NPC Natural Product Communications

2008
Vol. 3
No. 12
2003 - 2006

Comparative Analysis of Antimalarial Principles in *Artemisia annua* L. Herbal Drugs from East Africa

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Received: July 25th, 2008; Accepted: October 23rd, 2008

Malaria mortality continues to increase across the world and represents the most important parasitic disease of man. *Artemisia annua* L. (Asteraceae) has been used to treat fevers in China for over two millennia and recently the clinical efficacy of teas and decoctions derived from this species have been demonstrated, using high artemisinin-yielding plants. Therefore, it is important to verify the artemisinin levels in local cultivations in areas where malaria is endemic and to assess how different geographical and climatic conditions may affect the efficacy of traditional treatments. In this study, samples of the aerial parts of *A. annua* (ANAMED 3 hybrid) cultivated in three different locations in Burundi were compared for their content of active principles. Artemisinin levels in the plant materials ranged from 0.20% to 0.35%, while total flavonoid contents ranged from 0.32% to 0.80%.

Keywords: *Artemisia annua* L., Burundi, different cultivations, artemisinin, flavonoids, HPLC/DAD/MS.

Malaria is one of the oldest and most important life-threatening parasitic diseases in the tropical regions of the world. It causes more than 300 million acute illnesses and at least 1-2.7 million deaths annually. The majority of these deaths are due to cerebral malaria and other complications resulting from malaria-related anaemia, and the cost in human life, incapacity for work, programs of control and medical treatments are enormous [1a-1c]. Ninety per cent of those who die are in Africa, where malaria accounts for about one in five of all childhood deaths, mainly children under the age of five in sub-Saharan Africa. Burundi is among the African countries with a high incidence of malaria, which is probably the leading cause of death in this as well as other East African countries [1d]. The 2000-2001 epidemic of *Plasmodium falciparum* in Burundi, with an attack rate peak of 109% in the northern provinces of Kayanza, Karuzi, and Ngozi, is well documented [1e,1f].

Some non-governmental organizations and international agencies working in Burundi have offered to introduce to these regions the use of the plant *Artemisia annua* L. (sweet or annual wormwood) because its active constituent, artemisinin, has proven suitable for the control of malaria epidemics, including chloroquine- and quinine-resistant strains, and has shown a low propensity to induce resistance [2].

A. annua is an annual herb native to the northern parts of Chahar and Suiyuan provinces in China, where it is called "quinghao" and has been used as a remedy for chills and fevers for more than 2000 years [3,4].

In the most recent literature, clinical trials using teas or decoctions of *A. annua* leaves from high artemisinin-yielding plants (> 0.5% dried weight) grown in Central Africa, have shown a rapid disappearance of malaria parasites from the blood of patients treated with doses corresponding to the Chinese Pharmacopoeial recommendations [5-7]. Artemisinin plasma concentrations after intake of these *A. annua* traditional preparations were lower

than those achieved with modern artemisinin drugs used in malaria therapy, but still above 10 μg/L, the threshold for parasite growth inhibition.

Therefore, the locally grown herb may offer an additional tool for the control of malaria, especially in poor countries with scarce or no access to modern medicines or medical services. However, it is known that levels of artemisinin in *A. annua* may vary considerably (0.01-1.4% plant dry weight) with growing conditions, particularly climate and geographical location [8]. Furthermore, *A. annua* flavonoids have been shown to enhance the antiplasmodial activity of artemisinin *in vitro* [9a-9c]. Therefore, these components should be monitored in locally prepared *A. annua* herbal drugs in order to assess the quality of the drug used to treat malaria.

In this regard, we report herein the HPLC-DAD-MS analyses of different extracts of the aerial parts of *A. annua*, cultivated in malarial-endemic regions in East Africa. Finally, quantification of the active constituents in these plant materials offered an opportunity to assess the possible role of environmental conditions, such as altitude, in their biosynthesis, as the same *A. annua* cultivar, *Artemis* [8], was planted in three different locations in Burundi with distinct geographical and climatic conditions. The applied HPLC-DAD-MS method [9d] was specific for the detection of artemisinin and *A. annua* flavonoids, even if present only in trace amounts.

The plant material used was the cultivar *Artemis* [8], grown in different locations in Burundi, East Africa, namely Kyenzi, a prairie at 2300 m altitude in central Burundi, J1, a wooded area at ca. 1800 m near the border with Ruanda, and in a field near the hospital of Bubanza, a city situated in north-west Burundi, at 950 m altitude.

The *n*-hexane and dichloromethane extracts of each herbal drug sample were prepared, because it was known from previous investigations [9d] that the extraction efficiency for artemisinin and flavonoids is maximised using these solvents. Samples were analysed by HPLC-DAD-MS.

