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Changes in key constituents of clonally propagated *Artemisia annua* L. during preparation of compressed leaf tablets for possible therapeutic use

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ABSTRACT

Artemisia annua L., long used as a tea infusion in traditional Chinese medicine, produces artemisinin. Although artemisinin is currently used as artemisinin-based combination therapy (ACT) against malaria, oral consumption of dried leaves from the plant showed efficacy and will be less costly than ACT. Many compounds in the plant have some antimalarial activity. Unknown, however, is how these plant components change as leaves are processed into tablets for oral consumption. Here we compared extracts from fresh and dried leaf biomass with compressed leaf tablets of *A. annua*. Using GC–MS, 19 endogenous compounds, including artemisinin and several of its pathway metabolites, 9 flavonoids, 3 monoterpenes, a coumarin, and 2 phenolic acids, were identified and quantified from solvent extracts to determine how levels of these compounds changed during processing. Results showed that compared to dried leaves, artemisinin, arteannuin B, artemisinic acid, chlorogenic acid, scopoletin, chrysoplenetin, and quercetin increased or remained stable with powdering and compression into tablets. Dihydroartemisinic acid, monoterpenes, and chrysoplenol-D decreased with tablet formation. Five target compounds were not detectable in any of the extracts of this cultivar. In contrast to the individually measured aglycone flavonoids, using the $AlCl_3$ method, total flavonoids increased nearly fivefold during the tablet formation. To our knowledge this is the first study documenting changes that occurred in processing dried leaves of *A. annua* into tablets. These results will improve our understanding of the potential use of not only this medicinal herb but also others to afford better quality control of intact plant material for therapeutic use.

1. Introduction

Malaria affects nearly three billion people with almost a million deaths annually, especially in Africa and amongst children (WHO, 2012a). Artemisinin (AN; Fig. 1), extracted from *Artemisia annua* L., is delivered in concert with another antimalarial drug (artemisinin combination therapy; ACT) as the preferred treatment, but it is costly and supply is inadequate (Davis et al., 2013; O'Connell et al., 2011). We and others have proposed more direct consumption of this plant either as a tea infusion (Mueller et al., 2004; Räth et al., 2004; Silva et al., 2012; Suberu et al., 2013) or by oral consumption of the leaves (Elfawal et al., 2012; ICIPE, 2005; Onimus et al., 2013; Weathers et al., 2011). These approaches are similar to the traditional use by the Chinese for >2000 years, show therapeutic efficacy (see review by Weathers et al., 2014a), cost less, and can be produced and implemented locally. Recently we showed that in contrast to oral consumption of pure drug, the presence of plant material significantly enhanced appearance of AN in serum of healthy and *Plasmodium chabaudi*-infected mice (Weathers et al., 2014b). A 2012 WHO white paper stressed, among other things, the need to thoroughly examine production stability and therapeutic efficacy of herbal *A. annua* as an antimalarial remedy (WHO, 2012b).

Considerable information is already known about how AN changes when leaves of *A. annua* are dried prior to extraction (Charles et al., 1993; Ferreira and Luthria, 2010; Laughlin, 2002) with the MMV report by Simonnet et al. (2010; personal communication) being the most comprehensive. While artemisinic acid (AA) levels do not seem to change, AN increases as leaves dry (Charles et al., 1993; Laughlin, 2002) and dihydroartemisinic acid (DHAA) level drops (Ferreira and Luthria, 2010). DHAA is a direct precursor

Abbreviations: AA, artemisinic acid; AB, arteannuin B; AN, artemisinin; DHAA, dihydroartemisinic acid; DW, dry weight; GC–MS, gas chromatography mass spectrometry; pACT, dried *A. annua* leaves (SAM cultivar); ROS, reactive oxygen species.

