Flavonoid versus artemisinin anti-malarial activity in Artemisia annua whole-leaf extracts

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Author contribution statement

TC, MR DR, TW, TL, PH and IG. conceived and designed the research; TC, MR, DR, TW, DH, MF, MV, performed the experiments; TC, MR, TL, MF, MV, PH and IG analyzed data; and TC, MR, PH and IG wrote the manuscript.

Keywords

Malaria, Artemisia annua, Artemisinin, Flavonoids, Plasmodium falciparum, Chalcone isomerase

Abstract

Word count: 209

Artemisinin, a sesquiterpene lactone produced by Artemisia annua glandular secretory trichomes, is the active ingredient in the most effective treatment for uncomplicated malaria caused by Plasmodium falciparum parasites. Other metabolites in A. annua or related species, particularly flavonoids, have been proposed to either act as antimalarials on their own or act synergistically with artemisinin to enhance antimalarial activity. We identified a mutation that disrupts the CHALCONE ISOMERASE 1 (CHI1) enzyme that is responsible for the second committed step of flavonoid biosynthesis. Detailed metabolite profiling revealed that chi1-1 lacks all major flavonoids but produces wild-type artemisinin levels, making this mutant a useful tool to test the antiplasmodial effects of flavonoids. We used whole-leaf extracts from chi1-1 and mutant lines impaired in artemisinin production in bioactivity in vitro assays against intraerythrocytic P. falciparum Dd2. We found that chi1-1 extracts did not differ from wild-type extracts in antiplasmodial efficacy nor initial rate of cytocidal action. Furthermore, extracts from the A. annua cyp71av1-1 mutant and RNAi lines impaired in amorpha-4,11-diene synthase gene expression, which are both severely compromised in artemisinin biosynthesis but unaffected in flavonoid metabolism, showed very low or no antiplasmodial activity. These results demonstrate that in vitro bioactivity against P. falciparum of flavonoids is negligible when compared to that of artemisinin.

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Contribution to the field

The medicinal plant Artemisia annua produces the sesquiterpene artemisinin, the World Heath Organisation (WHO) recommended treatment for malaria. The discovery of this potent and rapidly cytocidal compound was recognised in the 2015 Nobel Prize winning work of Youyou Tu. Malaria continues to have major global impact, especially in Sub-Saharan Africa. Concerns about emerging resistance to artemisinin are justified with several cases of resistance being reported in the scientific literature. A number of studies have focussed on exploring whether other antimalarial compounds in the medicinal plant Artemisia annua might act synergistically with artemisinin. Flavonoids in particular have been singled out as likely synergistic compounds. Based on this idea, several publications have proposed to use whole plant preparations of A. annua to treat malaria, instead of the World Health Organization recommended artemisinin-based combination therapies consisting of pharmaceutical artemisinin derivatives co-formulated with other compounds that enhance treatment outcomes and minimise the risk of resistance emerging. Specifically, Weathers et al., 2014, Elfawal et al., 2015, and Daddy et al., 2017 develop this idea and explain how they would implement it. We directly address the question of whether other relevant antimalarials exist in A. annua in this manuscript. We have used whole plant extracts from the series of A. annua mutants in in vitro Plasmodium falciparum assays that monitor both potency and rate of initial cytocidal kill. We have described first A. annua mutant, chi1-1 strongly impaired in flavonoid production and provided detailed metabolite analysis of that mutant line. Moreover, whole leaf extracts from chi1-1 displayed in vitro antiplasmodial activity equivalent to the wild type, strongly suggesting that flavonoids do not contribute to the A. annua antimalarial activity. Furthermore, we used previously described transgenic and mutant lines of A. annua which have reduced or no artemisinin, but are not impaired in biosynthesis of flavonoids. Results of antiplasmodial assays using those two lines clearly showed no evidence of potent or rapid antiplasmodial activity of flavonoids. Our work also proves the function of the A. annua CHI1 as a gene that encodes a trichome specific chalcone isomerase enzyme. Our conclusions are supported by detailed chemical characterization of the chi1-1 mutant and thorough testing of plant extracts for antiplasmodial activity based on a recently described rate of kill assay using parasites genetically modified to express a bioluminescent reporter (Ullah et al., 2017). This is to our knowledge the first use of various A. annua lines with altered metabolite profiles to address the question of presence of antimalarials other than artemisinin in A. annua. With the advent of artemisinin resistance emergence worldwide, it is important to understand artemisinin and the chemicals in Artemisia annua correctly to offer proper responses and treatments and we believe that our contribution to a better understanding of the chemical composition of this medicinal plant will be of interest to the scientific community at large as well as people involved in developing policies to combat malaria around the world, but also more specifically to plant scientists studying the metabolism of medicinal plants and to the scientific community developing drugs and other solutions to malaria.

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Ethics statements

(Authors are required to state the ethical considerations of their study in the manuscript, including for cases where the study was exempt from ethical approval procedures)

Does the study presented in the manuscript involve human or animal subjects: No

Data availability statement

Generated Statement: All datasets generated for this study are included in the manuscript and the supplementary files.

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Running title: antimalarials in *Artemisia annua*

Keywords: malaria, *Artemisia annua*, artemisinin, flavonoids, *Plasmodium falciparum*, chalcone isomerase

Abstract

Artemisinin, a sesquiterpene lactone produced by *Artemisia annua* glandular secretory trichomes, is the active ingredient in the most effective treatment for uncomplicated malaria caused by *Plasmodium falciparum* parasites. Other metabolites in *A. annua* or related species, particularly flavonoids, have been proposed to either act as antimalarials on their own or act synergistically with artemisinin to enhance antimalarial activity. We identified a mutation that disrupts the CHALCONE ISOMERASE 1 (CHI1) enzyme that is responsible for the second committed step of flavonoid biosynthesis. Detailed metabolite profiling revealed that *chi1-1* lacks all major flavonoids but produces wild-type artemisinin levels, making this mutant a useful tool to test the antiplasmodial effects of flavonoids. We used whole-leaf extracts from *chi1-1* and mutant lines impaired in artemisinin production in bioactivity *in vitro* assays against intraerythrocytic *P. falciparum* Dd2. We found that *chi1-1* extracts did not differ from wild-type extracts in antiplasmodial efficacy nor initial rate of cytocidal action. Furthermore, extracts from the *A. annua cyp71av1-1* mutant and RNAi lines impaired in amorpha-4,11-diene synthase gene expression, which are both severely compromised in artemisinin biosynthesis but unaffected in flavonoid metabolism, showed very low or no antiplasmodial activity. These results demonstrate that *in vitro* bioactivity against *P. falciparum* of flavonoids is negligible when compared to that of artemisinin.

1 Introduction

Malaria is one of the most prevalent infectious diseases with 219 million cases and 435,000 deaths reported in 2017 (WHO, 2018). The World Health Organization recommends the use of artemisinin-based combination therapies (ACTs) for treatment of uncomplicated malaria caused by

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the *Plasmodium falciparum* parasite (WHO, 2018). ACTs consist of fast-acting and stable artemisinin derivatives, such as artesunate, co-formulated with a different class of drug to reduce the emergence of resistance and increase treatment efficacy (Petersen et al., 2011). The main source of the sesquiterpene artemisinin is currently the medicinal plant *Artemisia annua*, which has achieved a yield of 1.5% dry leaf weight through breeding (Townsend et al., 2013). Additionally, a semi-synthetic alternative has been developed through precursor biosynthesis in yeast and chemical conversion to artemisinin (Peplow, 2016).

A. annua accumulates artemisinin together with a wide range of secondary metabolites in the extracellular subapical cavity of glandular secretory trichomes, specialized 10-cell structures on the surfaces of aerial tissues (Brown, 2010; Lange, 2015; Czechowski et al., 2018). This wide range of metabolites has led to speculation that perhaps other compounds in *A. annua* or related species might act as antimalarials or potentially enhance the antimalarial activity of artemisinin. Therefore, several groups have tried to isolate and identify metabolites from *A. annua* and related species that might function as antimalarials (O'Neill M et al., 1985; Elford et al., 1987; Liu et al., 1989; Cubukcu et al., 1990; Liu et al., 1992; Mueller et al., 2000; Bhakuni et al., 2001; Kraft et al., 2003).

