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5 **Uptake, Accumulation and Impact of Antiretroviral and Antiviral Pharmaceutical**
6 **Compounds in Lettuce**

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14 **Abstract**

15 While the contamination of agroecosystems with pharmaceutical compounds has been
16 reported, the fate of these compounds, particularly uptake into plants remains unclear. This
17 lack of environmental fate data is evident for a critical class of pharmaceuticals, the antivirals
18 and antiretrovirals (ARVDs). Thus, this study evaluated the root uptake of the antiretroviral
19 compounds nevirapine, lamivudine and efavirenz, and the antiviral compound oseltamivir in
20 lettuce. The lettuce was hydroponically grown in a nutrient solution containing the four ARVD
21 pharmaceutical mixture in the 1-100 $\mu\text{g L}^{-1}$ concentration range. The measured
22 bioaccumulation showed that efavirenz and lamivudine accumulated to the highest and lowest
23 degree, at concentrations of 3463 ng g^{-1} and 691 ng g^{-1} respectively. The translocation factor
24 between the root and leaf for nevirapine was greater than 1. The highest concentration of the
25 pharmaceutical mixture had a physiological impact on the lettuce. Potential toxicity was
26 evidenced by a statistically significant 34 % ($p = 0.04$) mean reduction in root and leaf biomass
27 in the 100 $\mu\text{g L}^{-1}$ ARVD mix exposed lettuce, compared with the controls. This study advances
28 knowledge of the fate of ARVDs in agroecosystems, in particular, plant root – ARVD
29 interaction and the resulting potentially toxic effects on plants.

30 **Keywords:** Antiretroviral; Antiviral; Uptake; Pharmaceuticals; Accumulation; Toxicity

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43 **1 Introduction**

44 Plant uptake of active pharmaceutical ingredient (API) molecules has been reported in food
45 and non-food crops. Exposure to APIs can either be under a controlled environment
46 (hydroponic and pot trial experiments) (e.g. Goldstein et al., 2014; Ahmed et al., 2015; Hurtado
47 et al., 2016; Kodešová et al., 2019; Tian et al., 2019) or under field conditions on soils
48 amended with contaminated surface water, un/treated wastewater, sewage sludge and
49 biosolids (e.g. Prosser et al., 2014; Paz et al., 2016; Ben et al., 2018; Carter et al., 2018;
50 Bagheri et al., 2019). Uptake studies, however, have primarily focussed on antibiotics,
51 antidepressants, analgesics, anti-epileptics, hormones and non-steroidal anti-inflammatory
52 therapeutic based drugs. Past studies and reviews (Navarro et al., 2014; Wegst-Uhrich., 2014;
53 Wu et al., 2015; Bártíková et al., 2016a; Zhang et al., 2017; Madikizela et al., 2018; Christou
54 et al., 2019) reveal that there is limited information on an equally significant therapeutic class
55 of drugs, the antivirals and antiretrovirals (ARVDs). A global review of the environmental and
56 ecotoxicological effect of antiretrovirals by Madikizela et al., (2018), acknowledges this
57 deficiency, recommending plant ARVD uptake studies.

58 The environmental occurrence of ARVDs is ostensibly less frequent and manifests at trace
59 concentration levels in high and upper-middle-income countries and hence of minimal concern
60 (De Voogt et al., 2009; Sui et al., 2012, Funke et al., 2016). This scenario has presumably
61 contributed to the low number of associated ARVD plant uptake investigations as the majority
62 of uptake studies are undertaken in these countries. The consumption of ARVDs and the
63 extent of their contamination in the Sub-Saharan Africa region (particularly Kenya and South
64 Africa) compared with Europe is detailed by Fekadu et al., (2019) and Nannou et al., (2020)
65 in fresh surface waters and wastewaters respectively. Recent antiretroviral data from Lusaka,
66 Zambia, report on the occurrence of three antiretrovirals, lamivudine nevirapine and
67 zidovudine in the aquatic environment at concentrations in the range of 60 - 118,970 ng L⁻¹
68 (Ngumba et al., 2020). The three compounds were also detected in source-separated urine
69 at concentrations in the range 7740 – 12800 µg L⁻¹, which is several magnitudes higher than
70 measured environmental concentrations. Considering that source-separated urine is re-used
71 as fertilizer (Ledezma et al., 2015), it is imperative to gain knowledge on plant-ARVD
72 interaction.

73 The therapeutic objective of ARVDs is to counter a range of viruses in the host body (De
74 Clercq and Li, 2016; Nannou et al., 2020). Therefore, an untargeted wide-scale release of
75 ARVD into the environment may alter ecosystem functioning and potentially induce antiviral
76 resistance in microorganisms (Jain et al., 2013; Bártíková et al., 2016; Nannou et al., 2020).
77 Concerning ensuing activity and toxicity of ARVDs, a Q-SAR modelling study revealed that

78 ARVDs were highly potent. In a class of 50 therapeutic APIs, ARVDs were predicted to be
79 among the top eight most hazardous drugs (Sanderson et al., 2004). Besides the public health
80 concern, APIs may also induce toxic effects on plants by affecting their physiological and
81 development processes (Hillis et al., 2011; Liu et al., 2013; Christou et al., 2018; Sun et al.,
82 2018; Rede et al., 2019). For example, Sun et al., (2018) exposed cucumber to a mixture of
83 17 APIs at concentrations of up to 1000 $\mu\text{g L}^{-1}$ and found that the leaves exhibited burn-like
84 features. At present, there is a limited understanding of potential ARVD effects on plants. A
85 review of the impact of APIs on plants presented by Bártíková et al. (2016). It outlines the toxic
86 effects brought by antibiotics, anti-parasitic drugs, hormones, growth promoters and
87 antifungals on plants but contains hardly any information on the impact of ARVDs on plants.