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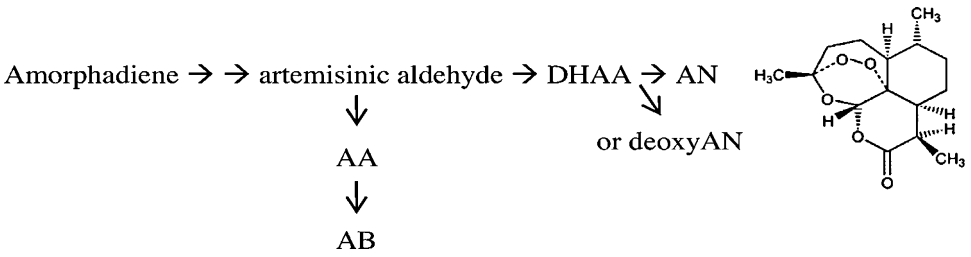

Fig. 1. Artemisinin structure and relationship to the other artemisinic compounds found in *A. annua* leaves and measured in this study. AA, artemisinic acid; AB, arteannuin B; AN, artemisinin; DHAA, dihydroartemisinic acid; deoxyAN, deoxyartemisinin.

Table 1
Compounds with antimalarial activity ± artemisinin vs. *P. falciparum* in vitro.

Compound	Compound IC_{50} (μM)[1]	Compound + artemisinin IC_{50} (μM)	Ref.
Artemisinic acid	77.8–61.6[a]	nd	Suberu et al. (2013)
Arteannuin B	3.2–4.8[a]	nd	
Dihydroartemisinic acid	21.1-17.7[a]	nd	
Artemisinin	–	0.023–0.021[a]	
alone	–	0.033	Liu et al. (1992)
Artemetin	26	0.026	
Casticin	24	0.026	
Cirsilineol	23	0.023	
Chrysoplenol-D	32	0.015	
Chrysoplenetin	36	0.016	
Eupatorin	65	0.030	
Quercetin	15, 14[b]	nd	Lehane & Saliba (2008)
Luteolin	11, 12[b]	nd	
Kaempferol	33, 25[b]	nd	
Myricetin	40, 76[b]	nd	
α-pinene[c]	0.1	nd	van Zyl et al. (2006)
1,8-cineole[c]	7.0	nd	
Nerolidol[c]	0.9	nd	

[a] Against chloroquine-sensitive (HB3) and chloroquine-resistant (Dd2) in vitro *P. falciparum*, respectively.
[b] Against chloroquine-sensitive (3D7) and a chloroquine-resistant (7G8) in vitro *P. falciparum*, respectively.
[c] Toxicity from van Zyl et al. (2006): nerolidol, 5.5 μM; 1,8 cineole, 69.1 μM; α-pinene, 172.2 μM; for quercetin, not toxic up to 2 g/kg in vivo (Utesch et al., 2008); see Radulović et al. (2013) for toxicities of *A. annua* essential oils.
nd, not determined.

of AN (Fig. 1), so the shift from DHAA to AN suggested that that there was biochemical activity during desiccation of the leaves, probably from accumulation of reactive oxygen species (ROS) that increased during water stress (Miller et al., 2010). The reaction from DHAA to AN likely involves ROS (Brown, 2010; Mannan et al., 2010). Drying method and conditions clearly exacerbate data interpretation and Simonnet et al. (2010) showed that relative humidity (RH) and temperature were critical for stabilizing AN content and long term leaf storage. For example, drying leaves at 40 °C resulted in AN losses within 2 h. At 30 °C and 10–30% RH, AN remained constant in the dried leaves for 12 months, but when the RH was 85%, even at 20 °C, AN content declined after about 6 months storage (Simonnet et al., 2010).

Because the dried leaves of *A. annua* have many other components, e.g. flavonoids and other terpenes, etc. that seem to impact either bioavailability or therapeutic efficacy (Table 1; also see recent review by Weathers et al., 2014a), it has been suggested that this endogenous mixture within the plant may provide its own combination therapy and has been termed plant artemisinin combination therapy (pACT; Elfawal et al., 2012). Although AN was not particularly affected, overall ferric reducing antioxidant power (FRAP) measured as Trolox equivalents decreased in sun- or shade-dried leaves compared to freeze-dried controls (Ferreira and Luthria, 2010). Flavonoids are important antioxidants and many also have weak antimalarial activity (Table 1), but to our knowledge changes in individual flavonoids produced in *A. annua* during drying have not been measured. Recently Suberu et al. (2013) showed that several other artemisinic compounds, namely dihydroartemisinic acid, artemisinic acid, and arteannuin B also have mild antiplasmodial activity and may synergize with AN. Some of the more volatile monoterpenes, e.g. α-pinene and 1,8-cineole also have weak antimalarial activity (van Zyl et al., 2006), but content declines when leaves are dried at temperatures >20–25 °C, while others like camphor and borneol are stable to 65 °C (Khangholi and Rezaeinodehi, 2008). Although camphor can comprise >30% of the essential oil of vegetative leaves of *A. annua* (Rana et al., 2013), thus far no antiplasmodial activity seems to be associated with it or borneol. Total essential oil yield declined in *A. annua* when drying temperature was increased in 10 °C intervals to 65 °C (Khangholi and Rezaeinodehi, 2008).

