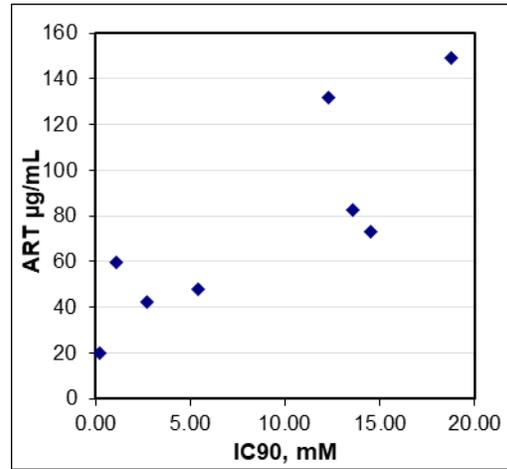


**Figure 4.** Comparison of *A. annua* SAM extracts and other antimalarial and artemisinin compounds against Vero E6 cells infected with SARS-CoV-2 USA/WA1 (MOI 0.1). A full concentration series for all samples except for the *A. annua* tea could not be fully tested due to solvent toxicity, which was also observed for *A. annua* in dichloromethane (DCM) at higher concentrations.

472 **ARTEMISININ CORRELATIONS**



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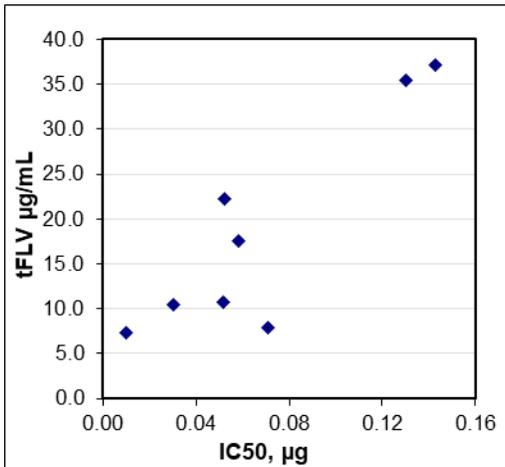


474 Spearman's Rho=0.90, P=0.002

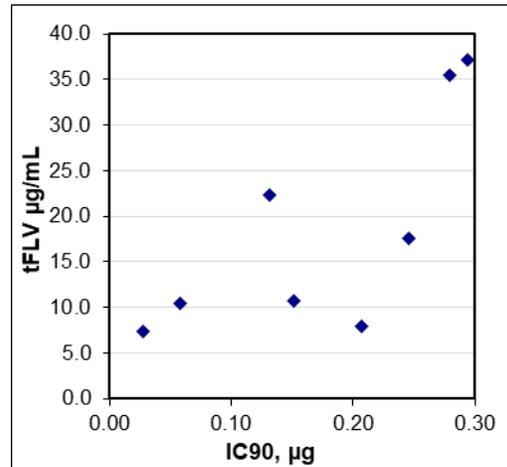
Spearman's Rho=0.83, P=0.010

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476 **TOTAL FLAVONOID CORRELATIONS**



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478 Spearman's Rho=0.74, P=0.037

Spearman's Rho=0.76, P=0.028

**Figure 5.** Spearman's correlation scatter plots between artemisinin concentration or total flavonoid levels vs. calculated IC<sub>50</sub> and IC<sub>90</sub> for the hot water extract of each cultivar from data in Table 2.

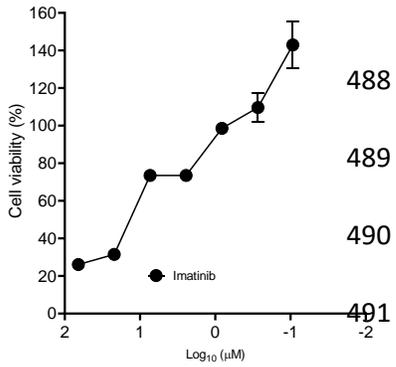
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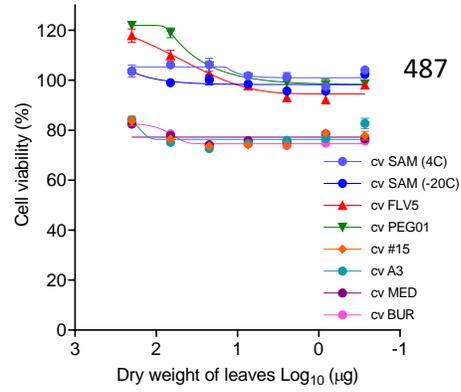
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**Figure 6.** Cytotoxicity of Vero 6 cells in response to imatinib (A) and *A. annua* hot water extracts (B).