88 Plant-API uptake studies with an African perspective include Amos Sibeko et al. (2019). They
89 detected the non-steroidal anti-inflammatory drugs (NSAIDs) naproxen, ibuprofen and
90 diclofenac in water hyacinth (*Eichhornia crassipes*) in river water in South Africa. Naproxen
91 showed the highest concentration (12 ng g^{-1}). Mlunguza et al., (2020) reported on the uptake
92 of three antiretrovirals (emtricitabine, tenofovir disoproxil and efavirenz) in water hyacinth in a
93 water reservoir in South Africa. The concentrations of antiretrovirals in the hyacinth reed varied
94 from 0.97 to 29.6 ng g^{-1} .

95 Although Mlunguza et al. (2020), reported on plant uptake of ARVDs, a structured
96 experimental approach is necessary to elucidate plant root-ARVD interactions
97 comprehensively. In this hydroponic study, the uptake of three antiretroviral APIs (nevirapine,
98 lamivudine and efavirenz) and one anti-influenza antiviral API (oseltamivir) into lettuce
99 (*Lactuca sativa*) was investigated. Selection of the APIs was based on their frequency of
100 detection from previous (Ngumba et al., 2016; K'oreje et al., 2016) studies. The advantage of
101 hydroponic experiments is that they provide test conditions to rapidly screen and identify
102 priority APIs that exhibit the highest uptake potential, which can inform in-depth plant-API fate
103 studies. The major drawback, however, is that hydroponic studies do not provide the
104 complexity of a real agroecosystem environment (Wu et al., 2015). Leafy vegetables
105 accumulate organic contaminants greatest in comparison to root vegetables, cereals and fruit
106 vegetables (Christou et al., 2019), hence the selection of lettuce. Uptake can be described
107 either as a passive or active process (Kumar and Gupta, 2016). This study focussed on active
108 processes (solute specific), i.e. it solely concentrated on the characteristic of the molecule
109 rather than the existing environmental conditions.

110 **2 Materials and methods**

111 **2.1 Reagents**

112 Methanol (MeOH), acetonitrile (MeCN), HPLC water and 0.1 % formic acid in water were
113 obtained from Fisher Scientific, UK, and ammonium hydroxide (NH₄OH) solution (25% v/v)
114 and formic acid from Sigma Aldrich, UK. Nevirapine (NVP) and lamivudine (LVD) standards
115 were obtained from Sigma Aldrich, UK, efavirenz (EFV) from Tokyo Chemical Industries, and
116 oseltamivir (OSV) from Fisher Scientific, UK. All antiviral API standards had a purity grade of
117 > 99 %.

118 **2.2 Standards and stock solutions**

119 Preparation and storage of stock solutions and standards followed the European Commission,
120 SANTE/11813/2017 (2018) guidelines. LVD and OSV stock solutions were prepared in 50 %
121 (v/v) MeOH: HPLC water solution. NVP and EFV stock solutions were made in 100 % MeOH.
122 Dilutions of the four ARVD mix were prepared at concentrations of 0.1 to 100 µg L⁻¹.

123 **2.3 Liquid Chromatography**

124 Analyte separation was performed by liquid chromatography (Dionex Ultimate 3000, Thermo
125 Scientific) on a PhenylHexyl (*Ace ultracore*) (2.5 µm × 100 mm) stationary phase. The column
126 temperature was isocratic at 50 °C. The analytical run time was 9.8 min, starting from a mobile
127 phase composition of 100 % 0.1% ammonia (in HPLC water) and 0.1 % formic acid solution
128 (1:1, v/v), adjusted to pH 8.4, before transitioning to 100 % MeOH. The equilibration time was
129 2 minutes and at a flow rate of 500 µL min⁻¹.

130 **2.4 High-resolution mass spectrometry (HRMS)**

131 An Orbitrap HRMS system (Thermo Scientific) was used for mass detection. Ionization was
132 via heated electrospray (HESI) at a vaporizer temperature of 300 °C. Nitrogen gas was used
133 as the auxiliary gas. Spray voltage was operated in positive mode at + 3.5 kV with a capillary
134 temperature of 270 °C. The scanning range covered the 100 -1000 *m/z* mass window and
135 mass resolution set at 17500 *m/z*. Mass calibration of the mass spectrophotometer was
136 performed 24 hours before analyses. An example chromatogram is provided in Figure 1.

