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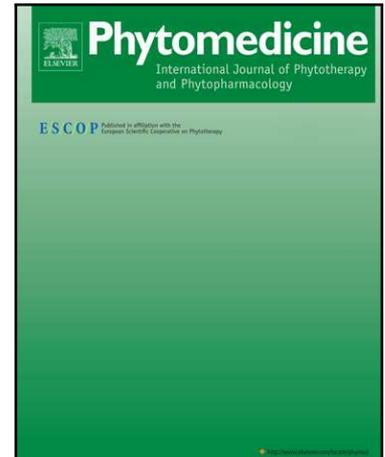
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***Artemisia annua* dried leaf tablets treated malaria resistant to ACT and i.v. artesunate:
case reports.**

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Author contributions.

NBD, physician attending patients receiving ACT or plant material, analyzed data; LMK, physician attending patients, ethical committee adviser, edited paper; PGB, pharmacist, supplied plant material tablets, edited paper; RLW, pharmacist, obtained plant material, had tablets cGMP manufactured, edited

paper; MJT, analyzed plant material, edited paper; PJW, analyzed plant material, analyzed data, wrote article.

ABSTRACT

Background Dried leaf *Artemisia annua* (DLA) has shown efficacy against *Plasmodium* sp. in rodent studies and in small clinical trials. Rodent malaria also showed resiliency against the evolution of artemisinin drug resistance.

Purpose This is a case report of a last resort treatment of patients with severe malaria who were responding neither to artemisinin combination therapy (ACT) nor i.v. artesunate.

Study Design Of many patients treated with ACTs and i.v. artesunate during the 6 mon study period, 18 did not respond and were subsequently treated with DLA *Artemisia annua*.

Methods Patients were given a dose of 0.5 g DLA *per os*, twice daily for 5d. Total adult delivered dose of artemisinin was 55 mg. Dose was reduced for body weight under 30 kg. Clinical symptoms, e.g. fever, coma etc., and parasite levels in thick blood smears were tracked. Patients were declared cured and released from hospital when parasites were microscopically undetectable and clinical symptoms fully subsided.

Results All patients were previously treated with Coartem® provided through Santé Rurale (SANRU) and following the regimen prescribed by WHO. Of 18 ACT-resistant severe malaria cases compassionately treated with DLA, all fully recovered. Of the 18, this report details two pediatric cases.

Conclusions Successful treatment of all 18 ACT-resistant cases suggests that DLA should be rapidly incorporated into the antimalarial regimen for Africa and possibly wherever else ACT resistance has emerged.

Background

The entire Democratic Republic of Congo (DRC) is at risk of malaria. In 2013 >25,000 deaths occurred; many were children under 5 [WHO, 2015]. In 2005 the DRC adopted free artemisinin combination therapy (ACT) for all ages in the public sector. ACTs provided by SANRU (Santé Rurale) via the Global Fund remain the front line treatment. Recently in the Goma area of the DRC, out of thousands of malaria cases successfully treated with ACTs, a few cases that did not respond began emerging, so a compassionate effort to save their lives ensued.

Artemisia annua L. qualifies as a generally recognized as safe (GRAS) plant [Duke 2001] from which artemisinin is extracted and purified to produce ACT. The plant has been safely consumed in various forms, e.g. as a tea infusion [Hsu, 2006] and leaf powder [Onimus et al. 2013], for millennia [Hsu 2006; Weathers et al. 2014a]. Furthermore, an increasing body of evidence has shown in both rodents and humans that *per os* consumption of the dried leaves is efficacious [Elfawal et al. 2012, 2015; ICIPE, 2005; Onimus et al. 2013; Weathers et al. 2014b], provides more bioavailable artemisinin [Weathers et al. 2011, 2014b; Desrosiers and Weathers 2016], may reduce emergence of artemisinin drug resistance [Elfawal et al. 2015], and is highly cost effective [Weathers et al. 2014a].

ACT-resistant cases of malaria were encountered in North Kivu Province, DRC, and those patients subsided into severe malaria. As defined by WHO [WHO 2014] the symptoms of severe malaria may include loss of consciousness, respiratory distress, convulsions, prostration, shock, pulmonary edema, abnormal bleeding, severe gastric distress, and jaundice. There were 18 patients who met the criteria for severe malaria, but who failed to respond to either ACT or i.v. artesunate. Compressed leaf tablets of *Artemisia annua* (dried leaf *Artemisia*, DLA) were used to treat those 18 patients, all of whom then recovered fully. Here we describe two of those 18 cases in detail.