Recent publications have reported that *A. annua* whole-plant preparations are more effective than artemisinin alone (although not ACTs) in treating rodent malaria (Elfawal et al., 2012) and reducing the development of resistance (Elfawal et al., 2015), and that whole-plant preparations may be effective in treating artesunate-resistant malaria patients (Daddy et al., 2017). These results suggest that *A. annua* produces metabolites that might act together with artemisinin and thus whole-plant preparations have been proposed as replacement treatments for ACTs (Weathers et al., 2014). In particular, flavonoids have been singled out as the likely synergistic metabolites (Elfawal et al., 2012; Weathers et al., 2014; Elfawal et al., 2015; Daddy et al., 2017) mainly because there is some evidence that they may improve the antimalarial activity of artemisinin *in vitro* (Elford et al., 1987; Liu et al., 1989; Liu et al., 1992; Ferreira et al., 2010).

Flavonoids are a diverse class of plant and fungal secondary metabolites with over 6500 different flavonoid products described from the secondary metabolism of various plant species (Ververidis et al., 2007). Flavonoid biosynthesis starts from primary metabolism precursors: phenylalanine and malonyl-CoA. Phenylalanine is used to produce 4-coumaroyl-CoA which is then combined with malonyl-CoA by chalcone synthase to yield the two-phenyl ring backbone common to all chalcones. A key step in flavonoid synthesis is the conjugate ring-closure of chalcones catalyzed by chalcone-flavanone isomerase (CHI), which results in the three-ringed structure of a flavone. The phenylpropanoid metabolic pathway contributes a series of enzymatic modifications that yield flavanones, dihydroflavonols, and eventually anthocyanins. Many products can be derived from this pathway including flavonols, flavan-3-ols, proanthocyanidins (tannins) and a host of other various polyphenolics (Ververidis et al., 2007). Flavonoids have been classified according to the position of the linkage of the aromatic ring to the benzopyrano moiety into four classes: major flavonoids (2-phenylbenzopyrans), which include flavonols, flavonones, flavanonols, flavones, anthocyanins and anthocyanidines; isoflavonoids (3-benzopyrans), which contain isoflavonons, isoflavanones and isoflavanonols; neo-flavonoids (4-benzopyrans) which include neoflavanenes and 4-arylcoumarines; and finally, minor flavonoids which include aurones, auronols, 2'OH chalcones and 2'OH dihydrochalcones (Ververidis et al., 2007).

In the present work we report an *A. annua* loss-of-function mutation of the trichome-specific *CHALCONE ISOMERASE 1* (*CHI1*) gene. Levels of all major flavonoids in the *chi1-1* mutant were reduced to undetectable levels. We used *chi1-1* to test the antimalarial effects of flavonoids. We

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81 extended the bioassays to include whole-leaf extracts from *A. annua* silenced in amorpha-4,11-diene
82 synthase (*AMS*), which encodes the enzyme that catalyzes the first committed step of artemisinin
83 biosynthesis (Catania et al., 2018), and the *cyp71av1-1* mutant, impaired in the second committed
84 step of artemisinin biosynthesis (Czechowski et al., 2016). The *AMS* silenced line has dramatically
85 reduced artemisinin production (5% of the wild-type levels) and accumulates the sesquiterpene
86 precursor farnesyl pyrophosphate in trichomes (Catania et al., 2018). *cyp71av1-1* completely
87 abolishes artemisinin production and redirects the artemisinin pathway to the synthesis of arteannuin
88 X, a novel sesquiterpene epoxide (Czechowski et al., 2016). Both the *AMS* silenced and *cyp71av1-1*
89 mutant lines produce wild-type levels of major flavonoids. We have performed a comparative
90 analysis of whole-leaf extracts from *chi1-1*, the *AMS* silenced line, *cyp71av1-1*, and wild-type *A.*
91 *annua* in *in vitro P. falciparum* Dd2 kill assays (Ullah et al., 2017) to determine the antiplasmodial
92 efficacy and initial cytocidal activity of these extracts.

93 **2 Materials and methods**

94 **2.1 Plant material**

95 For wild-type plant material we used the Artemis F1 hybrid variety of *Artemisia annua* developed by
96 Mediplant (Conthey, Switzerland), obtained by crossing C4 and C1 parental lines of East Asia origin
97 (Delabays et al., 2001). Seeds were sown in 4-inch pots filled with Levington modular compost and
98 grown in a glasshouse under long-day conditions (16-h day/8-h night) at 17-22 °C for 12 weeks.

99 **2.2 RNA isolation and semi-quantitative RT-PCR**

100 Total RNA was isolated from eight *A. annua* tissues: meristems, cotyledons, trichomes, young
101 leaves, expanded leaves, mature leaves, stems and flowers as previously described (Graham et al.,
102 2010) and quantified using the NanoDrop 8000 (NanoDrop products, Wilmington, USA). Five µg of
103 total RNA was digested with RQ1 RNase-free DNase (Promega, UK) according to the
104 manufacturer's protocol. First strand cDNA synthesis was performed using 2.5 µg of DNaseI
105 digested RNA with oligo dT(18) primers and SuperScript™ II Reverse Transcriptase (Thermo
106 Fisher, UK) according to manufacturer's protocols. 3 µL of the first strand cDNA was used for PCR
107 amplification using the following gene specific primers: CHI1_For: 5'-
108 TGGCAACACCACCTTCAGCTACC-3' (left), CHI1_Rev: 5'-GTTGTGAAGAGAATAGAGGCG-
109 3' (right), CHI2_For: 5'-ATGGCTAAGCTTCATTCCTCCAC-3' (left), CHI2_Rev: 5'-
110 CAGGTATGATACCATCTCTAGC-3' (right), CHI3_For 5'-CTGGAGCAATTCCCAGATCAG-3'
111 (left), CHI3_Rev 5'-AGAATGTTTGCCATCAACATCTC-3' (right), Ubiquitn_For: 5'-
112 GTCGGCTAATGGAGAAGACAAGAAG-3' (left) and Ubiquitn_Rev: 5'-
113 GAAAGCACGACCAGATTCATAGC-3' (right) using GoTaq™ *Taq* polymerase (Promega, UK).
114 The PCR program used to amplify the target sequences was: 94 °C, 2 min; followed by 10 cycles of
115 "touch down": 94 °C, 30 sec; 65 °C (-1 °C/cycle); 72 °C, 1 min, followed by 20 cycles of 94 °C, 30
116 sec; 55 °C, 30 sec; 72 °C, 1 min, followed by a final extension at 72 °C for 5 min. 10 µL of PCR
117 product was resolved on 1% agarose gels. Predicted product sizes for each gene are: 466 bp for
118 *CHI1*, 520 bp for *CHI2*, 507 bp for *CHI3*, and 454 bp for *UBQ*.

119 **2.3 *chi1-1* mutant isolation and characterization**

120 An ethyl methanesulfonate-mutagenized *A. annua* population was established as described before
121 (Graham et al., 2010; Czechowski et al., 2016). Screening of the self-fertilized M2 population was
122 performed as previously described (Czechowski et al., 2016) with the following modifications. DNA
123 was isolated from 30-50 mg of fresh leaf material harvested from individual 4 to 6-week-old M2

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125 plants, using the BioSprint 96 system (Qiagen, Hilden, Germany) according to the manufacturer's
126 protocol. DNA was quantified fluorometrically using Hoechst 33258 dye and a plate reader
127 (Fuoromax, UK). DNA samples were normalized to 5 ng/µL using the Freedom EVO® 200
128 workstation (Tecan UK Ltd) and arranged in four-fold pools for reverse genetic screening. The full-
129 length genomic DNA sequence of the Artemis *A. annua CHI1* gene for TILLING was obtained by
130 PCR using gene-specific primers designed based on Gene Bank-deposited sequence EZ246664. A
131 937-bp fragment of CHI1 was amplified in a two-step PCR reaction. The first step was carried out
132 with unlabeled primers: 5′-TGGCAACACCACCTTCAGCTACC-3′ (left) and 5′-
133 CTGTGGTTGCTTTCTCATCAAAATGG-3′ (right) on 12.5 ng of pooled gDNA in 10 µL volumes.
134 Nested PCR and labeling with IR dyes were performed on a 1/10 dilution of the first PCR with a
135 mixture of unlabeled M13-tailed primers (5'-
136 TGTAAAACGACGGCCAGTCGACAGCAACTAGTAATGGTAAACTG-3′ (left) and 5′
137 AGGAAACAGCTATGACCACATAAGATCTGAAAGTCTTGAAGCC ′ (right), and with M13 left
138 primer (5'TGTAAAACGACGGCCAGT3') labeled with IRDye700 and M13 right primer
139 (5'AGGAAACAGCTATGACCAT3') labeled with IRDye 800 (MWG, Ebersberg, Germany).
140 Heteroduplex formation, CEL I nuclease digestion and analysis on the LI-COR 4300 DNA sequencer
141 platform were carried out as previously described (Till et al., 2006). All mutants found on the
142 TILLING gels were verified by Sanger sequencing of both DNA strands of PCR-amplified fragments
143 using the following primers: 5'-GCAATAATGCTATGTGTTGGTGC-3' (left) and 5'-
144 CACAATGTTTGCAGCTTCAGGTATG-3' (right). Two segregating M3 mutant populations were
145 obtained by crossing M2 siblings that were heterozygous for the *chi1-1* mutation.