137 **2.5 Method validation/optimization**

138 The ultrasonic-assisted extraction (UAE) approach adapted from Wu et al., (2012) was
139 selected as the extraction method. Sample clean-up was achieved using solid-phase
140 extraction (SPE). Since the target analytes exhibited varying physicochemical characteristics

141 (**Error! Reference source not found.**), it was necessary to determine the optimal extraction-
142 pH.

143 The performance characteristics of the method were evaluated using extraction recoveries of
144 the analytes from plant material, instrument and method linearity, method limit of detection,
145 and quantification (MLOD and MLOQ) and evaluation of matrix effects (ME).

146 **2.5.1 Extraction pH and recoveries**

147 Fresh lettuce was obtained from a local grocer. The leaves were washed with high purity water
148 (HPW), chopped and freeze-dried. The freeze-dried sample was then ground to powder.
149 Recovery experiment was performed at two spiking levels, 10 ng g⁻¹ and 50 ng g⁻¹. Ground
150 lettuce (0.2 g) was placed in a 50 mL polypropylene centrifuge tube and spiked with the four
151 ARVD external standard mix stock to attain the two desired spiking levels. Spiked samples
152 were allowed to equilibrate for 24 hours. Ten mL of the extraction solvent, (MeOH: MeCN; 1:1,
153 v/v) was added to the sample, and the tube placed in an ultrasonic bath operated at 50 Hz for
154 15 min. The resulting supernatant was collected and centrifuged at 4000 rpm for 5 min. The
155 sample residue was re-extracted and the supernatants combined before solvent reduction to
156 ca 500 µL under a gentle stream of nitrogen gas. The reduced extract was diluted with 20 mL
157 HPW water and then filtered (GF/F, 0.7 µm). The optimal extraction pH was obtained by
158 extracting set of samples (n = 3) at four pH values: pH 2.4 (adjusted using 1 % (v/v) formic
159 acid), the native sample pH 5.4; pH 7.4 and 9.4 (1 M NH₄OH solution adjusted).

160 **2.5.2 Solid-phase extraction**

161 Solid-phase extraction was implemented using a 60 mL HLB sorbent (Waters, UK). Before
162 sample loading, the sorbent was conditioned sequentially with MeOH and HPLC water (2.5
163 mL aliquots). The 20 mL sample extract was loaded onto the cartridge at a rate of 2 mL min⁻¹.
164 After loading, the sorbent was dried and washed with 1 mL of HPLC water. Aliquots (2 x 2.5
165 mL) of the extracting solvent mix were used for sample elution. The 5 mL extract was
166 evaporated to almost dryness and reconstituted to 1 mL using 30 % MeOH (in HPLC water).
167 Finally, the 1 mL sample was filtered using a 0.22 µm PTFE filter and stored in at 4°C in the
168 dark before analysis.

169 **2.5.3 Linear range, MLOD, MLOQ and Matrix effect**

170 The LC-HRMS was calibrated at the 0.1 – 100 µg L⁻¹ concentration range using matrix-
171 matched (MM) standard solutions, prepared according to EU SANTE/11813/2017 (2018)
172 guidelines. It followed that the amount of the solute stock in the MM standard solution was 10
173 % of the total standard solution volume and the remaining 90 % comprised of the unspiked
174 plant extract. The MLOD and MLOQ were calculated according to the ICH guidelines (ICH,

175 1995), based on the calibration curve of the MM working standards at the native lettuce extract
176 pH (pH 5.4). Matrix effects (ME) was evaluated by comparing the slope of the calibration curve
177 in the matrix to the slope of the calibration curve using solvent (Eq 1).

$$178 \text{ Matrix effect (\%)} = \left(\frac{\text{Slope of calibration curve in matrix}}{\text{slope of calibration curve in solvent}} - 1 \right) \times 100$$

179 1

180 2.6 Hydroponic experiments

181 The hydroponic system was housed in a greenhouse. The experimental approach was
182 adapted from the OCSPPC 850.4800 Plant Uptake and Translocation Test guidelines (EPA,
183 2012). Young lettuce seedlings (10 days old) of the *Analora* genus were obtained from Defland
184 Nurseries, UK. Before the actual exposure test, the plants were exposed to a dilute water-
185 fertilizer solution for seven days to nurture and acclimatize the roots to a water-only
186 environment. Individual seedlings were each exposed to 400 mL of the four ARVD mix
187 standard nutrient solution, composed of water and commercial fertilizer (Flora Gro, NPK 3:1:6
188 at a concentration of 0.5 mL L⁻¹). The set up consisted of 6 replicates per exposure
189 concentration. In total, there were 24 lettuce plant samples. The sample containers (glass)
190 were covered with aluminium foil, and the top sealed to allow only the roots to be in contact
191 with the nutrient solution. The solution was automatically aerated for 10 minutes every hour.
192 The experiment was run for 21 days with the exposure solution renewed on days 7 and 14.
193 Loss of water due to evaporation and evapotranspiration during the growing period did not
194 exceed 20 % of the initial volume of the nutrient solution placed at the beginning of each
195 experiment.