Methods

Plant material: preparation and analysis. Leaves of Ethiopian-grown *A. annua* L. [Anamed A-3; <https://www.anamed-edition.com/en/artemisia-annua-anamed.html>] were harvested from field-grown plants in their vegetative stage, dried and then powdered. In March 2015, the powdered dried leaves were compressed without excipient [Weathers and Towler 2014] into 500 mg tablets by Ancient Formulas Inc., a cGMP facility in Wichita, KS, USA for Plesion International, Inc., a nonprofit, charitable NGO located in New Holland, PA, USA. Plesion is affiliated with the HEAL Africa Hospital in Goma, DRC. Samples of DLA tablets were re-powdered, extracted, and analyzed for artemisinin and other phytochemicals according to methods using GC-MS and spectroscopy [Towler and Weathers 2015]. The GC-MS method

for monoterpenes was modified as: 40°C held for 5 min, increased to 250°C at 4°C/min, increased to 300°C at 25°C/min, held for 5 min. Thin layer chromatography (TLC) fingerprints used 20 x 20 cm 60 F254 Si-Gel plastic plates, 10.5 µm particle size, 200 µm thickness. Artemisinin and terpenoids were stained with *p*-anisaldehyde reagent containing (v/v) 97:2:1 glacial acetic acid, sulfuric acid, *p*-anisaldehyde, and heated at 105°C for 10 min. Flavonoids were stained with 2% (w/v) aluminum chloride in methanol.

Patient selection and treatment: About 18% of malaria patients presented with severe malaria and were treated with ACT (Coartem®), in the Rwanguba Health Zone March-August, 2016. Typically for uncomplicated malaria, patients are treated for 3d with Coartem®, consisting of artemether-lumefantrine (AL; 20 mg artemisinin, 120 mg lumefantrine) with dosage defined by Novartis [2015]. The ACTs were purchased from the EU by SANRU and provided as validated drugs. Severe malaria is treated with intravenous (i.v.) artesunate [WHO 2012]. AL and i.v. artesunate were administered per WHO guidelines. Some patients (18 in all: 6 males, 12 females, ages 14 mon to 60 years) responded neither to AL nor i.v. artesunate and thus were treated with DLA ~24 h post i.v. artesunate, which had followed AL ACT treatment. Adults (>30 kg) were given one 500 mg DLA tablet (5.5 mg artemisinin/tablet) of DLA twice daily for 5d; this was 55 mg artemisinin *in toto* per adult. Children were apportioned tablets by weight: for those 5 to <15 kg, a quarter-tablet was given; for 15-30 kg, a half-tablet was administered. Total artemisinin per child was thus 13.75 mg and 27.5 mg for the quarter and half-tablet regimens, respectively. For those in coma or too young to swallow tablets, the dose was crushed, mixed with clean water, and delivered by nasogastric (NG) tube. Malaria infection was confirmed using a WHO approved Rapid Diagnostic Test (RDT) [WHO 2017]. Parasites in thick blood smears from all patients were tracked for 5d. Patients were released from hospital only after all clinical symptoms disappeared and parasites were undetectable in thick blood smears. For the two detailed cases, written, signed consent was obtained *ex post facto*. The Institutional Review Board for the Committee of Research at HEAL Africa granted permission for a retrospective evaluation of patient data regarding these case reports of patients treated with *Artemisia annua* tablets in the Rwanguba hospital.

Results and Discussion

After 3d of AL, all 18 patients still had blood parasites and fever. All subsequently progressed into severe malaria with symptoms beyond fever and headache: 7 had anemia; 4 had vomiting and diarrhea; 6 had convulsions, some with coma and fever >40°C; 1 had vomiting, diarrhea, and was comatose. After unsuccessful AL treatment, all 18 patients were subsequently treated with i.v. artesunate per WHO guidelines for severe malaria. When i.v. artesunate also failed, DLA was used. All 18 patients who were

treated with DLA tablets recovered fully with undetectable trophozoites (tr) in thick blood smears (tr/field). Similar to observations by ICIPE [2005], DLA tablets were well tolerated with no observed side effects. Two pediatric cases are described in further detail.