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504 **Supplemental Data:**

505 *Bioavailability of artemisinin from per os consumption of dried leaf Artemisia annua in a human*  
506 *subject.*

507 NB: PJW verified with the WPI IRB that no IRB approval is required for self-  
508 experimentation. One of the authors, PJW, age 71, 140 lb [63.6 kg]) consumed 3 g powdered,  
509 encapsulated dried *A. annua* SAM (2018 garden crop) and had 3 total blood draws: just prior to  
510 consumption; at 2 h post consumption, and a few weeks later, subject took another 3 g dose and  
511 blood was drawn 5 h post consumption. Serum was isolated from the blood and analyzed for  
512 artemisinin using gas chromatography mass spectrometry (GCMS) per Martini et al. 2020.  
513 Artemisinin (MW = 282.33) amount in the encapsulated material was 1.5% (15 mg/g), so amount  
514 consumed (delivered) was 45 mg artemisinin. Estimating 100% bioavailability, and that this human  
515 subject had a total volume of about 4.13 L blood  
516 (<https://reference.medscape.com/calculator/estimated-blood-volume>), the amount of delivered  
517 artemisinin/mL blood could not exceed 10.90 mg/L, or 10.90 µg/mL. Human blood is 55% serum  
518 (or 2.3 L for this human subject), so the highest serum concentration of artemisinin would actually  
519 be about 20 mg/L or 20 µg/mL.

520

| Table S1. Human pharmacokinetics of ART delivered from <i>p.o. Artemisia annua</i> <sup>521a</sup> |                      |   |
|--|----------------------|---|
| Time (h)   | ART in serum (µg/mL) | % of <i>A. annua</i> -ART consumed <sup>522</sup> |
| 0  | 0.0                  | 0 <sup>523</sup>                                  |
| 2  | 7.04                 | 36 <sup>524</sup>                                 |
| 5  | 0.16                 | 0.8 <sup>525</sup>                                |

526

527 <sup>a</sup> Consumed 3 g powdered dried leaf *A. annua* containing 10.90 mg ART *in toto*; maximum possible  
528 serum concentration at *p.o.* delivery = 19.62 µg ART/mL.

529

**Table S2.** Examples of comparative amounts of artemisinin metabolites in various cultivars of *A. annua*.

| Compound               | LUX        | BUR       | SAM1, <sup>1</sup>                      |
|------------------------|------------|-----------|---|
|                        | MNHNL17732 | LG0019527 | MASS 00317314                           |
| mg/g dry weight leaves |            |           |   |
| Artemisinin            | 1.34       | 1.70      | 10.94 <sup>2</sup> , 15.90 <sup>1</sup> |
| Arteannuin B           | 0.93       | ND        | 0.09 <sup>2</sup> , 2.32 <sup>1</sup>   |
| Artemisinic acid       | 0.86       | ND        | ND <sup>2</sup> , 0.37 <sup>1</sup>     |
| Deoxyartemisinin       | 0.32       | 0.39      | 0.83 <sup>2</sup> , NM <sup>1</sup>     |

530 ND, not detected; NM, not measured.

531 <sup>1</sup> Data from Weathers and Towler (2014) *Ind Crop Prod* 62:173-178; artemisinic acid is detectable  
532 in the dried leaves.

533 <sup>2</sup> Data estimated from fresh leaf analysis using dry weight/fresh weight ratio = 0.26 from Table 1 in  
534 Towler and Weathers (2015) *Ind Crop Prod* 67:185-191.

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