196 2.7 Accumulation and physiological effect of ARVDs on lettuce

197 After test termination, the plant samples were immediately washed with HPW and thoroughly
198 dabbed dry. Lettuce samples were separated into roots and leaf then weighed. After that, the
199 samples were freeze-dried before extraction and analysed according to the optimized method
200 described in the previous sections. Potential physiological effects on the plant were assessed
201 by comparing the biomass of the control (root and leaves) with the biomass ARVD exposed
202 samples.

203 Uptake of ARVDs in this study was characterized using four parameters: bioconcentration
204 factor (BCF), root concentration factor (RCF), leaf concentration factor (LCF) **Error!**
205 **Reference source not found.**, **Error! Reference source not found.** and **Error! Reference**
206 **source not found.** and translocation factor (TF). **Error! Reference source not found.** TF
207 quantifies the movement of the organic analyte from the root to above-ground tissues.

208 $BCF(mL/g) = C_{plant}/C_{exposure\ solution}$ (2)

209 $RCF(mL/g) = C_{root}/C_{exposure\ solution}$ (3)

211 $LCF(mL/g) = C_{leaf}/C_{exposure\ solution}$ (4)

212 $TF(L/g) = C_{leaf}/C_{root}$ (5)

213
214
215
216 Where C_{leaf} , C_{root} , C_{plant} , and $C_{exposure\ solution}$ is the API concentration in the leaf, root, plant
217 and nutrient solution, respectively (Goldstein et al., 2014; Hurtado et al., 2016; Emhofer et al.,
218 2018).

219 **2.8 Data analyses**

220 Data were analyzed using Ms Excel 2016 and IBM SPSS Statistic v 24 software. One-way
221 analysis of variance (ANOVA) and Dunnett's T3 test was used to measure statistical
222 differences between means at the 95 % confidence interval.

223 **3 Results and discussion**

224 **3.1 Method optimization**

225 **3.1.1 Recoveries and effect of pH**

226 The objective of this experiment was to select the optimal extraction pH for the target analytes.
227 Since the recovery of an analyte is concentration-dependent; while performing recovery
228 experiments an analytically appropriate spiking range should be selected as opposed to a
229 single spike level. (Thompson et al., 1999). Pharmaceuticals are typically detected at
230 nanogram level in the environment, hence 10 ng g^{-1} and 50 ng g^{-1} level were selected as they
231 were considered critical spike levels for the recovery test. The extraction recoveries were
232 tested at four pH values, ranging from acidic (pH 2.0) to alkaline (pH 9.4).

233 2 depicts the obtained analyte absolute extraction recoveries. The recoveries were
234 cumulatively evaluated across the two spike levels. Mean ARVD recoveries at the two spike
235 levels did not vary significantly ($p=0.55$), being $68 \pm 22\%$ (\pm SD) and $65 \pm 19\%$ for the 10 ng g^{-1}
236 and 50 ng g^{-1} level respectively. Individually, NVP exhibited the highest recoveries (mean 88
237 %). EFV and OSV had second and third highest recoveries at 75 % and 62 % respectively.
238 LVD yielded the lowest recoveries an average of 42 %. pH change least influenced NVP and
239 EFV recoveries. OSV recoveries were relatively consistent across three (pHs 2.0-7.4) with
240 only a minimal drop at highest pH. Contrastingly, LVD recoveries varied widely, which
241 ultimately contributed to its low mean recoveries.

242

243 Cumulatively, the highest ARVD extraction recoveries were obtained at pH 5.4 (mean of 72
244 %). At pH 2.0, 7.4 and 9.4, recoveries of 66 %, 66 % and 62 % respectively, were obtained.
245 While EFV and NVP recoveries at pH 5.4 had recoveries of > 70 %, LVD and OSV at the same
246 environment exhibited recoveries of < 65 %. Referring to EU SANTE/11813/2017 guidelines,
247 it states that satisfactory extraction recoveries may vary between 70-120 %. Nonetheless, it
248 clarifies that reproducible recoveries of 30-70 % are also acceptable. For this reason, pH 5.4
249 was selected as the optimal pH for subsequent extraction procedures. It is noteworthy to
250 mention that pH 5.4 was also the natural extraction pH, so working at this pH meant that
251 subsequent buffer additions were unnecessary.

252

253 **3.1.2 Linearity, MLoD and MLoQ**

254 A visual inspection of the plot of analyte signal vs concentration revealed the existence of a
255 linear relationship. Whereas the relationship between the analyte signal and the analyte in
256 solution is often linear, the relationship between analyte signal and the analyte in the matrix is
257 not routinely linear due to the influence of matrix components (Kruve et al., 2015a). For this
258 reason, linearity was assessed using analyte in the matrix. **Error! Reference source not
259 found.** shows that analyte in matrix linearity was satisfactory at $r^2 > 0.990$. Likewise, the limit
260 of detection is also matrix-dependent (Kruve et al., 2015b); accordingly, matrix-spiked
261 samples were used in the determination of MLoD and MLoQ. The MLoD of the ARVD analytes
262 varied from 1.51 to 5.61 ng g⁻¹ and the MLoQ from 5.13 to 18.7 ng g⁻¹ (**Error! Reference
263 source not found.**).