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146 **2.4 KASP™ SNP assay for *chi1-1* mutation status**

147 Twenty nanograms of DNA was used for 10-µL KASPar assay reactions containing: 1 × KASP V4.0
148 low ROX master mix (LGC Genomics); a concentration of 167 nM of each of the two allele-specific
149 primers: chi1-1_ForC: 5′-
150 GAAGGTGACCAAGTTCATGCTCAATGATACTACCATTAACTGGTAAGC-3′ and chi1-
151 1_ForT: 5′-GAAGGTCGGAGTCAACGGATTGACAATGATACTACCATTAACTGGTAAGT-3′
152 and 414 nM universal primer chi1-1_Rev 5′-CTCCAACGCACATTTCAGACACCTT-3′, according
153 to the manufacturer's recommendations. Allelic discrimination runs and allelic discrimination
154 analysis were performed on Viia7 system (Life Technologies Ltd.) according to the manufacturer's
155 recommendations.

156 **2.5 Metabolite Analysis by UPLC-and GC-MS**

157 Plants were grown from five cuttings from each genotype and metabolic profiles were generated
158 from 10-50 mg FW pooled samples of leaves at different developmental stages: 4-6 (counting from
159 the apical meristem) representing the young stage; leaves 11-13 representing the mature, expanded
160 stage and three leaves taken just above first senescing leaves representing old leaves. Fresh leaf
161 samples were stored at -80 °C. Trichome-specific metabolites were extracted as described previously
162 (Czechowski et al., 2016) and analyzed by UPLC-MS as previously described (Graham et al., 2010;
163 Czechowski et al., 2016). Dry leaf material was obtained from 14-week-old plants, cut just above the
164 zone of senescing leaves and dried for 14 days at 40 °C. Leaves were stripped from the plants, and
165 leaf material sieved through 5 mm mesh to remove small stems. Metabolite extractions from 10 mg
166 of the dry leaf material and UPLC-MS analysis were performed as previously described (Graham et
167 al., 2010; Czechowski et al., 2016). Number of the biological replicates measured was as follows:
168 young and mature wild-type leaves n=49, old wild-type leaves n=60, dry wild-type leaves n=21;
169 young-, mature- and old heterozygous *chi1-1* leaves n=94, dry heterozygous *chi1-1* leaves n=37;

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171 young, mature and old homozygous *chi1-1* leaves *n*=63, dry heterozygous *chi1-1* leaves *n*=32. The
172 experiments comparing trichome vs. whole-leaf metabolites were performed on leaves 14-16
173 harvested from five individuals grown from cuttings (*n*=5). Trichome-specific metabolites were first
174 extracted from the fresh mature leaves as described above. The remaining leaf material was washed
175 three times with 500 µL of chloroform and solvent removed by pipetting. Leaf tissue was ground to a
176 fine powder in TissueLyser II (Qiagen, UK), extracted and quantified by UPLC-MS. GC-MS
177 analysis was performed on the same dipped and ground-leaf extracts as described before
178 (Czechowski et al., 2016). To evaluate method suitability for detecting flavonoids, comparative
179 extracts of dry material were made with either 9:1 chloroform:ethanol (v/v; used throughout this
180 study) vs 85:15 methanol:water (v/v; typically used to extract polar flavonoids from plant material).
181 These extracts were then separated on an extended UPLC gradient (starting conditions modified to
182 100% of aqueous solvent A), to avoid any potentially highly polar flavonoids being lost in the void
183 volume.

184 **2.6 Whole-leaf extraction for *P. falciparum* kill rate assays.**

185 Fourteen-week-old plants were cut above the area of senescing leaves and dried for 14 d at 40 °C.
186 Leaves were separated from the rest of the dry plants and sieved through 5-mm mesh to remove
187 small stems. Dry leaves were stored long term in a humidity-controlled cabinet at 4 ºC. For whole-
188 leaf extracts, 1 g dry leaves was ground to a fine powder and extracted in 9:1 chloroform and ethanol
189 solution overnight, centrifuged at 4,700 rpm for 20 min and the supernatant was filtered through
190 Wattman paper. An aliquot was taken for quantification by UPLC-MS. The solvent was evaporated
191 until only an oily residue remained and re-suspended in DMSO to reach a final concentration of 5
192 mg/ml artemisinin (or to reach a casticin concentration equivalent to that of the wild type in the *AMS*
193 RNAi line, or equivalent to heterozygous *cyp71av1-1* in homozygous *cyp71av1-1*).

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194 **2.7 In vitro Plasmodium falciparum assays**

195 The *in vitro* screening of antiplasmodial activity of extracts was carried out starting with trophozoite
196 stage (24-32 hours post infection) intraerythrocytic stages of the *Plasmodium falciparum* Dd2 strain
197 using a 48hr (one complete cycle of intraerythrocytic development) Malaria Sybr Green I
198 Fluorescence assay as previously described (Smilkstein et al., 2004; Ullah et al., 2017). The mean
199 percentage growth ± StDev (n=9 from three independent biological repeats) was plotted against
200 $\log_{10}$-transformed drug concentration and a non-linear regression (sigmoidal concentration–
201 response/variable slope equation) in GraphPad Prism v5.0 (GraphPad Software, Inc., San Diego, CA,
202 USA) used to estimate the 50% effective concentration (EC_{50}) and the 95% confidence intervals.

203 Determination of the relative initial cytocidal activity against trophozoite stage intraerythrocytic
204 stages of the *Plasmodium falciparum* $Dd2^{luc}$ (Wong et al., 2011) were carried out using the
205 Bioluminescence Relative Rate of Kill assay as described (Ullah et al., 2017). All assays were carried
206 out over 6 h using a $9xEC_{50}$ to $0.33xEC_{50}$ concentration series. The mean ± StDev bioluminescence
207 signal, normalized to an untreated control, are plotted (n=9 from three biological repeats) and
208 compared to the benchmark standards of dihydroartemisinin (DHA), chloroquine (CQ), mefloquine
209 (MQ) and atovaquone (ATQ). Stock solutions of atovaquone (10 mM in DMSO), chloroquine (100
210 mM in deionized water), dihydroartemisinin (50mM in methanol), and mefloquine (50 mM in
211 DMSO) were made (Sigma-Aldrich) and stored at -20 ºC. In all experiments, the maximum final
212 concentration of solvent was 0.6% (v/v).

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213 **3 Results**

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216 **3.1 Isolation and characterisation of a *CHI1* mutant impaired in flavonoid biosynthesis**

217 Casticin and other polymethoxylated flavonoids accumulate in leaf and flower trichomes of the *A.*
218 *annua* Artemis variety, some to high levels comparable to those of artemisinin (Czechowski et al.,
219 2016; Czechowski et al., 2018). We previously identified three putative *CHI* genes using *A. annua*
220 transcriptome data (Graham et al., 2010). *CHI1* is expressed in young leaf and flower bud trichomes
221 whereas *CHI2* is expressed in young and mature leaf trichomes and *CHI3* is expressed most highly in
222 meristems and cotyledons (Graham et al., 2010). Further quantitative RT-PCR-based expression
223 profiling, extended to other tissues, revealed that *CHI1* expression is the most trichome specific of
224 the three genes tested, whereas *CHI2* is more generally expressed in several tissues and *CHI3*
225 expression is not detected in trichomes (Figure 1A). The 229 amino acid-long predicted protein
226 sequence for CHI1 is most similar to *CHI* characterized in other organisms than it is to the other two
227 putative *CHI* genes we previously identified from *A. annua* (Supplementary Figure 1A; Jez et al.,
228 2000). Amino acid sequence alignment of CHI homologs shows that CHI2 and CHI3 are missing a
229 number of highly conserved residues that including those required for substrate binding
230 (Supplementary figure 1A; Jez *et al*., 2000). In contrast CHI1 contains all of the conserved residues,
231 suggesting that it is the only one of the three CHI homologs from *A. annua* that produces a functional
232 chalcone isomerase enzyme.

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Deleted: The first 39 amino acids of CHI1 are predicted to encode a chloroplast transit peptide according to the TargetP 1.1 tool (Nielsen et al., 1997).