In the extracts, all polymethoxyflavonoids related to the antimalarial activity, such artemetin, chrysoplenetin, casticin, cirsilineol and eupatin were detected. However, as shown in Table 1, yields of each of these constituents varied significantly among the different samples. In the *n*-hexane extracts artemisinin yields ranged from 10.7% to 5.7% (w/w), while in the dichloromethane extracts the total flavonoid content ranged from 12.8% to 5.2%. Table 2 shows the flavonoid variability in the herbal drugs from different cultivations. Finally, table 3 reports the artemisinin and total flavonoid levels expressed as percentage, w/w, of herbal drug. We found that the *A. annua* plant materials obtained from the different cultivation areas of Burundi contained artemisinin the range 0.20%-0.35% of the herbal drug. These values are inferior to those reported for the same original cultivar (*Artemis*) after professional cultivation (0.5%-0.75%) [9d].

The lower yields of artemesinin found in the analysed herbal drugs could be a consequence of altered agricultural and collection practices operated at the local sites of production with respect to the established methods for attainment of high-yielding plants [10]. Furthermore, the diverse geographical and climatic conditions, or soil composition of the different fields in Burundi could be responsible for the observed yield variation in *A. annua* antimalarial constituents. In particular, we noticed that plants grown at higher altitude (i.e. at 2300 m in the Kyenzi region) were richer in artemisinin (0.35%, w/w, of herbal drug) than plants produced at either 1800 m (0.24%) or 950 m (0.20%), in the J1 area and in Bubanza, respectively. The beneficial influence of altitude on artemisinin yields in *A. annua* plants cultivated at tropical latitudes had been suggested previously [10] and the data collected in this work seems to indicate an analogous relationship. The total flavonoid percentage in the analysed samples ranged from 0.80% to 0.32%, w/w, of herbal drug, with the highest amount present in the samples produced in the fields at higher altitude.

In conclusion, we analysed samples of *A. annua* herbal drug obtained from cultivation at three different sites in Burundi, East Africa. All three samples analysed possessed detectable levels of artemisinin and flavonoids. However, the quantitative profiles of the antimalarial active compounds varied significantly in the different samples. Our results suggest that there could be a correlation between the content of artemisinin and flavonoids and the altitude of the growing site in the East African territory. Further investigations will need to be undertaken in order to assess the best conditions for growing high artemisinin and flavonoid-yielding *A. annua* plants in these conditions.

Table 1: Extraction yield and percentage of artemisinin and flavonoids in the *Artemisia annua* extracts (dm: dichloromethane, hx: *n*-hexane) from the Burundi cultivations of J1, Bubanza (Bb) and Kyenzi (Ky).

		Yield (mg)[a]	% total flavonoids[b]		% artemisinin[b]	
			average value	stand. dev.	average value	stand. dev.
1	J1_dm	622.0	5.2	0.025	3.9	0.396
2	J1_hx	287.6	2.4	0.040	8.3	1.048
3	Bb_dm	762.0	6.1	0.026	2.6	0.355
4	Bb_hx	351.8	2.1	0.026	5.7	0.807
5	Ky_dm	627.0	12.8	0.042	4.8	0.632
6	Ky_hx	329.2	3.3	0.042	10.7	1.261

[a]Dried extract obtained from 10,0 g herbal drug; [b]Percentage content in the dried extract.

Table 2: Percentage content (w/w) of individual flavonoids in the *Artemisia annua* herbal drugs from the Burundi cultivations of J1, Bubanza (Bb) and Kyenzi (Ky). Percentage content in the dried extract (w/w); *n*=3 samples.

Extract		% Eupatin		% Cirsilineol		% Casticin and Chrysoplenetin		% Artemetin	
		average value	stand. dev.	average value	stand. dev.	average value	stand. dev.	average value	stand. dev.
1	J1_dm	2.49	0.010	0.06	0.003	2.52	0.018	0.13	0.006
2	J1_hx	0.25	0.003	-	-	1.95	0.032	0.25	0.003
3	Bb_dm	4.35	0.033	0.20	0.003	7.60	0.050	0.62	0.016
4	Bb_hx	0.19	0.003	-	-	2.63	0.036	0.47	0.007
5	Ky_dm	2.13	0.027	0.10	0.004	3.55	0.041	0.28	0.011
6	Ky_hx	0.23	0.002	-	-	1.58	0.055	0.27	0.010

Table 3: Percentage content (w/w) of artemisinin and flavonoids in the *Artemisia annua* herbal drugs from the Burundi cultivations at J1, Bubanza (Bb) and Kyenzi (Ky).

	% total flavonoids[a]		% artemisinin[b]	
	average value	stand. dev.	average value	stand. dev.
J1	0.32	0.000	0.24	0.029
Bb	0.46	0.000	0.20	0.023
Ky	0.80	0.000	0.35	0.044

[a]Percentage of the dichloromethane extract; *n*=3 samples. [b]Percentage of the *n*-hexane extract; *n*=3 samples.