264 **3.1.3 Matrix effects (ME)**

265 Ultrasonic extraction mechanically breaks down the sample matrix allowing the release of the
266 target analytes (Schantz, 2006; Tadić et al., 2019). However, a drawback is its lack of
267 selectivity necessitating intensive subsequent clean-up processes (Ros et al., 2016). For this
268 reason, it was necessary to evaluate the effect of matrix on the detection of analytes. As
269 indicated in **Error! Reference source not found.**, ion suppression predominated. LVD
270 exhibited the lowest ion suppression at ≤ 30 %. NVP, OSV, and EFV, on the other hand,
271 showed ion suppression ranging from 46–50 %. Classification of the ME was made according
272 to Barreales-Suárez et al., (2018), i.e. ME of < 20 % considered as low, ME 20 - 40 % as a
273 medium, ME 40-60 % as high and ME as 60 % as very high. In general, ME lay in the medium
274 to high-level region, indicating significant signal suppression. Varying levels of ME following
275 the UAE-SPE method have been reported. For example, ME of 26 to 29 % were noted during
276 the analyses of antibiotics in four vegetable matrices (Tadić et al., 2019) and ion suppression
277 of 30 to 60 % was reported in the analyses of 7 antibiotics in lettuce (Albero et al., 2019).

278 Overall, the data from this study is consistent with Furey et al. (2013) and Tadić et al. (2019)
279 who suggest that signal enhancement or suppression due to the matrix is unpredictable. It
280 presents itself unsystematically and also indiscriminately and therefore is unique for each
281 analysis.

282 **3.2 Presence of APIs in the lettuce plant**

283 3A shows that the ARVDs present in the nutrient solution accumulated at varying
284 concentrations in the roots and leaves of the lettuce plant. The four ARVDs studied had a
285 molecular weight < 400 Da; molecules of this size may penetrate the root via the epidermis
286 into the bulk of the root (Miller et al., 2015). At the lowest exposure concentration ($1 \mu\text{g L}^{-1}$),
287 the accumulation of LVD, OSV, and EFV in the leaf was below the MLoD and was not
288 quantified, whereas, for NVP and LVD, root concentrations were below the MLoD.

289 The overall whole plant accumulation of the ARVDs in the lettuce varied from < MLoQ to 3463
290 ng g^{-1} and < MLoD to 1647 ng g^{-1} for the root and leaf respectively across the three exposure
291 levels. EFV exhibited the highest total tissue biomass accumulation (3463 ng g^{-1}) measured
292 in the $100 \mu\text{g L}^{-1}$ exposed sample. This accumulation was five times higher than the
293 concentration of the lowest accumulated ARVD, LVD (691 ng g^{-1}) in the same treatment level.
294 NVP and OSV accumulations were 2625 ng g^{-1} and 1541 ng g^{-1} , respectively in the $100 \mu\text{g L}^{-1}$
295 exposed samples.

296 The extent of accumulation over the three exposure levels were such that the higher the
297 concentration of the API in the nutrient solution, the higher the measured accumulation. This
298 observation is consistent with Al-Farsi et al. (2017) and González García et al. (2018). They,
299 in a separate hydroponic API exposure experiments, reported that the magnitude of
300 accumulation was directly associated with the concentration of the pharmaceutical in exposure
301 solution. In the current study, the mean plant concentration factor (CF) rise between the $1 \mu\text{g L}^{-1}$
302 and $10 \mu\text{g L}^{-1}$ treatment was $17.7 \pm 9.32 (\pm 1 \text{ SD})$, while the CF between the $10 \mu\text{g L}^{-1}$ and
303 $100 \mu\text{g L}^{-1}$ treatment was $11.7 \pm 5.7 (\pm 1 \text{ SD})$. Cumulatively, the mean increased CF in the
304 plant matrix between the lowest ($1 \mu\text{g L}^{-1}$) and highest exposure solution treatment ($100 \mu\text{g L}^{-1}$)
305 was $189.8 \pm 87.5 (\pm 1 \text{ SD})$.

306 The RCF, BCF and TF values discussed herein are the means across the $10 \mu\text{g L}^{-1}$ and 100
307 $\mu\text{g L}^{-1}$ exposure levels. 3B illustrates that > 80 % of the OSV and LVD API mass fractions
308 predominantly accumulated in the roots whereas for EFV root accumulation was > 95 %.
309 Accordingly, the RCFs were such that $\text{EFV} > \text{OSV} > \text{NVP} > \text{LVD}$ ($0.043 > 0.013 > 0.08 > 0.05$
310 mL g^{-1} , respectively). BCF (mL g^{-1}) was evaluated to determine accumulation into the bulk of
311 the plant. The mean BCF across the $10 \mu\text{g L}^{-1}$ and $100 \mu\text{g L}^{-1}$ exposure region showed that

312 EFV had the highest whole plant tissue accumulation. The ascending order with regards to
313 BCF was $EFV > NVP > OSV > LVD$ at $(0.044 > 0.025 > 0.016 > 0.005 \text{ mL g}^{-1})$.

314 Regarding transport within the plant, NVP exhibited the highest TF values, with 4 indicating
315 that it was readily translocated to the leaves. Typically, a TF (L g^{-1}) > 1 suggests that a
316 molecule can indeed migrate from the roots and its largest fraction accumulate in the above
317 root tissues (Chuang et al., 2019). Therefore, a TF > 2 implied that NVP was two times more
318 likely to bioaccumulate in the above root tissues, specifically the lettuce leaf in this study.