To measure biological responses it is important to use fresh weight analyses to determine real time results; however, for controlled therapeutic use, dried plant materials are used and often have to be powdered in order to compress into tablets or fill capsules. Making *A. annua* dried leaf tablets is preferred because a recent simulated digestion study of pACT encapsulated either in a cellulose or gelatin capsule showed >50% loss of its AN as recovered from the liquid phase of an intestinal digestate (Weathers et al., 2014c). Here we describe how the leaf composition of a high yielding *A. annua* cultivar changes after being dried, processed into powder, and then compressed into a tablet.

2. Materials and methods

2.1. Plant material and cultivation conditions

Artemisia annua L., SAM cultivar (Weathers and Towler, 2012; voucher MASS 00317314), was clonally grown in soil in the lab at room temperature under a vegetative photoperiod of 16 h light, 8 h dark. Shoots were harvested and dried in a 25 °C warm room at 30–50% RH under ambient room light. Dried leaves were removed from stems and sieved as subsequently described; multiple harvests were pooled for further processing and tablet formation. To normalize the study material, all plant material used in the study came from a single large pool of dried leaves harvested from several crops of lab-grown plants over about 12 months. For fresh leaf analysis, the first four leaves 15 cm from the apical meristem were harvested from some of the above vegetatively-grown plants and immediately extracted as subsequently described.

2.2. Sample sieving and tablet preparation

After harvest and drying, plant leaves were prepared in two sizes by subsequently passing them through a 2 mm and then a 0.6 mm brass sieve (Fig. 2). Powdered samples (100–150 μm) were produced from 2 mm sieved leaves by three successive 3 s pulses in a KitchenAid coffee grinder. All were prepared at room temperature under ambient light.

Fig. 2. Three granulation sizes of *A. annua* leaves; two were pressed into tablets. Granulation size L to R: 2.0 mm (could not form into tablets), 0.6 mm into tablets, ~100–150 μm into tablets. Tablet weights are ~0.17 and ~0.30 g, respectively; tablet dimensions were 10 mm diameter, 3 mm maximum depth.

Tablets were made from 100% dried leaves of either the 0.6 mm sieved or powdered material by SMI (SMI Inc., Lebanon, NJ, USA) using the SMI Minipress single station tablet press and The Director data acquisition system (http://www.smitmc.com/products.php?catid=1&ItemID=3&page=1, accessed February 6, 2014). Compaction and ejection forces were about 23 kN and 122 N, respectively. No excipient was required to obtain robust tablets. No tablets were made from unpowdered 2 mm material because it had inconsistent flow properties and periodically plugged the Minipress entry port. Tablet dimensions were: 10 mm diameter × 3 mm maximum depth. Average tablet weights were 0.17 ± 0.0009 and 0.30 ± 0.002 g for the 0.6 mm and powdered leaf materials, respectively (Fig. 2).

2.3. Harvest, extraction, and analysis of metabolites

Metabolites were extracted with methylene chloride as described in Weathers and Towler (2012). For chlorogenic and rosmarinic acids, tissue was extracted as above, but with 100% methanol. Ratio of solvent to biomass was 4 mL to 25 mg DW.

Artemisinin (AN), arteannuin B (AB), artemisinic acid (AA), dihydroartemisinic acid (DHAA), and scopoletin used the following GC–MS method: GC, Agilent 7890A; MS, Agilent 5975 C; 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; transfer line, 150 °C for AN and AB, 280 °C for AA, DHAA, and scopoletin; oven temperature, 125 °C held for 1 min, and then increased to 300 °C at 5 °C/min. AA, DHAA, and scopoletin were analyzed separately from the other metabolites. Samples for AN and AB analysis were resuspended in 100 μL pentane prior to analysis. AA, DHAA, and scopoletin were derivatized prior to injection by resuspending in 20 μL of 1:1 pyridine:bis(trimethylsilyl) trifluoroacetamide (Sigma 270407 and Restek 35605, respectively) plus 50 μL pentane. Nerolidol was analyzed using the same GC-MS system and column using a modified method of Bouwmeester et al. (1999): ion source temperature, 180 °C; inlet, 250 °C; transfer line, 290 °C; oven temperature, 45 °C held for 1 min, then increased to 220 °C at 10 °C/min and held for 5 min.