An underweight (17 kg) 14 yr old female was treated at a rural dispensary with ACT and then at home for 3d. Fever persisted, so she was transferred to hospital with convulsions. She lost consciousness twice en route. At hospital she was given i.v. artesunate (2.4 mg/kg) for 5d, but fever persisted and her blood still showed 0-2 tr/field. Day 5 she was given DLA (half-tablet twice a day for 3d) *per os*; on day 8 her fever was gone, her blood was tr negative, and she was released.

A 5 yr old male was treated with ACT at home with no success, then transferred to hospital with fever, anemia, respiratory infection, and convulsions leading to coma. He was treated for 5d with ampicillin (200 mg/kg/day) and gentamicin (4 mg/kg/day). Artesunate i.v. was also begun: 2.4 mg/kg twice on day 1, then once daily to complete 5d. Concurrent nonmalarial infections were halted, so the other antibiotics were stopped. The patient still had malaria (0-7 tr/field) and now was comatose, so *A. annua* treatment began. He received a half tablet DLA crushed in water delivered by NG tube, twice daily for 3d. Day 8 after admission to hospital, his parasite count dropped to 0-2 tr/field and he was out of coma. DLA treatment was continued for 2 more days (5d total), after which parasites were undetectable. The boy subsequently was able to resume normal activities, so he was discharged the next day.

This study is consistent with earlier rodent malaria studies that showed DLA was successful in eliminating artemisinin resistant parasites [Elfawal et al. 2015]. Although patient follow-up was not done 28 d after treatment to determine recrudescence, the results of these case studies suggested that consumption of dried leaf *A. annua* tablets thwarted ACT-resistant malaria.

DLA tablets contained (per g DW) (Table 1) 10.97, 2.54, 0.89, 1.30, 3.91, and 18.32 mg, respectively, of artemisinin, deoxyartemisinin, arteannuin B, artemisinic acid, dihydroartemisinic acid, and total flavonoids (as quercetin equivalents). Although artemisinin and flavonoids have reported antimalarial activity in the nanomolar and micromolar range, respectively, Suberu et al. [2013] observed that arteannuin B, artemisinic and dihydroartemisinic acids also are weak antimalarials. Each also demonstrated synergism that is seemingly dependent on their concentration relative to artemisinin [Suberu et al. 2013].

Flavonoids are another important group of phytochemicals in *A. annua* that also have antimalarial activity, but weaker than artemisinin. Indeed, *A. annua* can produce as many as 40 different flavonoids [Ferreira et al. 2010]. To our knowledge only five flavonoids frequently found in *A. annua* cultivars have been studied for synergism with artemisinin. Of these, chrysoplenetin, chrysoplenol-D, and eupatorin, showed a significant improvement in their IC₅₀ in the presence of artemisinin [Liu et al. 1992]. Of the total flavonoids measured, only chrysoplenetin and chrysoplenol-D were specifically identified via TLC and GC-MS in the DLA tablets (Figure 1; Table 1).

While not an exhaustive list, other phytochemicals measured in DLA tablet extracts are listed in Table 1 including the coumarin, scopoletin, the phenolic acid, chlorogenic acid, and other terpenoids, e.g. the monoterpene camphor and diterpene alcohol phytol. Of these, only chlorogenic acid and phytol have demonstrated antimalarial activity [Suberu et al. 2013; Grace et al. 2012].

When leaves are dried prior to DLA tablet formation, many volatile monoterpenes are eliminated or severely reduced except for camphor [Weathers and Towler 2014]. TLC fingerprints of DLA extracted with two solvents are shown in Figure 1 and demonstrate via differential staining the plethora of terpenoids and flavonoids present in the DLA used to treat these patients. Many of these extracted compounds are as yet unidentified in this cultivar. Many of the phytochemicals found in *A. annua* also have not been thoroughly tested for synergism with artemisinin.