319 As noted in Section 1, there is little information on the plant uptake of ARVDs. For this reason,
320 discussion in this study will refer and relate to pharmaceutical compounds of different
321 therapeutic groups but of comparable physicochemical characteristics to the ARVDs studied
322 here.

323 **3.3 pH - adjusted octanol-water partitioning coefficient (Log Dow) influence on** 324 **uptake**

325 An API's solubility, hydrophobicity, molecular weight and ionization tendencies may influence
326 its uptake potential (Al-Farsi et al., 2017; Chuang et al., 2019). Of all factors, the octanol-
327 water partitioning coefficient (log Kow) is possibly the most extensively investigated API
328 property influencing uptake. For non-ionic compounds, log Kow is linearly related to uptake
329 (Collins et al., 2006; Prosser et al., 2014). For ionizable compounds, however, log Kow is
330 adjusted to log Dow to reflect the environmental pH. The present study was conducted at a
331 constant nutrient solution pH of 6.5.

332 Characterization of the relationship between log Dow and uptake of the APIs was achieved by
333 plotting log Dow against log BCF, RCF, and TF, respectively (5). For BCF, exhibiting an R^2
334 value of 0.69 as indicated in 5A, linearity was influenced mainly by the hydrophobic and neutral
335 NVP and EFV (see Table 1 for the Physico-chemical properties of the ARVDs of interest here).
336 The contribution to linearity by the hydrophilic LVD and OSV (log Dow = -1.1 and -1.44,
337 respectively) however could not be decisively defined. A closer inspection revealed that had
338 the log Kow of OSV had remained unchanged, i.e. not transformed by a change in pH (as was
339 with NVP and EFV), stronger linearity of $R^2 = 0.97$ value would have been realized. The
340 observation further shows that log Dow of hydrophilic ionizable molecules (in this study OSV
341 and LVD), potentially may not accurately describe uptake in plants. Thus, OSV's relatively low
342 log Dow value may not be precise in describing its minimal accumulation in the lettuce.

343 The relationship between the RCF and log Dow, $R^2 = 0.47$, is shown in 5B. It is a relatively
344 weaker relationship compared with BCF's ($R^2 = 0.69$), so possibly other factors besides
345 hydrophobicity appear responsible for the root API uptake (or its lack thereof). Hence, it may
346 be more logical to relate whole plant accumulation (BCF) with hydrophobicity rather than RCF.

347 Chuang et al., (2019) also reported a weak relationship ($R^2 = 0.293$) between the RCF and
348 log Dow in lettuce exposed to 13 ionizable APIs via hydroponic growth. This observation is
349 consistent with Miller et al., (2015) who, in an extensive review on plant API uptake, concluded
350 that no statistically significant relationship exists between LCF and hydrophobicity. It may be
351 plausible to extend this inference to RCF.

352 Regarding TF, 5C shows that no relationship existed between log Dow and TF ($R^2 = 0.017$).
353 This absence of a relationship is consistent with Chuang et al., (2019) and Li et al., (2019).
354 Their studies established that no statistically significant relationship between hydrophobicity
355 and TF for APIs with log Dow between -3 and 4.

356 **3.4 Speciation of ARVD compounds and influence on plant uptake**

357 The cell walls of plant root hairs are negatively-charged. The root cell wall is approximately
358 0.4 μm thick and composed of polysaccharides which naturally reduce the cell wall
359 permeability to solutes. As a result, uptake of anionic molecules is constrained by the
360 electrostatic repulsion by the root hair cells (Trapp, 2000; Miller et al., 2015; Christou et al.,
361 2019). Physiologically the cell vacuole is larger than the cytoplasm, occupying up to 95 % of
362 a plant cell's volume. It has a pH of 5.5 compared to pH 7 of the cytoplasm. Nonetheless, it
363 is the cytoplasm that is in contact with the root cell wall (Trapp, 2000; Goldstein et al., 2014)
364 and transport of molecules is predominantly via the symplastic pathway (through the cell's
365 cytoplasm) rather than the apoplastic pathway (Goldstein et al., 2014; Pan et al., 2014;
366 Prosser et al., 2014; Al-Farsi et al., 2017).

367 OSV was the only ARVD fully ionized at the pH of the growing medium (pH 6.5), being 99.8
368 % in the cationic form (**Error! Reference source not found.**). OSV was thus potentially
369 electrostatically attracted to the negatively charged root hairs. In contrast, LVD, NVP, and EFV
370 were predominantly neutral at the exposure pH. Strong retention due to sorption, however,
371 could have impeded the permeation of the highly cationic OSV further into the roots. One may
372 relate the characteristics of OSV to the antibiotic trimethoprim (TMP). At pH 5.8 (approximately
373 1 pH unit lower than this study pH), TMP was chiefly cationic (95%) and with a log Dow of -
374 0.43 In Chuang et al. (2019). Lettuce was exposed to $50 \mu\text{g L}^{-1}$ of TMP in a hydroponic-
375 based experiment. The concentration of TMP in the leaf in was < 25 % of the API's total
376 accumulation, which is similar to the OSV leaf accumulation measured (< 20 %) in this study
377 (3B). TMP also exhibited a TF value of 0.1, demonstrating its limited ability to be translocated
378 to the above root tissues. Referring to 4, OSV had a comparable TF of approximately 0.1.