233 Using an established ethyl methanesulfonate-mutagenized population of *A. annua (Czechowski et al.,*
234 *2016)* we performed a TILLING screen of the single-copy *CHI1* gene that resulted in an allelic series
235 of five mutants, including three with intronic mutations, one with a silent mutation and one with a
236 nonsense mutation that created a C1567 to T transition in the third exon of *CHI1* (Figure 1B and
237 Supplementary Figure 1B). The latter mutation, which we designate *chi1-1*, gave a predicted change
238 of amino acid Gln107 in the polypeptide to a stop codon that would result in a major truncation of the
239 enzyme and loss of most of the putative substrate-binding site (Figure 1C, Supplementary Figure
240 1A). CHI is a functional monomer and residues that are important for substrate binding and the active
241 site in other species lie beyond the residue corresponding to *A. annua* CHI1 Q107 (Figure 1C and
242 Supplementary Figure 1A; Jez et al., 2000), which suggested the truncation would result in a
243 complete loss of CHI function.

244 In order to investigate the effects of the *chi1-1* mutation on artemisinin and flavonoid biosynthesis
245 we analyzed three leaf developmental stages: young (leaves 4-6 as counted down from the apical
246 meristem), mature (leaves 11-13) and old (three leaves preceding the first senescing leaves). To
247 generate material for this analysis we performed two crosses of heterozygous *chi1-1* M2 siblings
248 originating from a self-fertilized M1 individual and performed DNA marker-based selection of wild
249 type (WT) and heterozygous and homozygous *chi1-1* individuals from the segregating M3 population
250 using the KASP™ SNP assay. We observed a strong segregation distortion from the expected 1:2:1
251 (WT:heterozygous:homozygous mutation) in both M3 populations. The first cross resulted in 30
252 individuals of which 24 were heterozygous and 6 homozygous for the *chi1-1* mutation whereas the
253 second cross resulted in 54 individuals of which 36 were heterozygous and 18 homozygous for the
254 *chi1-1* mutation, but we could not identify segregating wild-type individuals. Such segregation
255 distortion is not unusual for *A. annua*, which naturally outcrosses, and has been reported for the
256 populations coming from self-fertilized individuals (Graham et al., 2010). In the absence of any
257 segregating M3 wild–type individuals we used non-mutagenized *Artemis* F1 as wild type for
258 metabolic profiling.

259 *CHI* disruption or suppression has previously been reported to result in discoloration and/or
260 decreased flavonol levels in *Arabidopsis thaliana*, petunia, carnation, onion and tobacco (van Tunen

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267 et al., 1991; Shirley et al., 1992; Itoh et al., 2002; Kim et al., 2004; Nishihara et al., 2005) whereas
268 petunia *CHI* overexpression leads to increased flavonol accumulation in tomato (Muir et al., 2001).
269 *A. annua* produces the polymethoxylated flavonoids casticin, artemetin, chrysoplenetin,
270 chrysosplenol-D, and cirsilineol (Bhakuni et al., 2001). Whereas the wild type and the *chi1-1*
271 heterozygote produced similar amounts of casticin, chrysoplenol C, dehydroxycasticin and artemetin
272 none of these flavonoids were detectable in homozygous *chi1-1* individuals (Figure 2Ai, ii, iii and
273 iv). These results demonstrate that *chi1-1* is a null allele. Flavonoids in the wild type and
274 heterozygous *chi1-1* are most abundant in young, followed by mature and old leaves (Figure 2A).
275 Noteworthy, *chi1-1* accumulated a compound with an m/z ratio of 273.0757 that was not detectable
276 in the wild type or the *chi1-1* heterozygote (Figure 2Av). The UPLC-MS profile of this compound
277 suggests it represents the molecular ion of naringenin chalcone (MW = 272.26 g/mol), the substrate
278 of chalcone isomerase, which would be expected to accumulate in the *chi1-1* null mutant.

279 We initially devised our chloroform:ethanol extraction method to be optimal for artemisinin
280 extraction, which has a logP of 2.8. Chloroform has a logP value of ~2.3, which is also quite closely
281 matched to the calculated logP values of *A. annua* methoxylated flavonoids (2.1-3.4, using structures
282 reported by Ferreria et al 2010). We compared our 9:1 chloroform:ethanol extraction method used
283 throughout this study with a solvent more typically used for flavonoid extraction (85:15
284 methanol:water) by extracting WT and homozygous *chi1*-1 dry material (Supplementary Table 4).
285 The UPLC method was also extended so that the elution conditions at the start of the run were much
286 more aqueous, to ensure that any polar flavonoids (if present) were not eluted in the void volume.
287 Peaks were picked and identified according to our standard high-resolution accurate mass protocols,
288 and additionally matched against formula hits for 40 previously reported flavone and flavonol
289 compounds from *A. annua* (Ferreira *et al*., 2010). The results show, from dry material, that 142 peaks
290 could be resolved of which only six potential flavonoids could be identified; all six of these
291 compounds were extracted in both solvent systems, and were in fact best extracted in our standard
292 chloroform:ethanol solvent (Supplementary Table 4). As expected, highly polar phenolic compounds
293 such as scopolin and scopoletin (PubChem xlogP values of -1.1 and 1.5, respectively) extracted
294 better in the methanolic solvent and could be resolved using the adapted UPLC method. All 40
295 flavonoids reported by Ferreria et al (2010) have predicted xlogP values in the range -1.3 to 3.5, so
296 we would expect to detect these in the modified UPLC method, if present in any of the extracts.
297 From this comparison we conclude that our chloroform:ethanol extraction solvent is sufficient to
298 extract the full suite of flavonoids present in the various *A. annua* genotypes used in the present
299 study, which all derive from the F1 Artemis commercial variety (Delabays *et al*., 2001) which serves
300 as the wild type in the current study. In a detailed metabolite analysis of high- and low- artemisinin-
301 producing chemotypes of *A. annua,* which involved both MS and NMR based detection and
302 identification we found similarly low numbers of flavonoids (Czechowski *et al*., 2018). We note that
303 the much larger number of flavonoids reported in the review by Ferreria et al., (2010) are based to an
304 extent on HPLC-UV analysis of *A. annua* material obtained from Yunnan Herbarium, China (Lai et
305 al., 2007). Future work involving comparative metabolite analysis of different cultivars grown under
306 identical conditions should help establish the basis of the difference in the numbers of flavonoids
307 being reported in these different studies.

308

309 Finally, artemisinin levels were consistently decreased in all homozygous *chi1-1* leaf material types
310 compared to heterozygous *chi1-1* and the wild type (Figure 2Avi). DHAA levels were
311 simultaneously reduced in young leaves of homozygous *chi1-1* when compared to heterozygous
312 *chi1-1* and the wild type (Supplementary Table 1). We also observed a mild reduction in the level of

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315 dihydroartemisinic acid tertiary allylic hydroperoxide in all leaf types of homozygous *chi1-1* when
316 compared to heterozygous *chi1-1* and the wild type. On the other hand, levels of DHAA-derived
317 11,13-dihydroamorphanes such as dihydroepideoxyarteannuin B, deoxyartemisinin, arteannuin I/J,
318 arteannuin M/O and 11-hydroxy-arteannuin remained unchanged in homozygous *chi1-1*
319 (Supplementary Table 1).

320 To further confirm the specificity of the effects of the *chi1-1* mutation on trichomes, we analyzed
321 metabolites in trichomes and leaves separately. Fresh mature leaves were dipped in chloroform to
322 disrupt the trichomes and release the contents (dip), as previously described (Graham et al., 2010),
323 and the remaining leaf material was ground, extracted and analyzed separately (ground leaves).
324 Known trichome-specific compounds such as artemisinin, DHAA or camphor were found in extracts
325 from the dip treatment but not in the post-dip ground leaf extracts (Figure 2Bi and iii, Supplementary
326 Table 2), consistent with previous morphological studies (Duke et al., 1994) and the trichome-
327 specific expression of the relevant biosynthetic pathway enzymes (Olsson et al., 2009; Graham et al.,
328 2010; Olofsson et al., 2011; Soetaert et al., 2013). Casticin, chrysoplenol C/D, dehydroxycasticin and
329 artemetin were also found in extracts from dip treatments but not in post-dip ground leaf extracts
330 from heterozygous *chi1-1* or the wild type, but were completely absent in homozygous *chi1-1* dip
331 and post-dip ground leaf extracts (Figure 2Bii, Supplementary Table 2). β-farnesene, germacrene-D,
332 trans-caryophyllene and squalene were found mostly in post-dip ground leaf extracts (Figure 2Biv,
333 Supplementary Table 2, Supplementary Table 3). This is consistent with the previous metabolite
334 studies on gland bearing *v*s glandless biotypes of *A. annua* (Tellez et al., 1999) and with the
335 ubiquitous expression of the relevant terpene synthases in *A. annua* (Graham et al., 2010; Olofsson et
336 al., 2011) . A principal component analysis for 83 of the UPLC-MS (Figure 2C) and 58 of the GC-
337 MS (Figure 2D) detectable metabolites revealed that homozygous *chi1-1* more strongly diverged
338 from the wild type and heterozygous *chi1-1* in extracts from dip treatment, but less so in post-dip
339 ground leaf extracts, where ground material clustered together. These findings suggested that the
340 *chi1-1* mutant is mainly disrupted in trichome metabolism and that *CHI1* is needed for flavonoid
341 synthesis specifically in trichomes.

