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The effect of *O*-methylated flavonoids and other co-metabolites on the crystallization and purification of artemisinin

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ABSTRACT

Methoxylated flavonoids casticin, artemetin and retusin were identified as putative causative factors for low crystallization yields of artemisinin from extracts. Comparative profiling of biomass grown in different countries found elevated levels (~60% higher) of artemetin in the East African biomass, which also demonstrates poor crystallization yields. The single compound and the combined doping experiments at 0, 25 and 50 μg mL^{-1} doping levels showed that artemetin (at 50 μg mL^{-1}) caused a reduction in the amount of artemisinin crystallized by *ca.* 60%. A combination of the three flavonoids at 50 μg mL^{-1} almost completely inhibited crystallization, reducing the yield by 98%. Treatment of extracts by adsorbents efficiently resolves the problem of low crystallization yield.

1. Introduction

Natural products are a major source for drug discovery (Newman and Cragg, 2007) and a continued source of actives for the bio-pharmaceuticals industry. Isolation of the anti-malaria agent artemisinin from Chinese medicinal plant *Qinghao* (*Artemisia annua* L.), is a typical example. Artemisinin, Scheme 1, is a sesquiterpene lactone with a unique endo-peroxide bridge, which is key to its bioactivity (Haynes and Krishna, 2004; Meshnick, 2002; Meshnick et al., 1996; O'Neill et al., 2010; Qu et al., 2010; Webster and Lehnert, 1994). In 2002 World Health Organization (WHO) recommended the use of artemisinin in a combination therapy (ACT) as a first-line treatment of uncomplicated malaria (Qu et al., 2010; Shretta and Yadav, 2012). Since then, global delivery of ACT treatment courses to the public and private sectors has been increasing and rose sharply from 11 million in 2005 to 278 million in 2011 (WHO, 2012). This trend is projected to continue against the challenge of demand and supply imbalances resulting from widespread fluctuations in the price and global ACT shortages in some cases (Shretta and Yadav, 2012).

In 1987 Arvey et al. discovered the total synthesis of artemisinin (Avery et al., 1987) and seven years ago an engineered artemisinin metabolic pathway was successfully inserted into microbes to produce artemisinic acid, the biosynthetic precursor to artemisinin (Ro et al., 2006; Withers and Keasling, 2007). Chemical conversion of artemisinic acid to artemisinin was also recently developed (Levesque and Seeberger, 2012). The announcement in 2012 by Sanofi of the first industrial-scale bio-engineered production of artemisinic acid that could be converted to over 40 million treatment courses *via* the Berkeley process will help to ease shortages but also could add to the problem of fluctuation in price for the growers of the plant (Peplow, 2013).

Presently the bulk of artemisinin used in ACT and other treatments comes from the plant. East Asia (mainly China and Vietnam) cultivated approximately 80% of global output in 2012 while the rest 20% came from East Africa and Madagascar (Cutler, 2011). This is an increase over the previous year for Madagascar and East Africa, when the latter contributed just 9%, or 15 metric tons, to the global output (A2S2, 2011). This trend is significant in the light of the reported problem of variable recovery rates in the

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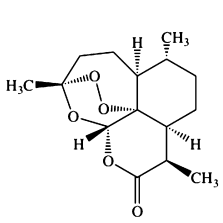
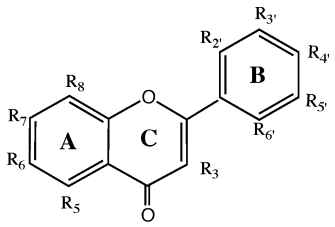


artemisinin



methoxylated flavonoids

Compounds	Substituent on rings									
	R_3	R_5	R_6	R_7	R_8	$R_{2'}$	$R_{3'}$	$R_{4'}$	$R_{5'}$	$R_{6'}$
Artemetin	OCH_3	OH	OCH_3	OCH_3	H	H	OCH_3	OCH_3	H	H
Casticin	OCH_3	OH	OCH_3	OCH_3	H	H	OH	OCH_3	H	H
Chrysoplenetin	OCH_3	OH	OCH_3	OCH_3	H	H	OCH_3	OH	H	H
Chrysosplenol-D	OCH_3	OH	OCH_3	OCH_3	H	H	OH	OH	H	H
Cirsilineol	H	OH	OCH_3	OCH_3	H	H	OCH_3	OH	H	H
Eupatorin	H	OH	OCH_3	OCH_3	H	H	OH	OCH_3	H	H
Retusin	OCH_3	OH	H	OCH_3	H	H	OCH_3	OCH_3	H	H

Fig. 1. Chemical structures of artemisinin and some methoxylated flavonoids of *A. annua* L. extract.

processing of East African biomass into the active ingredient (Henfrey, 2013).