379 In contrast to OSV, NVP (log Dow 2.48, neutral) had the highest TF of the four ARVDs, being
380 > 1. NVP lies in the region of moderate hydrophobicity ($1 < \log \text{Dow} < 3$). Organic molecules

381 lying within this hydrophobic window region exhibit the highest predisposition to be transported
382 to above root tissues (Tanoue et al., 2012; Kumar & Gupta, 2016; Li et al., 2019). Kumar &
383 Gupta (2016) illustrated that a sigmoidal relationship exists between the transpiration stream
384 factor (the ratio of the amount of the contaminant in the xylem to exposure medium) and log
385 K_{ow} whose maxima lies in the range between log K_{ow} 2-2.5, as shown in 6. This phenomenon
386 thus provides the most definitive account in describing the measured NVP's high TF value that
387 certainly originated from enhanced mobility from the transpiration stream.

388 This observation is consistent with Chuang et al. (2019), who examined carbamazepine (CBZ)
389 uptake in lettuce under hydroponic conditions. CBZ which has comparable characteristics to
390 NVP (i.e., log D_{ow} 2.5 and neutral charge), displayed a TF pattern that was analogous to NVP,
391 i.e. it had the highest TF of the 13 APIs studied. González García et al. (2018) reported that
392 the concentration of CBZ was 82 % higher in leaves compared to roots in three varieties of
393 lettuce irrigated with CBZ-spiked treated wastewater. Similarly, Shenker et al. (2011) found
394 that 76-84 % of CBZ accumulated in the leaf compartment of cucumber plants compared to
395 roots in a hydroponic and pot trial experiment.

396 EFV showed the greatest whole plant bioaccumulation in lettuce in comparison with the other
397 ARVDs (Figure 3B). However, > 95 % of its mass was retained in the roots, consequently
398 presenting the highest RCF value. Characteristically, EFV was unionized and remained
399 neutral at the experimental pH. Organic compounds with log D_{ow} > 4.5 hardly experience any
400 significant translocation to above-ground tissues (Kumar & Gupta, 2016). However, for a better
401 comprehension of the high accumulation tendencies of EFV on the roots, it is vital to carry out
402 root EFV sorption experiments. Sorption tests enable the quantification of the fraction of EFV
403 that adsorbed onto the root surface and the actual fraction that permeates into the bulk of the
404 roots. Boxall et al., (2006) and Miller et al., (2015) for example, reported on a significant
405 amount of APIs being detected on the exterior of root crops following analyses of the peels
406 rather than the core of the plant. For this study, supposing that the bulk of the EFV permeated
407 into the root, it then implies that the API did not migrate to reach the vascular tissues (i.e., the
408 phloem and xylem). According to Collins et al., (2006), neutral non-ionizable organic
409 compounds with log K_{ow} (> 4), are primarily retained by the lipid cell components in the
410 endodermis and do not reach the vascular tissues for subsequent transport to above root
411 tissues. As EFV was in its neutral form and its log D_{ow} = log K_{ow} = 4.3 (**Error! Reference**
412 **source not found.**), it therefore implied that it was highly unlikely that it partitioned into the
413 vascular tissue and was largely retained at the endodermis. This interaction between EFV and
414 the plant root is analogous to diclofenac (DCF). DCF, a non-steroidal anti-inflammatory drug
415 (NSAID) with a log D_{ow} of 4.5 is similar to EFV but differs in terms of pK_a . González García
416 et al., (2018) measured and found that 89 % of DCF was retained on lettuce roots. Likewise,

417 Zhang et al., (2012) reported higher root accumulation factors ($0.40\text{-}1.36\text{ mL g}^{-1}$) of DCF in
418 the roots of the macrophyte, *Scirpus validus*, compared with the shoot accumulation (0.17-
419 0.51 mL g^{-1}). These two studies further affirm that the roots retain highly hydrophobic APIs
420 ($\log D_{ow} > 4$).

421 LVD exhibited the lowest bioaccumulation in lettuce (3B). LVD also had the lowest octanol-
422 water partition level coefficient (-1.1) before pH correction. As with OSV, minimal accumulation
423 of LVD could be attributed to its hydrophilic nature. Low hydrophobicity i imply minimal
424 permeation into the lipophilic root cell membranes. LVD was primarily uncharged at the test
425 pH. LVD uptake can be compared with caffeine, which is highly soluble in water and has a log
426 K_{ow} value of -0.77 . Accumulation of caffeine in cucumber leaves was observed to be lower
427 than in the root (Goldstein et al., 2014). In contrast, Chuang et al. (2019), reported a TF value
428 of > 1 for caffeine in lettuce. These two contrasting degrees on uptake highlight the need for
429 more in-depth investigations into the uptake of LVD and other similar hydrophilic molecules
430 into vascular plant tissues.