To avoid loss of volatile monoterpenes, α-pinene, eucalyptol, and camphor were extracted and analyzed directly with the same GC–MS system and column using a modified method of Tzenkova et al. (2010): inlet, 250 °C; transfer line, 280 °C; oven temperature, 40 °C held for 5 min, then increased to 100 °C at 4 °C/min, then increased to 300 °C at 25 °C/min and held for 5 min.

Individual flavonoids, plus chlorogenic and rosmarinic acids were analyzed using the same GC–MS system and column, but with a method modified from Li et al. (2009): inlet, 280 °C; oven temperature, 60 °C for 1 min, then increased to 130 °C at 30 °C/min and held for 2 min, then increased to 205 °C at 20 °C/min, held for 1 min, then increased to 275 °C at 30 °C/min and held for 15 min. Prior to injection, aliquots of plant tissue extract were derivatized with 60 μL acetone and 40 μL BSTFA + TMCS 99:1 (Supelco 33149-U).

Total flavonoids were assayed using the $AlCl_3$ method of Arvouet-Grand et al. (1994) with quercetin as standard as detailed in Weathers et al. (2014c). Total flavonoid content of each sample extract was calculated using the quercetin standard curve, and results expressed as quercetin equivalents.

AN, nerolidol, monoterpenes, and scopoletin identification was via NIST library and validated standards. Identification of AB, AA, chlorogenic and rosmarinic acids, and target flavonoids was via validated standards. DHAA was identified using an authentic standard purified from *A. annua* (Mannan et al., 2010) and quantified as AA equivalents. Authentic standards of AN, camphor, chlorogenic acid, eucalyptol, trans-nerolidol, α-pinene, rosmarinic acid, scopoletin were from Sigma–Aldrich (St. Louis, MO, USA); artemetin, casticin, eupatorin, quercetin were from Chromadex (Irvine, CA, USA); chrysoplenetin, chrysoplenol-D were from ChemFaces (Wuhan, PRC); artemisinic acid and arteannuin B were gifts of Dr. Nancy Acton from the Walter Reed Army Institute of Research.

2.4. Statistical analysis

Each condition had at least 4 replicates. Results were averaged and means statistically analyzed for significance against the dried 2 mm sieved leafy biomass using Student's *t* test.

3. Results and discussion

3.1. Rationale for analysis of the compounds targeted in this study

Previously we showed that oral ingestion of the dried leaves of this *A. annua* cultivar (SAM) was more effective than pure AN in quashing parasitemia in *P. chabaudi*-infected mice (Elfawal et al., 2012). Ingestion of dried *A. annua* leaves delivered >45-fold more AN into the serum than ingestion of the pure drug (Weathers et al., 2011) and the presence of dried plant material was crucial to the high delivery level of AN into the serum (Weathers et al., 2014b). Most of the 19 compounds targeted for analysis in this study were chosen because they are known to be present in various cultivars of *A. annua* (Ferreira et al., 2010) and have reported antiplasmodial activity (Table 1). Camphor was added mainly because it often comprises a considerable amount of the essential oil (Bhakuni et al., 2001), up to 30 + % of the total extractable oil of many *A. annua* cultivars (Rana et al., 2013). To our knowledge camphor has no antiplasmodial activity, but it may impact bioavailability. Scopoletin, also present in *A. annua*, was targeted because of its immunomodulatory activity and potential indirect

Table 2
Artemisinin content in dried leaves of *A. annua* from different developmental stages of plants grown over several years under different cultivation regimes.