342 **3.2 Flavonoids do not contribute antimalarial activity in whole-leaf extracts**

343 The *chi1-1* line allowed for a direct comparison of *A. annua* extracts with and without flavonoids to
344 evaluate the contribution of the cytocidal effects of these compounds on *Plasmodium* parasites *in*
345 *vitro*. To evaluate whether there were changes from the potent and rapid cytocidal effects expected
346 from artemisinin-containing extracts, the metabolites from wild type, and heterozygous and
347 homozygous *chi1-1* extracts were quantified and re-suspended to the same artemisinin concentration
348 (Table 1). The antiplasmodial activity against asexual intraerythrocytic stages of *Plasmodium*
349 *falciparum* indicated that the effective concentration required to inhibit growth by 50% (EC_{50}) was
350 essentially the same, between 15-35 ng/ml for the wild type and heterozygous and homozygous *chi1-*
351 *1* (Figure 3A, Table 1). We also performed an evaluation of the initial cytocidal activity of the same
352 extracts using a Bioluminescence Relative Rate of Kill (BRRoK) assay (Ullah et al., 2017). Here,
353 asexual intraerythrocytic stages of *P. falciparum* are exposed to multiples (0.33 to 9X) of EC_{50} of
354 extract, or benchmark antimalarial drugs of a known order of rate of kill, for 6 hours. This assay
355 allows a compound/extract to be compared to fast cytocidal drugs like artemisinin, the derivative
356 dihydroartemisinin and chloroquine; slower cytocidal drugs like mefloquine; and cytostatic drugs
357 such as atovaquone (Ullah et al., 2017). When performing BRRoK assays, the three samples were
358 indistinguishable from one another and most similar to dihydroartemisinin (the active metabolite of
359 artemisinin compounds) in the concentration *v.* loss of bioluminescence plot (Figure 3B). These

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360 results indicate that flavonoids in the wild-type extracts did not alter the fast cytocidal activity of
361 artemisinin in the samples.

362 **3.3 Artemisinin-reduced whole-leaf extracts lack potent and rapid antiplasmodial activity**

363 To test for the potential antiplasmodial activity of artemisinin-unrelated compounds in *A. annua*, we
364 used the artemisinin-reduced *AMS* silenced plant line (Catania et al., 2018). Samples from this line
365 were prepared alongside the other genetic variants and re-suspended to match the wild-type casticin
366 levels, which resulted in a 100-fold reduction in artemisinin levels compared to the wild-type (Table
367 1). Determination of the EC_{50} in the *AMS* silenced line revealed a greater than 20-fold reduction in
368 potency when compared to the wild-type (Figure 3A). Moreover, samples from the *AMS* silenced line
369 in the BRRoK assay lacked the rapid initial cytocidal activity of the wild type and heterozygous and
370 homozygous *chi1-1* samples and were only apparently cytocidal at concentrations above $3xEC_{50}$
371 (Figure 3B).

372 We also used a *cyp71av1-1* mutant shown to be completely deficient in the synthesis of artemisinin
373 (Czechowski et al., 2016) to investigate potential antiplasmodial effects of flavonoids (and other *A.*
374 *annua* compounds) in the absence of artemisinin. As a control we used heterozygote *cyp71av1-1* that
375 accumulates wild-type artemisinin levels. In extracts from *cyp71av1-1* antiplasmodial activity was
376 reduced ~300 fold compared to extracts from heterozygous *cyp71av1-1* (Figure 3C). The initial
377 cytocidal activity of the control heterozygote cyp71av1-1 extracts were comparable to those of the
378 wild-type and chi1-1 extracts, whereas cytocidal activity was reduced in the cyp71av1-1 mutant
379 (Figure 3D). It is noteworthy that extracts from *cyp71av1*-1 homozygous lines are among the highest
380 in total flavonoid content of the material used for anti-palsmodial assays (Table 1). Taken together
381 these results represent convincing evidence that *A. annua* flavonoids do not exhibit anti-plasmodial
382 activity in *in-vitro* assays. These results also suggest that the sesquiterpene epoxide artennuin X, one
383 of the most abundant metabolites produced by *cyp71av1-1* in the absence of artemisinin (Czechowski
384 et al., 2016), also does not have appreciable antiplasmodial activity. This is not really surprising as
385 arteannuin X does not carry an endoperoxide bridge (Czechowski et al., 2016), which is thought to be
386 crucial for antiplasmodial activity of sesquiterpene lactones such as artemisinin.

387 **4 Discussion**

388 **4.1 CHI1 is necessary for trichome-specific flavonoid synthesis**

389 We report the identification and characterization of an *A. annua* mutant in *CHI1*, which encodes the
390 enzyme that catalyzes the second committed step of the flavonoid biosynthesis pathway. The *chi1-1*
391 mutation is predicted to result in a truncation that would preclude a sizable portion of the CHI1
392 functional monomer, including sections that may interact with the product naringenin (Figure 1C and
393 S1*A*). Indeed, *chi1-1* failed to produce all four major polymethoxylated flavonoids, usually detected
394 in young, mature and dry *A. annua* leaves (Figure 2Ai, ii, iii and iv). Flavonoid levels in
395 heterozygous *chi1-1* were comparable with wild type (Artemis), which indicates that *chi1-1* is a
396 recessive mutation (Figure 2Ai, ii, iii and iv). Expression profiling in various tissues of wild-type *A.*
397 *annua* demonstrated that *CHI1* seems to be specifically expressed in trichomes (Figure 1A). In fact,
398 we showed that the effect of the *chi1-1* mutation on metabolite levels is clearly trichome-specific
399 (Figure 2B, C, and D and Supplementary Table 2) which is consistent with the *CHI1* expression
400 pattern (Figure 1A). The fact that two other *CHI* gene homologs expressed in *A. annua* (*CHI2* and
401 *CHI3*) did not compensate for the lack of flavonoids in trichomes of *chi1-1* strongly suggests that
402 CHI1 is the main enzyme responsible for flavonoid biosynthesis in *A. annua* trichomes.

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410 The precursors of all secondary or specialised metabolites in higher plants are derived from primary
411 metabolism. Phenylpropanoid biosynthesis leading to flavonoids relies on the synthesis of L-
412 phenylalanine from chorismate, sourcing carbon precursors from the pentose phosphate pathway of
413 primary metabolism. Terpenoid biosynthesis on the other hand starts from the common precursors
414 supplied by the plastidic MEP and the cytosolic mevalonate pathways, which both rely on carbon
415 sourced from glycolysis. Crosstalk between the phenylpropanoid and terpenoid biosynthetic
416 pathways occurs, therefore, at the level of early carbon precursors, such as glyceraldehyde 3-
417 phosphate and acetyl-CoA, and with reducing power provided by NAD(P)H and energy released
418 from ATP hydrolysis. We had therefore speculated that artemisinin biosynthesis may be improved by
419 specific blockage of flavonoid biosynthesis in *A. annua* trichomes, due to more carbon precursors
420 becoming available for farnesyl pyrophosphate biosynthesis. However, we did not observe any
421 increase in levels of artemisinin or related precursors in homozygous *chi1-1* mutants disrupted in
422 flavonoid production (Figure 2Aiv and Figure 2Bi). On the contrary, artemisinin levels in all *chi1-1*
423 leaf ages were lower when compared to heterozygous *chi1-1* and the wild type (Figure 2Aiv and
424 Figure 2Bi). The reduction of artemisinin levels in *chi1-1* might be explained by lower DHAA levels
425 (Supplementary Table 1), which could be due to either decreased DHAA synthesis or enhanced
426 DHAA degradation, but the connection to the *chi1-1* mutation is unclear. The crosstalk between
427 phenylpropanoid and terpenoid metabolism is further highlighted by the report that overexpression of
428 the *A. annua CINNAMYL ALCOHOL DEHYDROGENASE* results in an increase in lignin and
429 coumarin and a reduction in artemisinin and other sesquiterpenes (Ma *et al*., 2018).

