Published in final edited form as:

Planta Med. 2012 June ; 78(10): 1024–1026. doi:10.1055/s-0032-1314949.

The flavonoids casticin and artemetin are poorly extracted and are unstable in an *Artemisia annua* tea infusion

PJ Weathers¹ and MJ Towler

Department Biology and Biotechnology, Worcester Polytechnic Institute, 100 Institute Rd, Worcester, MA 01609 USA

Abstract

A number of flavonoids including casticin and artemetin from *Artemisia annua* have shown synergism with artemisinin against *Plasmodium falciparum*, but it is unclear if flavonoids are also extracted into a tea infusion of the plant. Using a tea infusion preparation protocol that was reported to be highly effective for artemisinin extraction, we measured casticin and artemetin extraction. There was only a 1.8% recovery of casticin in the infusion while artemetin was undetectable. After 24 hr storage at room temperature, casticin yield declined by 40%. These results show that although a tea infusion of the plant may extract artemisinin, the polymethoxylated flavonoids, casticin and artemetin, are poorly extracted and lost with storage at room temperature and thus, the tea infusion appears to lose synergistic value.

Keywords

casticin; artemisinin; malaria; stability; tea infusion; *Artemisia annua*; Asteraceae; Compositae

Artemisia annua L. (Asteraceae) produces the sesquiterpene lactone, artemisinin, and many other medicinally active compounds including flavonoids such as casticin and artemetin (Figure 1). A tea infusion of *A. annua* has been used for millennia to treat malaria and other ailments [1], and has recently been of interest again to treat malaria victims in locations without healthcare access [2]. Artemisinin and its derivatives as Artemisinin-based Combination Therapies (ACT) are currently the best treatment against malaria [2], but they have also been shown effective against a number of viruses, a variety of human cancers, other human parasites [3, 4], and a number of livestock diseases [5].

Of the many flavonoids in *A. annua* [6], at least six, artemetin, casticin (Figure 1), chrysoplenetin, chrysoplenol-D, cirsilineol, and eupatorin, have shown *in vitro* therapeutic efficacy against malaria [7, 8]. When each of these six methoxylated flavones was combined individually with artemisinin, the IC₅₀ of artemisinin against *P. falciparum* decreased by 20–50%, demonstrating an apparent synergy with the sesquiterpene lactone, artemisinin [8]. Interestingly, Elford et al. [7] also showed that while casticin showed synergism with artemisinin vs. *P. falciparum*, casticin did not synergize with chloroquine, suggesting a different interactive mechanism. Casticin and artemisinin did, however, inhibit parasite-mediated transport systems controlling influx of L-glutamine and myo-inositol in erythrocytes infected with human and murine malaria. This apparent synergistic action

¹Corresponding author: weathers@wpi.edu, FAX: 508-831-5936.

Conflict of Interest

The authors declare no conflicts of interest.

between artemisinin and flavonoids suggests that flavonoids would likely be important for efficacious use of *A. annua* tea infusion.

Recently van der Kooy and Verpoorte [9] showed that in a systematic study of different preparations of this therapeutic tea infusion, and under certain conditions, nearly 93% of available artemisinin can be extracted from dried *A. annua* leaves. More importantly, they also showed that when stored at room temperature, the artemisinin concentration in the tea infusion did not significantly decrease, which is important for people in developing countries where malaria is endemic and where there is little or no refrigeration. Typically a liter of tea infusion is prepared daily and consumed in equal aliquots throughout the day [9].

Although Carbonara et al. [10] detected 0.06 mg/g DW of cirsilineol in an *A. annua* tea infusion, they did not report the original starting concentration in the leaves to indicate recovery from original material. To our knowledge, there have been few studies examining the extraction and stability of the other five of the six reportedly active flavonoids in *A. annua* tea infusion. Here we provide evidence that using an extraction method reported as optimal for artemisinin, the polymethoxylated flavonoids casticin and artemetin are poorly extracted and not particularly stable at room temperature.