Harvest identity	Developmental stage	AN (mg g DW^{-1})
Lab – April 2011	Vegetative	1.46 ± 0.06
Lab – Oct 2011		1.62 ± 0.18
Lab – Feb 2012		1.39 ± 0.24
Lab – March 2012		1.48 ± 0.12
Lab – Jan 2013		1.41 ± 0.22
Field July 2, 2012 (2H1)		1.18 ± 0.19
Field Sept 23, 2012 (1H1)	Reproductive; floral bud formation stage	1.40 ± 0.17
Field Sept 23, 2012 (2H2)		1.45 ± 0.23
PW's home garden Sept 2011		1.21 ± 0.93
PW's home garden Sept 2013		1.51 ± 0.18
Overall:		1.39 ± 0.22

Each value in the table is the mean ± standard deviation (n = 4–16).

interaction with the parasite infection process (see review by Weathers et al., 2014a). Although artemisia ketone can be a common constituent of *A. annua*, often comprising >60% of the essential oil fraction (Bhakuni et al., 2001; Radulović et al., 2013), it is absent in some cultivars (Rana et al., 2013) including SAM, the one used in this study, and thus was not measured.

3.2. Consistency of artemisinin content in a clonal cultivar of *A. annua*

The SAM cultivar of *A. annua*, isolated in 2009, has been grown clonally either via tissue culture or by rooted cuttings in soil. Numerous harvests from several years of field and lab-grown plants (Table 2) showed AN was relatively consistent averaging 1.39% ± 0.22 (w/w) for 10 crops. These crops were all from rooted SAM cuttings harvested from 30 to 3000 cm plants that were in either vegetative or reproductive developmental stages grown under a variety of field and lab-grown conditions (Table 2).

3.3. Drying altered the composition of *A. annua* constituents

Not surprisingly, the composition of *A. annua* leaves changed after the leaves were dried (Table 3). As reported by others, AN levels increased from ~1.14% in freshly extracted leaves expressed on a dry weight basis to ~1.6% in 2 mm sieved dry leaves. In contrast to observations by Ferreira and Luthria (2010), DHAA was undetectable in the fresh leaves, but increased to about 0.19% after drying. This discrepancy may be explained because the 2 mm dried leaf material also contains all of the apical shoots of the plant. The DHAA level in the shoot tips of the plant (~0.3 cm down from each shoot tip) was also high (~0.4%, Table 3) compared to that in mature leaves (not detectable, Table 3), so higher DHAA levels in the dried leaves is reasonable. A similar argument can be made for AA, which in the apical meristems is ~0.2%, but undetectable in mature leaves. On the other hand, the oxidation product of AA, AB (Fig. 1), was not detectable in the apical meristems and only at low levels (~0.01%) in the mature leaves, so its relatively high level (~0.2%) in the dried leaves must be the result of some biochemical process that occurred during drying.

Although the three targeted monoterpenes were usually present in *A. annua*, only two, eucalyptol and camphor, were found in this cultivar with highest levels in shoot tips at 0.11 and ~1.1%, respectively. Mature leaves had about a third of that measured in shoot tips (Table 3). Once leaves were dried, eucalyptol levels remained about the same as in mature leaves, but camphor increased substantially to double that found in the shoot tips and six-fold that in mature leaves (Table 3). In sage, camphor exists both as the aglycone and as the mobile 1,2-campholide (Croteau et al., 1987). It is possible that the campholide also exists in *A. annua* and that the stress of desiccation activated glucosidases that cleaved the glucose moieties yielding an increase in the aglycone form that our analytical method measured.

Two of the nine targeted flavonoids, chrysoplenetin and chrysoplenol-D, were found at detectable levels in freshly extracted mature leaves. Both increased in the dried leaves to a level midway between that of the shoot tips and the mature leaves. In fresh shoot tips there was also a small detectable amount of casticin, but it was not detectable in the dried leaves. We measured only flavonoid aglycones, so it is possible that desiccation may have altered the ratio between the glycone and aglycone levels of flavonoids in *A. annua* as was observed in a study with red clover (Swinny and Ryan, 2005). In another instance, desiccation of buckwheat leaves increased rutin glucosidase activity yielding increased quercetin (Suzuki et al., 2005).

For the other target compounds, rosmarinic acid was usually not detectable in most samples in this cultivar. Chlorogenic acid, however, comprised 0.6% of the dry weight of the mature leaves, but ~1.08% of shoot tips (Table 3). After drying the overall chlorogenic acid content dropped to 0.065% (Table 3), a response also observed during desiccation in *Ramonda* for chlorogenic and other phenolic acids (Sgherri et al., 2004). Scopoletin was present in extracts of fresh mature leaves at ~0.13% with four times more in shoot tips (0.55%, Table 3). After drying, however, scopoletin in the 2 mm sieved tips and leaf biomass also dropped to <0.001% suggesting that like chlorogenic acid, there was biochemical alteration of the coumarin during drying.