431

432 **3.6 Impact of ARVD exposure on plant physiology**

433 Accumulation of APIs in plants may induce toxicity as (at high concentration) or hormesis (at
434 lower concentration levels) (Christou et al., 2018). Hormesis is a positive, non-distress effect
435 experienced by a plant when exposed to small doses of xenobiotics, characterized by a non-
436 linear dose-response relationship (Agathokleous et al., 2018). Hormesis may be characterized
437 by an increase in the length of roots and number or size of leaves. Toxicity, on the other hand,
438 presents as perturbations in plant growth, e.g. lowered germination rates, chlorosis, tissue
439 deformation, reduced length or mass of the root and shoot. Also, reduced reproduction rate,
440 and enzymatic activity (Liu et al., 2009; Hillis et al., 2011; Furtula et al., 2012; Liu et al., 2013;
441 Bártíková et al., 2016a; Christou et al., 2018, Sun et al., 2018). The current study focussed on
442 the visible physiological effects specifically on the measurable mass of the leaf and root.

443 7 shows the root and leaf mean wet weight ($n=6$) across the four concentration exposure levels
444 (including the control, i.e. unspiked nutrient solution). The mean biomass (root and leaf mass)
445 of the control sample differed (Dunnett's T3 test, $p=0.039$) with the $100\text{ }\mu\text{g L}^{-1}$ exposed sample.
446 Likewise, the 1 and $10\text{ }\mu\text{g L}^{-1}$ treatment contrasted with the $100\text{ }\mu\text{g L}^{-1}$ treated sample
447 (Dunnett's T3, $p=0.012$ and $p=0.07$ respectively). The mean root and leaf mass of the control
448 was $1.80 \pm 0.26\text{ g}$ ($\pm 1\text{ SD}$) and $3.10 \pm 0.34\text{ g}$ respectively, while for the $100\text{ }\mu\text{g L}^{-1}$ exposed
449 lettuce was $1.25 \pm 0.32\text{ g}$ and $2.02 \pm 0.53\text{ g}$ respectively, representing a mean 34 % reduction in
450 mass.

451

452 The significant difference in tissue mass between the control sample and the ARVD exposed
453 samples was likely an indicator of potential physiological effect induced by the ARVDs. Hillis
454 et al. (2011), reported that root elongation of lettuce was inhibited when exposed to a mixture
455 of 10 antibiotics at concentrations of 1, 10, 100, 1000 and 10000 $\mu\text{g L}^{-1}$. Wei et al., (2009)
456 indicated that tetracycline applied at 1, 10 and 100 mg kg^{-1} impacted the mass of the root of
457 ryegrass inducing a 40 % decline in weight. In the same study, shoot (leaf) inhibition was less
458 impacted compared to the root. The physiological impacts in Wei et al. (2009) however differ
459 with this study, in that most significant biomass variation occurred in the shoots (leaves) rather
460 than with the roots.

461 Contrasting the phytotoxic effect observed in the 100 $\mu\text{g L}^{-1}$ treatment, a possible hormetic
462 influence was exerted on the 1 $\mu\text{g L}^{-1}$ and 10 $\mu\text{g L}^{-1}$ treated samples. An inspection of 7 shows
463 that the two sets of treated lettuce samples had a relatively higher mean biomasses (though
464 not significantly different, $p=0.70$ and $p=0.96$ respectively) of 18 % and 8 % respectively
465 compared with the control. A comparable response was exhibited by *Phragmites australis*
466 (common wetland plant) when exposed to a mixture of 3 antibiotics, ciprofloxacin,
467 oxytetracycline and sulfamethazine in the 0.1 -1000 $\mu\text{g L}^{-1}$ concentration range (Liu et al.,
468 2013). Hormetic response (on root activity) was evident at the lower exposure level (0.1 - 10
469 $\mu\text{g L}^{-1}$) and not in the $> 100 \mu\text{g L}^{-1}$ treatment range. Root activity in the 0.1 - 10 $\mu\text{g L}^{-1}$ exposed
470 plants displayed a negative rate of inhibition. In contrast, toxicity was dominant on the 100 and
471 1000 $\mu\text{g L}^{-1}$ exposed plants, depicting a positive inhibition of root activity at 18 % and 36 %
472 compared to the control.

473

474 **Conclusion**

475 This study provides an optimized protocol for determining ARVDs in biological matrices. It also
476 provides evidence of ARVDs uptake in plants. Moreover, it shows that ARVDs interaction with
477 plant roots can be related to other APIs of similar physical-chemical characteristics. Uptake is
478 primarily influenced by molecules' hydrophobicity of the ARVDs, however, along the confines
479 sigmoidal relationship. At low and high concentration levels, a mixture of ARVDs induces both
480 hormetic and toxic effects to plants.

481

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487

488 **Conflict of interest declaration**

489 The authors declare NO conflicts of interests associated with any of this work.

490

491 **4 Reference**

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706 **Highlights**

- 707 • Antiretroviral accumulation up to 3463 ng g⁻¹
- 708 • Highest accumulation by hydrophobic compounds
- 709 • Toxic effects in plants exhibited by reduced biomass

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