Results in Table 1 confirm the work of van der Kooy and Verpoorte [9], which showed the artemisinin level in *A. annua* tea infusion was stable for 24 hr at room temperature. Although stable for 24 hr in frozen samples, artemisinin stability declines with longer storage at room temperature (Table 1). When compared to other cultivars in hand, the SAM clone of *A. annua* was determined to produce the highest levels of both artemisinin and the flavonoids, artemetin and casticin (Table 2). A batch of SAM clonal plants allowed to flower was used for tea infusion using the optimum artemisinin method described by van der Kooy and Verpoorte [9], as displayed in Table 3. The content of casticin and artemetin in the tea infusion was analyzed and shown to yield only ~0.5% of the casticin found in the dried material; artemetin was not detectable (Table 3). As before, artemisinin was stable at room temperature after 24 hr; however, the casticin level declined even further, indicating it was not stable (Table 3). Thus, although artemisinin was well extracted from an *A. annua* tea infusion and stable at room temperature for 24 hr (Tables 1 and 3), the flavonoids artemetin and casticin were both poorly extracted and relatively unstable. Therefore it seems unlikely that use of *A. annua* tea would provide the synergistic benefits of the flavonoids described as being most effective in the *in vitro* studies with *P. falciparum* [7, 8]. Alternatively, after additional animal and human studies confirm such effectiveness, ingestion of dried, pulverized encapsulated *A. annua*, as we previously proposed [11], may prove more useful than tea infusion preparations in providing adequate amounts of synergistic flavonoids along with artemisinin.

Materials and Methods

Four cultivars of *Artemisia annua* L. were used in the study: a nonclonal Chinese variety stemming from the seeds of an F2 generation of line PEG01 received from, and identified by, Chunzhao Liu (Chinese Academy of Science, Beijing, China); two clones, SAM and #15, isolated in our lab from the F2 generation; the fourth cultivar was FLV5 received as seed from, and identified by, Pedro Melillo de Magalhães (CPQBA-UNICAMP, Paulínia-SP, Brazil). *A. annua* used for tea infusion and analyses was from plants grown in soil, harvested and dried under light at room temperature. Dried leaves were pulverized through a series of brass sieves ending with 600 µm meshed powder, which was homogenized and used to prepare a tea infusion as described by van der Kooy and Verpoorte [9] for optimal artemisinin extraction, 9 g DW *A. annua* leaves per liter water at boiling point for 5–10 min while stirring. For the initial artemisinin stability study, dried nonclonal Chinese plants were

used and the infusion was boiled with stirring for 10 min. Aliquots of the infusion were filtered through 0.45 µm filters and placed in glass vials, and either stored at room temperature in the dark or frozen at −80°C. For analysis, samples were thawed if necessary and then partitioned with an equal volume of toluene. In all other experiments, the SAM clonal line was used, and the infusion was boiled for 5 min with stirring. For flavonoids, the same leaf concentration was extracted (9 g DW/L) in water at boiling point for 5 min. After filtration through 0.45 µm filters, half of the samples were extracted immediately and half were stored at room temperature in the dark for 24 hrs and then extracted. Partition of artemisinin or flavonoids from the infusion was achieved by adding an equal volume of toluene or methylene chloride, respectively. Solvents were separated, dried, and analyzed as described below.

Artemisinin was extracted from dried leaves of the various cultivars at a ratio of 6 mg dried leaves per mL pentane. After sonicating in a water bath at room temperature for 30 min, solvent was decanted and dried under N₂, then transferred to GC/MS sample vials; all samples were resuspended in 100 µL of pentane prior to analysis. GC/MS conditions were: GC, Agilent 7890A; MS, Agilent 5975C; column, Agilent HP-5MS (30 m × 0.25 mm × 0.25 µm); carrier gas, He at 1 mL/min; injection volume, 1 µL in splitless mode; ion source temperature, 280°C; inlet, 250°C; oven temperature, 125°C held for 1 min, and then increased to 300°C at 5°C/min. Artemisinin identification was via NIST library and validated standard. Flavonoids casticin and artemetin were quantitated by GC/MS by sonicating dried plant tissue in glass test tubes with methylene chloride (6 mg/mL) for 30 min. Solvent was decanted and dried under N₂. Residue was derivatized in GC/MS sample vials by adding 50 µL pyridine and 50 µL BSTFA+TMCS and heating at 70°C for 4 hr. GC/MS conditions are as above with the following oven program: ion source temperature, 275°C; inlet, 275°C; oven temperature, 110°C for 2 min, increased to 200°C at 10°C/min, then increased to 280°C at 5°C/min and held for 9 min. Casticin and artemetin were identified by comparing retention times and signature ion peaks in the plant extracts with purchased standards from Chromadex (Irvine, CA). All samples were analyzed at least in triplicate and statistically compared for significance using Student's t test.

Acknowledgments

This work was funded in part by NIH 2R15GM069562-03.