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430 **4.2 Flavonoids had no effect on the *in vitro* antiplasmodial activity of *A. annua* extracts**

431 Flavonoids have been suggested as candidates for increasing antiplasmodial activity and potentially
432 slowing the emergence of resistance in whole-plant preparations, relative to artemisinin alone
433 (Weathers et al., 2014; Elfawal et al., 2015). It has been proposed that these attributes may arise due
434 to flavonoids enhancing artemisinin action by increasing artemisinin solubility in water (Mueller et
435 al., 2000) or through the action of some flavonoids, such as casticin, in increasing artemisinin
436 binding to hemin, one potential target of artemisinin action (Bilia et al., 2002). Artemisinin action *in*
437 *vitro* against intraerythrocytic stages of *P. falciparum* typically provides an EC_{50} of 3-5 nM (Liu et
438 al., 1992; Hasenkamp et al., 2013). Casticin, the most abundant flavonoid in *A. annua*, has an EC_{50} of
439 65 µM and 5 µM casticin reduced the artemisinin EC_{50} some 3-5 fold (Liu et al., 1992). Artemetin
440 also reduces the artemisinin EC_{50}, although to a lesser degree than casticin (Elford et al., 1987). In
441 another report, the flavonoids artemetin, casticin, chrysoplenetin, chrysosplenol-D, cirsilineol and
442 eupatorin have an IC_{50} that is 100 times that of artemisinin (Liu et al., 1992). When combining 5 µM
443 of these flavonoids with artemisinin, the artemisinin IC_{50} is reduced to as much as half (Liu et al.,
444 1992). However, the interactive mode of action of these compounds is unclear. In an isobologram
445 analysis of compound interactions, casticin has an antagonistic antimalarial activity with artemisinin
446 in a 3:1 combination (Suberu et al., 2013) but is apparently synergistic at a 10-10,000:1 combination
447 (Elford et al., 1987; Liu et al., 1992). Therefore, additional compounds in whole-plant preparations
448 could have synergistic or antagonistic effects with artemisinin depending on the relative
449 concentration in the plant. Results of our *in-vitro* antiplasmodial activity assays using *Artemisia*
450 whole-leaf preparations do not show any synergistic effects between flavonoids and artemisinin, in
451 contrast to previous reports (20, 41). We observed no appreciable differences between the
452 artemisinin-producing heterozygous *chi1-1* (flavonoid containing) and homozygous *chi1-1*
453 (flavonoid lacking) in terms of their EC_{50} potency or initial rate of cytocidal activity (Figure 3B). We
454 therefore conclude that flavonoids do not appreciably contribute to the *in-vitro* antiplasmodial
455 activity beyond that provided by the artemisinin content, at least in the concentrations at which they
456 are present in leaves of Artemis, a commercial F1 hybrid of *A. annua* (Delabays et al., 2001).

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4.3 The *in vitro* antimalarial activity of *A. annua* extracts is predominantly due to artemisinin

Several groups have investigated compounds in *A. annua* extracts to find new sources of antimalarial activities other than artemisinin, or explore the possibility that *A. annua* compounds aid artemisinin (O'Neill M et al., 1985; Elford et al., 1987; Liu et al., 1989; Liu et al., 1992; Mueller et al., 2000; Bhakuni et al., 2001). *A. annua* compounds having antimalarial activity have been reported but with EC_{50} values that are over three orders of magnitude higher than artemisinin (Suberu et al., 2013). In *in vitro* assays, arteannuin B and artemisinic acid have been shown to have additive antimalarial activity with artemisinin, whereas DHAA has antagonistic antimalarial activity with artemisinin (Suberu et al., 2013). Furthermore, some artemisinin precursors isolated from *A. annua* tea, including 9-epi-artemisinin and artemisitene, while being reported to have antimalarial activity themselves, can act antagonistically with artemisinin, possibly because they could have similar molecular targets in the malarial parasite (Suberu et al., 2013). However, artemisinin related compounds reported to either act by themselves or aid artemisinin are present in *A. annua* at much lower concentrations than required for antimalarial activity based on the EC_{50} (Elford et al., 1987; Bhakuni et al., 2001; Suberu et al., 2013), and therefore would perhaps not be expected to have an effect in whole-leaf extracts.

Our data suggests that the artemisinin-reduced extracts prepared so that they have wild-type casticin levels (Table 1), and likely the same concentration of non-artemisinin related compounds as wild-type extracts, had no *in vitro* antiplasmodial activity beyond that provided by the residual artemisinin in the homozygous *chi1-1* extracts (Figure 3A and 3B). We extended our studies to include the use of *cyp71av1-1* mutant extracts, which has been shown to completely lack artemisinin (Czechowski et al., 2016). Whereas the *cyp71av1-1* heterozygote control extract was essentially indistinguishable from those of the wild type and the *chi1-1* homozygote (Figure 3C and 3D), extracts of the *cyp71av1-1* homozygote were some 350-1000 fold less potent in their antiplasmodial activity. Whilst the *cyp71av1-1* homozygote did demonstrate a moderate to good initial cytocidal activity (Figure 3D), the BRRoK assay of these extracts used at least 10 times a greater concentration of extract than any other sample by virtue of these assays using multiples of the EC_{50}.

While our results clearly demonstrate that flavonoids from *A. annua* plant extracts do not play a role in enhancing antiplasmodial activity relative to artemisinin in *in-vitro* assays, the possibility remains that these compounds could have *in-vivo* effects (Elfawal et al., 2012; Elfawal et al., 2015). It has been postulated that flavonoids could increase artemisinin solubility or inhibit activity of the cytochrome P450s responsible for degradation of artemisinin (Elfawal et al., 2012). *A. annua* extracts have been shown to result in higher artemisinin concentration in mice blood than the same concentration of artemisinin alone and this effect was attributed to arteannuin B (Cai et al., 2017). However, it should be noted that artemisinin is known to dissolve poorly in water and has a short serum half-life (Elfawal et al., 2012). Consequently, artemisinin is typically chemically converted to dihydroartemisinin, artesunate or artemether to improve solubility and increase its half-life in human serum (Petersen et al., 2011). These improved artemisinin-based compounds are combined with a companion drug from a different class to formulate ACTs - the World Health Organisation recommended method of treatment for patients with malaria. Companion drugs include lumefantrine, mefloquine, amodiaquine, sulfadoxine/pyrimethamine, piperaquine and chlorproguanil/dapsone. This combination contributes to high efficacy, fast action and reduction in the likelihood of resistance developing for ACTs. *In-vivo* investigations into the effectiveness of whole plant extracts for the treatment of malaria should use approved artemisinin-related compounds with improved solubility and lifetime in human serum or indeed ACTs, rather than artemisinin alone, as a proper comparator in studies to investigate the potential of whole-leaf extracts from *A. annua*. We conclude that

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502 endogenous flavonoids present in whole-leaf extracts of *A. annua* have no appreciable effect on the
503 antimalarial activity of artemisinin as determined by quantitative *in vitro* assays.

504 5 Conflict of Interest

505 The authors declare that the research was conducted in the absence of any commercial or financial
506 relationships that could be construed as a potential conflict of interest.

507 6 Author Contributions

508 TC, MR DR, TW, TL, PH and IG. conceived and designed the research; TC, MR, DR, TW, DH, MF,
509 MV, performed the experiments; TC, MR, TL, MF, MV, PH and IG analyzed data; and TC, MR, PH
510 and IG wrote the manuscript.

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663 ## 10 Figure legends

664 **Figure 1: Discovery and characterization of the *chi1-1* mutation. (A)** *A. annua CHI1*, *CHI2* and
665 *CHI3* expression in meristems, cotyledons, young leaf trichomes, young leaves, fully expanded
666 leaves, mature leaves, stems, flowers, and no template control (NTC) were determined by semi-
667 quantitative PCR. *UBQ* (Putative ubiquitin-like protein, GQ901904) was used as a loading control.
668 **(B)** Gene schematic of *CHI1* indicates the position of the *chi1-1* mutation. **(C)** The *A. annua* CHI1
669 protein structure was modelled by I-TASSER (Yang et al. 2015) on the 10 most closely related
670 structural analogues. The parts of the structure expected to be missing in the *chi1-1* mutant are
671 highlighted in yellow, naringenin (enzyme product) bound to CHI1 is shown in blue.