Although there is the possibility of a synergistic effect between DLA and residual artesunate from the i.v. treatments, the pharmacokinetics of artesunate makes that unlikely as by the time the DLA was given, about 24 h post i.v. artesunate, the drug had already been long eliminated from the body. The T_{1/2} of i.v. artesunate in human patients with severe or moderately severe malaria is 2.3 or 4.3, min, respectively, and depending on how the drug was delivered, plasma levels for both were undetectable after 6 h, all well before any DLA treatment began [Davis et al. 2001]. Artesunate, however, is considered a prodrug for dihydroartemisinin (DHA), the metabolic product of artesunate. DHA persists longer in the serum. The T_{1/2} of DHA produced after i.v. artesunate delivery in human patients with severe or moderately severe malaria is ~40 or 64 min, respectively [Davis et al. 2001], and again depending on length of drug delivery, extrapolation of DHA plasma levels in patients with severe malaria showed that it was undetectable ≤ 9 h later [Davis et al. 2001]. Considering that DLA was administered about 24 h after last i.v. artesunate, synergism between LA and residual DHA also seems unlikely. Taken together, the results of this study suggest that DLA alone successfully treated artemisinin-resistant malaria in these patients.

Conclusions

To our knowledge this is the first report of dried-leaf *Artemisia annua* controlling ACT-resistant malaria in humans. These 18 cases occurred over six months (Figure 2). They represented ~0.09 % of total ACT-treated patients in the same time and location, and demonstrated that oral consumption of dried leaf tablets of *A. annua* has possible utility in rescuing patients from ACT and i.v. artesunate failures. More comprehensive clinical trials on patients with ACT-resistant malaria are warranted and should include dosing studies with DLA containing different ratios of, e.g. artemisinin and flavonoids, and also patient follow-up through 28d to track recrudescence.

List of abbreviations

ACT, artemisinin combination therapy; AL, artemether-lumefantrine; DLA, dried leaf *Artemisia annua*; i.v., intravenous; tr, trophozoites

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Competing interests.

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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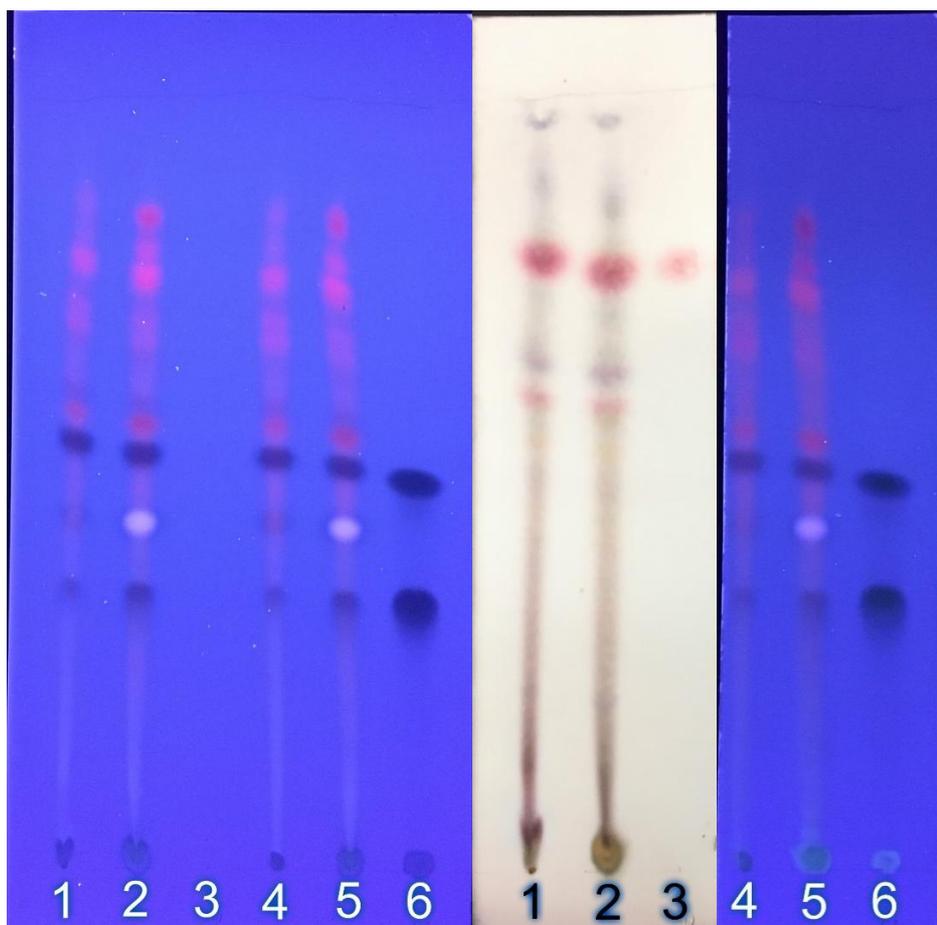