Experimental

Chemicals: Artemisinin (98%) was purchased from Sigma (Sigma-Aldrich S.r.l., Milan, Italy) and rutin (99.5%) from Merck (Darmstadt, Germany). Solvents for extraction and HPLC analysis (*n*-hexane, dichloromethane, MeOH and acetonitrile) were HPLC grade and were purchased from Merck. 85%. Formic acid was purchased from Carlo Erba (Milan, Italy). Water for HPLC analysis was purified by a Milli-Q_{plus} system from Millipore (Milford, MA).

Plant material: The seeds of *Artemisia annua* cv. *Artemis* were provided by Anamed in Germany (seeds "ANAMED A3"). Cuttings of germinated plants were planted in June 2006 by local farmers at three different locations in Burundi, namely Kyenzi, J1 and Bubanza, in areas exposed to sunlight. Leaves were harvested in November 2006 and dried in the air at temperatures below 40°C. Samples of dry aerial parts were sent to Europe in packages of 100 g. Plant material was cultivated and collected under the supervision of Paolo Monti, working for the Artemisia Project of the Medical Foundation for Africa and supported by the Department for the fight against malaria of the Ministry of Health of Burundi.

Preparation of extracts: *Artemisia annua* dried aerial parts were cut into small pieces and the leaves were separated from the branches and stems. Only leaves and flowering tops (herbal drug) were used for the analyses (Figure 1). Samples of 10 g herbal drug were exhaustively extracted at room temperature by maceration with 100 mL of either *n*-hexane or dichloromethane for 72 h. The eluates were subsequently dried under vacuum to obtain the crude extracts.

HPLC sample preparation: Five mg or each *n*-hexane or dichloromethane dried extracts were accurately weighed and suspended in acetonitrile (1.0 mL) in a volumetric flask. The suspensions were sonicated for 20 min, then filtered through a cartridge-type filtration unit with a polytetrafluoroethylene (PTFE) membrane (d = 13 mm, porosity 0.45 µm, Lida manufacturing Corp., Kenosha, WI) and immediately injected.

HPLC analyses: Artemisinin and flavonoid (Figure 2) contents of the dried extracts were determined by high performance liquid chromatography (HPLC) coupled with mass spectrometer (MS), according to [9d]. HPLC analyses were performed using a HP 1100 Liquid Chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a HP 1040 Diode Array Detector (DAD), an automatic injector, an auto sampler and a column oven, and managed by a HP 9000 workstation (Agilent Technologies, Palo Alto, CA, USA). The HPLC system was interfaced with a HP 1100 MSD API-electrospray (Agilent Technologies).

Separations were performed on a reversed-phase column of Purospher® Star RP-18, namely Hibar® pre-packed column RT (250 x 4.6 mm), with particle size 5 μm (Merck, Darmstadt, Germany). Eluents were: water adjusted to pH 3.2 with formic acid (A) and acetonitrile (B). The mobile phase was isocratic 50% A and 50% B for 15 min, following by gradient from 50% to 100% B in 5 min, at a flow-rate of 1.0 mL/min. The system was operated with an oven temperature at 26°C; the injection volume was 20 μL. Chromatograms were recorded both at 350 nm to detect the flavonoids and at 210 nm to detect artemisinin and any other constituents, with a peak threshold of 0.1 mAu. The following mass spectrometry operating conditions were used: gas temperature 350°C at a flow-rate of 10 L/min, nebulizer pressure 30 psi, quadrupole temperature 30°C, and capillary voltage 3500 V. Full scan spectra from *m/z* 100 to 800 in the positive ion mode were recorded (scan time 1 s).

Calibration and quantitative analyses: Calibration curves of artemisinin and rutin were obtained from stock solutions of each standard in acetonitrile (artemisinin 0.170 mg/mL and rutin 0.092 mg/mL) and used to quantify the artemisinin and flavonoid contents, respectively, in the samples of *A. annua* extracts. For artemisinin calibration, HPLC injection volumes of 10, 15, 20, 25 and 30 μL of the artemisinin stock solution were used and the peak areas in the MS were recorded. For flavonoid calibration, HPLC injection volumes of 2, 4, 6, and 8 μL of the rutin stock solution were used and the peak areas in the UV chromatogram at 350 nm were measured. Linear regression was used to establish the calibration curve. Each HPLC sample of *A. annua* extract was injected three times and the artemisinin and flavonoid contents were calculated on the basis of the peak areas in the mass spectra (for artemisinin) or in the UV spectra at 350 nm (for the flavonoids).

Acknowledgments - We are thankful to Mr Paolo Monti, responsible for the Artemisia Project in Burundi, for sending us the plant materials. The financial support of Ente Cassa di Risparmio di Firenze is gratefully acknowledged.

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