672 **Figure 2: Effects of the *chi1-1* mutation on the metabolite profile of *Artemisia annua*.** Box and
673 whisker plots showing levels of **(A)** four major flavonoids, putative naringenin chalcone and
674 artemisinin as measured by UPLC-MS in young (leaves 1-5 as counted from the apical meristem),
675 mature (leaves 11-13), old (3 leaves above first senescing leaf) and dry (oven-dried) leaf material
676 harvested from 12 to 14-week-old plants of the *Artemis* wild type (black), heterozygous (blue) and
677 homozygous *chi1-1* mutant (red) and **(B)** selected flavonoids, sesquiterpenes and monoterpenes in
678 the extracts from dipped (dip) or ground leaf material for the wild type (black) and heterozygous
679 (blue) and homozygous (red) *chi1-1* mutant. Metabolite concentrations measured by GC- or UPLC-
680 MS **(A and B)** are expressed as a proportion of the residual dry leaf material following extraction.
681 Groups not sharing letters representing Tukey's range test results indicate statistically significant
682 differences ($p<0.05$). Each box is represented by minimum of 20 **(A)** or by five **(B)** biological
683 replicates. **(C,D)** Principal component analysis of 83 UPLC-MS identified peaks **(C)** and of 58 GC-
684 MS identified peaks **(D)** from dipped and ground leaf material from wild type (black) and
685 heterozygous (blue) and homozygous *chi1-1* (red). Dip leaf extracts are represented by circles and
686 ground leaf extracts by triangles. Principal component analysis was performed on log-scaled and
687 mean-centered data.

688 **Figure 3: Comparison of *in-vitro* antiplasmodial activity of leaf extracts from *A. annua* wild
689 type, mutant and antisense lines with altered flavonoid and artemisinin content. (A,C)** Log
690 concentration-normalized response curves of *P. falciparum* parasites after 48 h of treatment with
691 extracts used to determine the EC_{50} (50% effective concentration of extract needed to inhibit growth
692 of the *P. falciparum* parasites) of the indicated extracts. **(B,D)** Bioluminescent Relative Rate of Kill
693 (BRRoK) assays to determine the initial (6 h) cytocidal action, compared to an untreated control after
694 exposure to extracts of wild type, heterozygous and homozygous *chi1-1*, and the *AMS* silenced line
695 **(B)** or heterozygous and homozygous *cyp71av1-1* **(D)** at multiples of the EC_{50} alongside
696 dihydroartemisinin (DHA) > chloroquine (CQ) > mefloquine (MQ) > atovaquone (ATQ) benchmark
697 controls. Error bars represent standard deviations from the means of three biological replicates.

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699 **Table 1: Artemisinin and flavonoid levels and antimalarial efficacy of plant extracts.** Mean
700 concentrations and standard deviations from the mean of five technical replicates are shown. Total
701 detected flavonoid is the sum of the five listed flavonoids. Letters represent Tukey's range test results
702 after one way ANOVA for each metabolite or total detected flavonoids. Genotypes not sharing letters
703 indicate statistically significant differences ($p<0.05$). EC_{50} is the 50% effective concentration of
704 extract needed to inhibit growth of the *P. falciparum* parasites. Artemisinin concentrations have been
705 normalized to 5 mg/ml in wild type, *chi1-1* het, *chi1-1* hom and *cyp71av1-1* as detailed in Materials
706 and Methods section 2.6.

707

	artemisinin (mg/mL)	casticin (mg/mL)	dehydroxycasticin (mg/mL)	cirsilineol (mg/mL)	chrysoplenol C (mg/mL)	artemetin (mg/mL)	total detected flavonoid (mg/mL)	EC_{50} (ng/mL) [95% CI]
Wild type	5.00 ± 0.80 ^{b}	0.51 ± 0.07 ^{c}	0.09 ± 0.01 ^{c}	0.11 ± 0.04 ^{b}	0.004 ± 0.003 ^{a}	0.00 ^{a}	0.71 ± 0.12 ^{c}	15.6 [14.5-16.8]
chi1-1 het	5.00 ± 0.28 ^{b}	0.36 ± 0.02 ^{b}	0.042± 0.009 ^{b}	0.00 ^{a}	0.005 ± 0.002 ^{a}	0.00 ^{a}	0.40 ± 0.03 ^{b}	34.6 [31.7-37.9]
chi1-1 hom	5.00 ± 0.44 ^{b}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	25.7 [25.1-26.4]
AMS silenced line	0.062 ± 0.007 ^{a}	0.50 ± 0.06 ^{c}	0.021 ± 0.007 ^{ab}	0.006 ± 0.002 ^{a}	0.00 ^{a}	0.12 ± 0.01 ^{c}	0.65 ± 0.08 ^{c}	350.4 [303.1-405.1]
cyp71av1-1 het	5.00 ± 0.55 ^{b}	0.69 ± 0.04 ^{d}	0.15 ± 0.02 ^{d}	0.00 ^{a}	0.074 ± 0.012 ^{b}	0.00 ^{a}	0.91 ± 0.05 ^{d}	14.1 [13.5-14.7]
cyp71av1-1 hom	0.00 ^{a}	0.61 ± 0.14 ^{cd}	0.31 ± 0.05 ^{e}	0.00 ^{a}	0.00 ^{a}	0.08 ± 0.01 ^{b}	1.00 ± 0.19 ^{d}	4220 [3820-4665]

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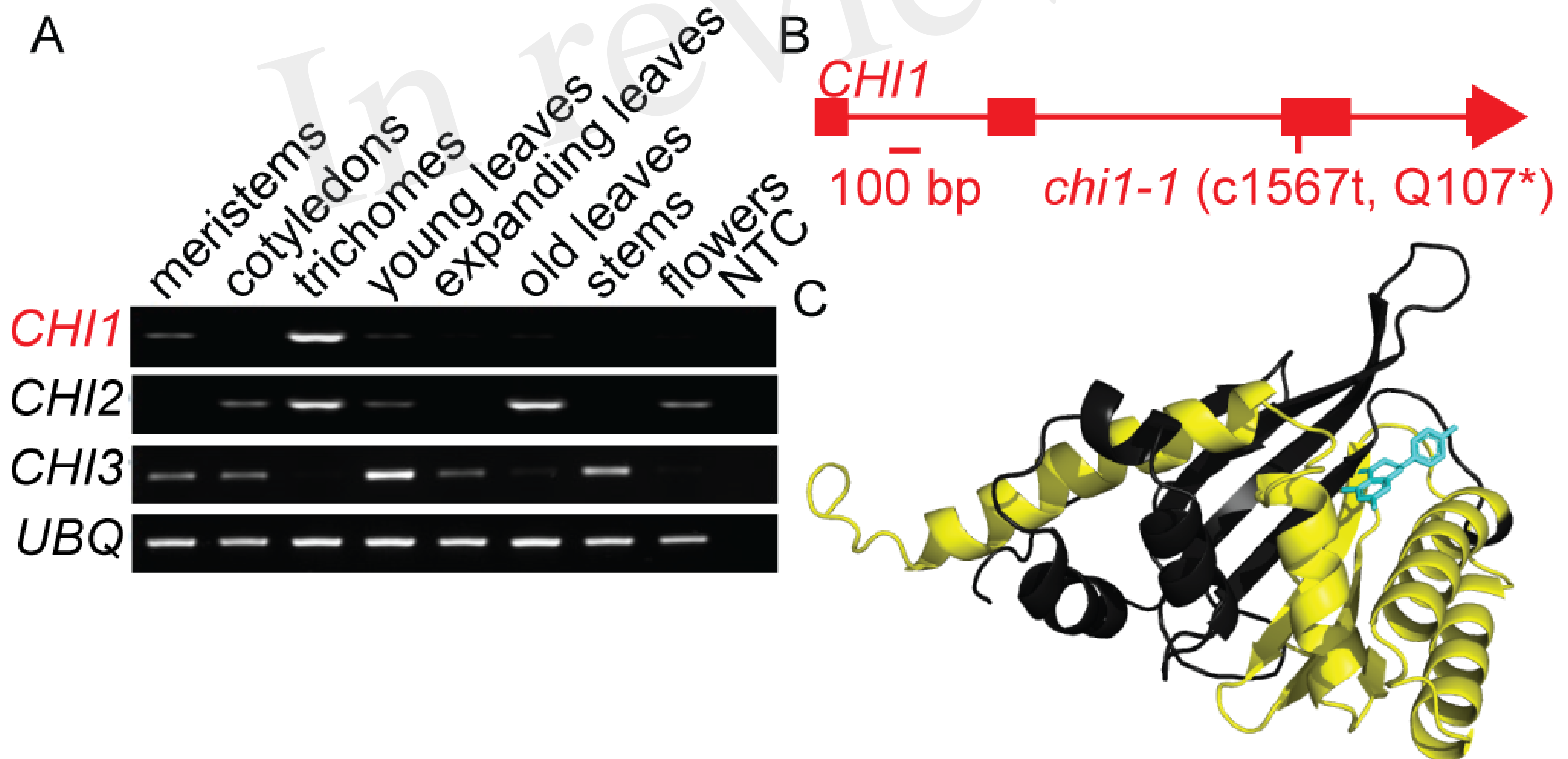