Figure 1. TLC fingerprint of methanol (MeOH) and methylene chloride (MeCl₂) extracts of the Anamed A-3 Ethiopian grown *Artemisia annua* dried leaf tablets used in the study. Lane identification: 1: MeCl₂ extract of DLA tablet powder (2 mg); 2: MeOH extract (2 mg); 3: artemisinin standard (5 mg); 4: MeCl₂ extract; 5: MeOH extract; 6: (top to bottom) chrysoplenetin, chrysoplenol-D, chlorogenic acid standards (10 mg). Left photo, unstained TLC under UV illumination; middle photo, *p*-anisaldehyde stain under visible light; right photo, aluminum chloride stain under UV illumination.

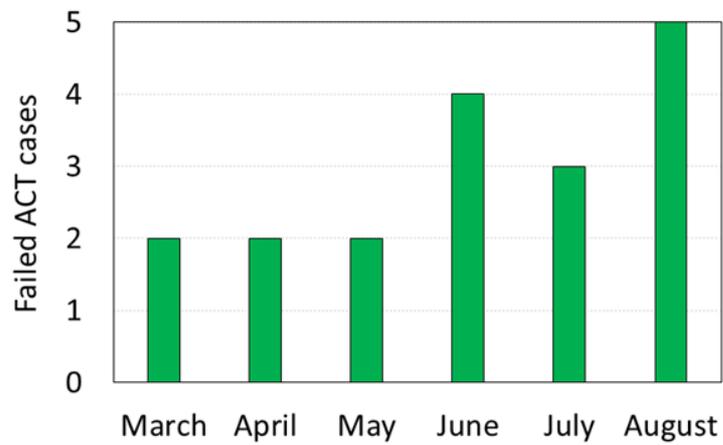


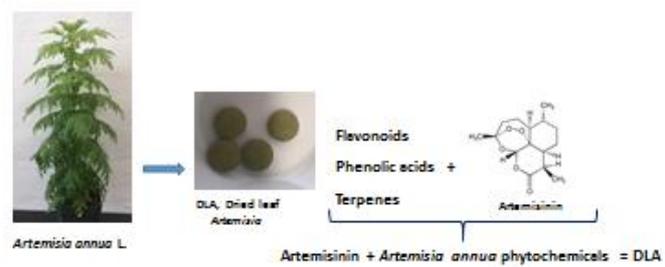
Figure 2: ACT and i.v. artesunate failure cases out of thousands of successfully ACT-treated malaria cases over 6 months in 2016 in North Kivu province, Democratic Republic of Congo.

Table 1. Phytochemical content of DLA tablets used in this study.

Phytochemical	Concentration (mg/g DW)
Artemisinin compounds	
Artemisinin	10.97
Artemisinic acid	1.30
Dihydroartemisinic acid	3.91
Arteannuin B	0.89
Deoxyartemisinin	2.54
Phenolic acids	
Chlorogenic acid	10.94
Rosmarinic acid	nd
Coumarins	
Scopoletin	2.47
Flavonoids	
Total flavonoids	18.32
Artemetin	nd
Casticin	nd
Chrysopenetin	0.50
Chrysopenol D	0.35
Eupatorin	nd
Isovitexin	nd
Kaempferol	nd
Luteolin	nd
Myrcetin	nd
Quercetin	nd
Nonartemisinin Terpenes	
1,8 cineole (eucalyptol)	nd
β -pinene	nd
β -neoclovene	nd
Borneol	nd
Camphor	0.27
Caryophyllene	nd
Caryophyllene oxide	nd
Phytol	0.56
Spathulenol	nd
Thujone	nd

nd, not detected

Graphical abstract



Of 18 severe malaria cases unresponsive to ACT and iv artesunate,
100% were cured by DLA

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