Production of artemisinin from the plant is a multi-step process starting with the drying of the plant parts, in most cases the leaves and the shoots. This is followed by extraction, which on a commercial scale involves soaking, percolation or continuous flow in warm (30–40 °C) organic solvents of low polarity like hexane, toluene, petroleum ether, *etc.* (ElSohly et al., 1990; Haynes, 2006; Lapkin et al., 2006). To improve solubility of artemisinin, a modifier like ethyl acetate is sometimes used. Up to three to four extraction cycles may be employed in the batch process and each cycle takes between 10 and 48 h, depending on the intensity of mixing employed (Lapkin et al., 2006). Simultaneously, extraction of essential oils, flavonoids, waxes and pigments occurs in the process, necessitating the separation of artemisinin from the raw extract by sequential crystallization, typically from ethanol (Malwade et al., 2013; Qu et al., 2009). To our knowledge, literature evidence on the effect of these co-extracted metabolites on the solubility of artemisinin in the extraction liquor and its crystallization and processing is lacking. Lapkin et al. reported the effect of eight co-metabolites in *Artemisia* plant extract on the solubility of artemisinin based on *a priori* computational screening (Lapkin et al., 2010). They found that some co-metabolites increased solubility of artemisinin by up to 7.5%. At a given concentration, casticin and its glycosylated form had the greatest impact on the solubility of artemisinin among the metabolites tested. On this basis we hypothesized that specific flavonoids present in East African biomass may be responsible for the reported problems in crystallization of artemisinin from extracts.

Casticin is one of a group of *O*-methylated flavonoids found in *A. annua* (Baeva et al., 1988). There are over 17 reported methoxylated flavonoids found in the plant (Bhakuni et al., 2001; Ferreira et al., 2010; Shilin et al., 1989). The major ones are artemetin, casticin, chrysoplenetin, chrysosplenol-D, cirsilineol, eupatorin and retusin (Liu et al., 1992). Methoxylated flavonoids are low molecular weight bioactive polyphenolics based on a C_{15} (C_6–C_3–C_6) carbon skeleton containing two aromatic rings (A and B) linked by a chroman ring (C), see Fig. 1 (Sisa et al., 2010). In plants the *O*-methylated (methoxylated) flavonoids are more widely distributed than the *C*-methylated compounds and the methoxy groups may be present on the flavone nucleus in positions 2′, 3′, 4′, 5′, 3, 5, 6, 7, and 8 (Bandyukova and Avanesov, 1971). The maximum number of methoxy groups is seven with molecules generally containing hydroxyl groups also. Methoxylated flavonoids are often present as *O*-glycosides or *C*-glycosides with the *O*-binding more abundant in plants (Bandyukova and Avanesov, 1971; de Rijke et al., 2006). The substituent sugar (commonly arabinose, galactose, glucose or rhamnose) of the *O*-glycosides usually binds to the hydroxyl of the aglycone at position 3 or 7, while in the *C*-glycosides this is usually with the carbon of the aglycone at 6 or 8 (de Rijke et al., 2006).

In the plant, methoxylated flavonoids are considered to participate in chemical defense due to the particular structural and absorptive features. They also participate in stress protection and as plant development regulators (Sisa et al., 2010). One major role proposed for these compounds and some other flavonoids is that of natural filters for solar UV radiation. This is supported by the observation that exposure to UV radiation induces higher levels of flavonoids in plants. One such example is the work of Caldwell, which showed that Alpine plants at high altitude and tropical plants from regions of intense UV radiation have a higher flavonoids content than the plants from other regions (Caldwell, 1971). Cuadra et al. obtained over a 40% increase in the amount of UV absorbing flavonoids between control and the UV irradiated *Gnaphalium* plants (Cuadra and Harborne, 1996).

Consequently, we suspect that the levels of flavonoids in various *A. annua* biomass samples will differ according to the climatic geography of the growth location and therefore be partly or wholly responsible for the differences observed in their processibility. The waxes and pigments content in the plants have also been suggested as possible reasons for poor artemisinin crystallization through verbal communications with industrial artemisinin extractors.