References

1. Hsu E. The history of *qing hao* in the Chinese material medica. *Trans Roy Soc Trop Med Hyg.* 2006; 100:505–508. [PubMed: 16566952]
2. de Ridder S, van der Kooy F, Verpoorte R. *Artemisia annua* as a self-treatment for malaria in developing countries. *J Ethnopharmacol.* 2008; 120:302–314. [PubMed: 18977424]
3. Efferth, T. Herbal drugs: ethnomedicine to modern medicine. Germany: Springer-Verlag, Berlin Heidelberg; 2009. Artemisinin: a versatile weapon from traditional Chinese medicine; p. 173-194.
4. Firestone GL, Sundar SN. Anticancer activities of artemisinin and its bioactive derivatives. *Expert Rev Mol Med.* 2009 Oct 30.11:e32. [PubMed: 19883518]
5. Ferreira JFS, Luthria DL, Sasaki T, Heyerick A. Flavonoids from *Artemisia annua* L. as antioxidants and their potential synergism with artemisinin against malaria and cancer. *Molecules.* 2010; 15:3135–3170. [PubMed: 20657468]
6. Ferreira J, Peaden P, Keiser J. *In vitro* trematocidal effects of crude alcoholic extracts of *Artemisia annua*, *A. absinthium*, *Asimina triloba*, and *Fumaria officinalis*. *Parasitol Res.* 2011; 109:1585–1592. [PubMed: 21562762]
7. Elford BC, Roberts MF, Phillipson D, Wilson RJM. Potentiation of the antimalarial activity of quinghaosu by methoxylated flavones. *Trans Roy Soc Trop Med.* 1987; 81:434–436.

8. Liu KC-SC, Yang S-L, Roberts MF, Elford BC, Phillipson JD. Antimalarial activity of *Artemisia annua* flavonoids from whole plants and cell cultures. *Plant Cell Rep.* 1992; 11:637–640.
9. van der Kooy F, Verpoorte R. The content of artemisinin in the *Artemisia annua* tea infusion. *Planta Med.* 2011; 77:1754–1756. [PubMed: 21544776]
10. Carbonara T, Pascale R, Argentieri MP, Papadia P, Fanizzi FP, Villanova L, Avato P. Phytochemical analysis of a herbal tea from *Artemisia annua* L. *J Pharmaceut Biomed Anal.* 2012; 62:79–86.
11. Weathers PJ, Arsenault PR, Covello P, McMickle A, Reed D, Teoh KH. Artemisinin production in *Artemisia annua* - studies *in planta* and results of a novel delivery method for treating malaria and other neglected diseases. *Phytochem Rev.* 2011; 10:173–183. [PubMed: 21643453]

Figure 1.
Structures of [1] artemisinin; [2], casticin (R=H); [3], artemetin (R=CH₃).

Table 1

Artemisinin concentration in *A. annua* tea infusion stored at room temperature or frozen.

time (d)	artemisinin (mg/g DW)		artemisinin % change at room temperature compared to frozen samples
	infusion frozen	infusion at room temp	
starting material pentane extract	9.63±1.36		
tea day 0	3.57±0.73		
day 1	4.91 _a	4.60 _a	−6.1
day 7	4.40 _a	2.79 _a	−36.3
day 14	5.89 _a	2.04 _b	−66.1

Used dried shoot material of nonclonal Chinese variety of *A. annua* in the vegetative stage. Letters indicate statistical difference between frozen and room temperature tea for each time point at P 0.05.

Table 2

Artemisinin and flavonoid content of various *A. annua* cultivars.

Artemisinin, artemetin, and casticin, in dried <i>A. annua</i> measured using GC/MS.				
<i>A. annua</i> cultivar (WPI cultivar reference name)	Cultivar voucher reference	Compound, mg/g DW (% w/w)		
		artemisinin	artemetin	casticin
FLV5	Artemisia 5' CPMA-UNICAMP 1246	6.79 (0.68)	0.019 (0.0019)	0.141 (0.0141)
Clone 15	MASS 00317313	5.71 (0.57)	0.080 (0.008)	0.211 (0.021)
SAM	MASS 00317314	14.89 (1.49)	0.210 (0.021)	0.410 (0.041)

Cultivar contents shown are an average for artemisinin, artemetin, and casticin for multiple analyses; although not detectable, eupatorin was also measured. All dried shoot material was from plants grown in the vegetative stage.

Table 3

Artemisinin and flavonoid content of *A. annua* tea infusion.

Artemisinin, artemetin, and casticin in dried <i>A. annua</i> and tea infusion measured using GC/MS.			
<i>A. annua</i> cultivar	compound, mg/g DW (% w/w)		
	artemisinin	artemetin	casticin
SAM	10.39 (0.10)	0.115 (0.012)	0.678 (0.068)
SAM infusion : immediate extraction	3.76 ^a (0.38)	nd	0.012 ^a (0.0012)
SAM infusion : 24 hr at rm temp	4.33 ^a (0.43)	nd	0.007 ^b (0.0007)

Used dried shoot material of *A. annua* SAM clonal line at full flowering. Letters indicate statistical significance between immediate extraction and room temperature storage of infusion tea at P 0.05; nd, not detected.