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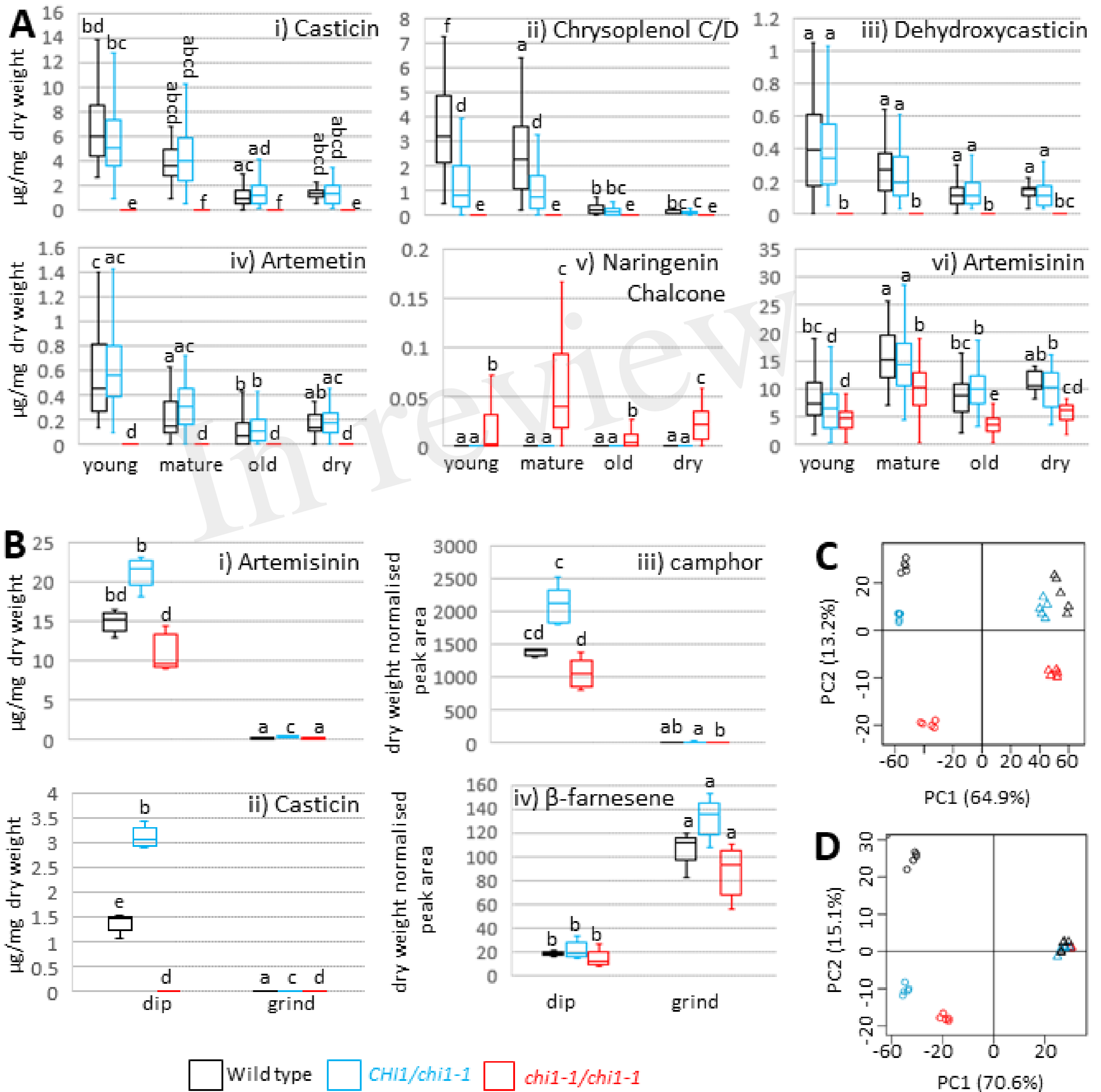
Figure 2.TIF



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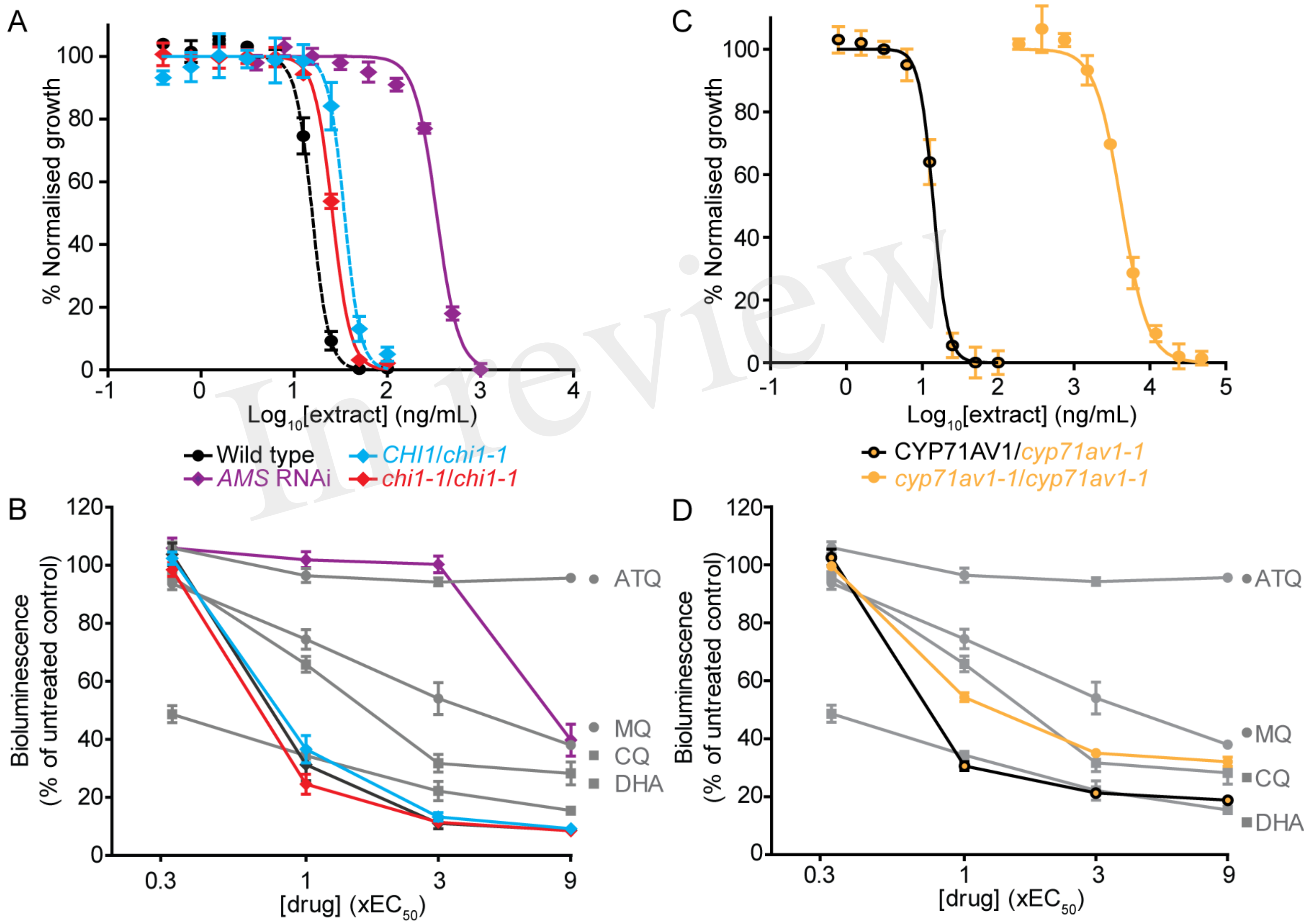

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