3.4. Composition further changed when leaves were processed into tablets

Once dried, leaves have to be further processed into a powder to fill capsules or to compress into tablets. *A. annua* is a bitter tasting herb, so to obviate the bitter taste one would consider encapsulation. However, a recent simulated digestion study showed that when encapsulated in either gelatin or cellulosic capsules, more than 50% of the AN in the dried leaves was lost by the end of the intestinal stage of digestion (Weathers et al., 2014c). Consequently, this study focused on how AN and other compounds in the plant changed with processing of dried leaves into a powder that was then compacted into a tablet.

Of the artemisinic compounds analyzed, only AN and AB remained constant as tablets were formed from dried leaves (Table 3). DHAA, however, decreased from ~0.2% in dried leaves to ~0.04% in the tablets. This was not surprising as DHAA is quite labile (Brown, 2010). AA, on the other hand, remained constant after the 2 mm dried leaves were powdered, and then increased >300% after tablet formation (Table 3). Monoterpenes all decreased with tablet formation with only camphor remaining, but it was less than 10% of that in dried leaves. While chlorogenic acid declined about 90% with drying to ~0.07%, it increased to 0.3% after powdering and to 0.5% in tablets, a near full recovery of the original amount measured in the freshly extracted mature leaves where it had seemed lost as a result of drying the biomass.

Total flavonoids also increased significantly with tablet formation, doubling with each step in processing dried leaves into powder and then tablets (Table 3). Although we do not know the full complement of flavonoids in the dried leaves, those targeted in this study were reported to have some antimalarial activity (Table 1) and were subsequently measured. Of those, only three were present in the tablets. As dry leaves were powdered, there was no significant change in either chrysoplenol-D or chrysoplenetin. Previously undetectable quercetin, however, appeared with

Table 3
Effect of drying, granulation, and tablet formation on key compounds in *A. annua* SAM leaves.

Compound	Freshly extracted leaves (μg g^{-1} DW)*		Dry extracted leaves (μg g^{-1} DW)		
	Shoot tips	Mature leaves	2 mm sieved	Powdered leaves	
				Leaf powder	Tablets
Artemisinin	8,188.92 ± 2,817.70a	11,380.32 ± 2,587.92b	15,897.23 ± 1,040.55x	17,306.24 ± 1,397.72y	17,180.86 ± 782.00y
Dihydroartemisinic acid	40,210.40 ± 6,414.62	nd	1,857.43 ± 737.22x	1,286.23 ± 205.24x	369.04 ± 32.00y
Arteannuin B	nd	96.76 ± 183.52	2,323.12 ± 246.97x	2,460.68 ± 246.86x	2,450.95 ± 202.63x
Artemisinic acid	2,209.12 ± 449.88	nd	367.39 ± 45.81x	345.30 ± 57.04x	1,328.84 ± 546.39y
α-Pinene	nd	nd	nd	nd	nd
Eucalyptol (1,8 cineole)	1,101.64 ± 106.39a	301.72 ± 51.52b	261.16 ± 42.35x	278.31 ± 44.34x	nd
Camphor	10,121.24 ± 1,141.62a	3,569.88 ± 318.76b	21,017.68 ± 1,600.64x	16,671.84 ± 1,260.17y	1,870.23 ± 214.98z
Chlorogenic acid	10,839.65 ± 3,004.50a	6,473.12 ± 981.72b	673.37 ± 138.64x	2,735.68 ± 487.12y	5,217.48 ± 528.44z
Rosmarinic acid#	nd	nd	2,261.19 ± 3,414.68#	nd	nd
Scopoletin	5,516.05 ± 905.12a	1,319.44 ± 454.96b	36.08 ± 16.43x	84.44 ± 47.58x,y	139.27 ± 47.22y
Artemetin	nd	nd	nd	nd	nd
Casticin	10.67 ± 28.92	nd	nd	nd	nd
Chrysoplenol-D	703.48 ± 201.90a	211.063 ± 99.44b	413.43 ± 56.59x	402.35 ± 30.55x	323.21 ± 28.61y
Chrysoplenetin	310.10 ± 76.13a	83.62 ± 40.67b	153.88 ± 23.95x	154.69 ± 13.41x	153.87 ± 11.26x
Eupatorin	nd	nd	nd	nd	nd
Kaempferol	nd	nd	nd	nd	nd
Luteolin#	nd	nd	206.61 ± 294.38#	128.39 ± 183.09#	nd
Myricetin	nd	nd	nd	nd	nd
Quercetin	nd	nd	nd	8.64 ± 0.55x	7.13 ± 4.25x
Total flavonoids ($AlCl_3$ method)	6,058.77 ± 419.96a	1,554.28 ± 439.60b	2,782.51 ± 274.19x	5,047.18 ± 671.02y	10,967.78 ± 656.35z

Each value in the table is the mean ± standard deviation (n = 4–6). Letters within a row indicate statistically significant data with a,b for fresh leaves and x,y,z for sieved/ground/tableted; $p \leq 0.05$; nd, not detectable; *DW calculated using DW/FW ratio of 0.25; #, only two of six replicates had rosmarinic acid or luteolin; shown is average of all 6 samples. DW, dry weight.

further processing of the dried leaves. This appearance can possibly be attributed to hydrolysis of one of its glycosides, rutin, which is known to be present in *A. annua* (Ferreira et al., 2010). While the other two flavonoids remained constant, quercetin was the only flavonoid that increased after tablet formation (Table 3). Although not detected in any of the comparative samples used in this study, trace amounts of kaempferol were occasionally detected in this cultivar.

The large difference between the total flavonoid levels measured using $AlCl_3$ and those measured separately by GC–MS is a dilemma. Although this species may contain up to 40 different flavonoids (Ferreira et al., 2010), we only measured those of already recognized therapeutic importance, so others are likely present. We also measured only the aglycone and not the glycone of the target flavonoids and this could account for the difference. The total flavonoid assay uses quercetin as standard, so we considered that other flavonoids or other compounds may react differently with the reagent. At the same concentration, each of the nine measured flavonoids varied in assay absorbance by at most twofold from that of quercetin; most were similar to quercetin, so that did not explain the results. Likewise, none of the targeted artemisinic compounds or phenolic acids reacted with the reagent. The only monoterpene of any consequence that we measured was camphor, but it was decreasing and not increasing once tablets were formed and thus could not account for the large increase in measured total flavonoids. Sterols and fatty acids would also be present in the methylene chloride extract used to assay total flavonoids. However, when cholesterol and linolenic acid were tested, neither compound reacted with the $AlCl_3$ reagent.

Powdering decreased plant material particle size from 2 to 0.1–0.150 mm. Decreased particle size increases the surface to volume ratio of the plant material 13–20 fold, which could enhance biochemical surface reactions in the material. When tablets are formed in a compression die, heat is generated, albeit for only a fraction of a second; this aids particle adherence and increases with the force of compaction (Bechard and Down, 1992; Zavaliangos et al., 2008). Compaction force of 20 kN can raise tablet surface temperature 5–30 °C (Bechard and Down, 1992) with even greater increases during actual production as heat accumulates in the machinery during a long production run. Although this transient heat of compression during tablet formation could enhance some of the smaller biochemical changes within the leaf material during compaction, e.g. artemisinic acid, it seemed too slight and transient to account for the especially large increases in total flavonoids and chlorogenic acid. Compaction heat could also account for loss of the volatile monoterpenes, a negative outcome from the therapeutic standpoint. Heat, again slight and for less than 10 s, is also produced during the powdering process. To determine if the heat generated during the brief powdering process altered composition, dried leaves were powdered along with small pieces of dry ice, but there were no significant changes in AN or flavonoids with cooling (unpublished results). Further work on the biochemical processes involved in the plant material during these steps involving drying and processing operations would prove useful.

4. Conclusions

The biochemical composition of either fresh or dried biomass is not a reliable measurement of either powdered dry biomass or compressed leaf tablets of the medicinal herb, *A. annua*. For preparations of the herb that are to be delivered either as an encapsulated powder or tablet, the final product has to be analyzed. One cannot assume that compounds in the dried leaves are the same as in a tablet or capsule. The results of this study will better inform the herbal industry as well as future purveyors of *A. annua* tablets and capsules of the potential changes and necessary measurements that must be conducted in order to validate quality.

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