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Tests confirm suitability of Ugandan soils for commercial growing of *Artemisia annua* Linn

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The content of artemisinin from *Artemisia annua* Linn plants grown in different geographical regions of Uganda was investigated. Two analytical methods; high performance liquid chromatography (HPLC) and an ultraviolet (UV) spectrophotometric analytical procedure were used and the results were compared. The HPLC method was modified and revalidated before it was used for analysis of the plant samples. The spectrophotometric procedure was used without modification. Seven districts including Kapchorwa, Kabale, Kaberamaido, Kabarole, Wakiso, Rakai and Rukungiri were selected for the study. With both methods, higher content of artemisinin was obtained in plant samples from Kapchorwa (1.2%, w/w, HPLC) and Kabale (1.4%, w/w, HPLC) than in samples from Kaberamaido (0.47%, w/w, HPLC) and Wakiso (0.36%, w/w, HPLC). In the other districts, the content varied between 0.57 and 0.93%, w/w. Fortunately, even the low content of artemisinin that was observed in Kaberamaido and Wakiso compared very well with values reported in other parts of the world where the plant is widely grown for commercial and for experimental purposes. This suggested that Ugandan soils are adequately suitable for commercial production of *A. annua*.

Key words: *Artemisia annua*, content of artemisinin, geographical region, Uganda, high performance liquid chromatography (HPLC), ultraviolet (UV) spectroscopy

INTRODUCTION

Artemisinin is an anti-malarial drug and also a starting compound for a class of highly active anti-malarial drugs namely artesunate, artemether, and dihydroartemisinin, which are currently used as components of Artemisinin-based Combination Therapies (ACTs). The latter drugs are formed by a semi-synthetic process which transforms the artemisinin molecule, first into dihydroartemisinin and then into artemether or artesunate (WHO, 1986; Haynes, 2006).

Artemisia annua has several other potential uses namely, as an insect repellant (Tripathi, 2000), in the treatment of schistosomiasis and gastrointestinal tract infections (Ferreira et al., 2006), treatment of coccidiosis in poultry (Ebiamadon et al., 2008) and treatment of cancer (Efferth et al., 2001; Ferreira, 2004). An infusion of the leaves is used for malaria prophylaxis and in the treatment of fevers, colds, diarrhea and some other illinesses, while *Artemisia* oil is used in perfumery, cosmetics and dermatology (Bhutani et al., 2003; Ogwang et al., 2011, 2012).

With the numerous potential uses of artemisinin and its derivatives, the future demand for the compound will continue to rise. However, chemical synthesis of artemisinin is an expensive and difficult multistep process that is accompanied by a low yield. Therefore, *A. annua* plant remains a major and economically viable commercial

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source of artemisinin (Sushil et al., 2004; Sushil and Suchi, 2005).

In December 2008, the United Nations General Assembly adopted the New Draft Resolution on the Decade to Roll Back Malaria, urging the financing and scaling up of artemisinin production and procurement, as appropriate; to meet the increased need (MediaClubSouthAfrica.com, 2009). Efforts have therefore been put in many countries to crop the *A. annua* plant on large scale in China, India, Vietnam, Turkey, Iran and Afghanistan (WHO, 2006b). It is also now grown in the highlands of some African countries such as South Africa, Madagascar, Nigeria, Mozambique, Tanzania and recently in Uganda (Horton, 2006).

In Uganda, *A. annua* is mainly cultivated in the districts of Kapchorwa, Kabale, Kaberamaido**,** Kabarole and Rukungiri and some low lying areas of Wakiso and Rakai districts for both commercial and experimental purposes. The differences in the geographical location of the farmlands may be a significant factor on the quality of artemisinin grown in the different parts of the country.

Although many Ugandan farmers are picking interest to grow *A. annua* plants for commercial purposes due to the high demand by pharmaceutical companies, there is inadequate information about which part of Uganda has the best *A. annua* with regard to the content of artemisinin. This knowledge gap by the farmers as well as other investors interested in commercial growing of *A. annua* creates a situation of unpredictable economic viability because of the likely risk of growing this medicinal plant in areas that would give poor yields in terms of content, which may eventually translate into business losses.

In addition, cultivation of *A. annua* plants for production of artemisinin requires affordable and reliable test methods to analyze for artemisinin content in the quality control of artemisinin herbal extracts and plant materials containing this medicinal active ingredient. Thus, two analytical methods have been used for determination of artemisinin content and the results for both methods are reported. Quality control determination is one of the key problems facing the artemisinin market and investment in Uganda due to expensive and sophisticated analytical methods and laboratory equipment that are required for analysis.

Although it is important that the content of artemisinin in *A. annua* should be tested before harvesting, there are no approved quality control programs for monitoring artemisinin levels in the plants cultivated in Uganda to know whether harvesting is also done at the right time. In fact, the purchase of dry *A. annua* leaves is based on the trust of the producers who are paid based on the weight of dry leaves yet the international price of artemisinin is based on the percentage assay (w/w) of artemisinin content in the dry leaves.

As the demand for artemisinin remains high around the world, finding suitable geographic regions for *A. annua* is also a critical research area for the World Health Organization (WHO, 2006a). The main objective of this research therefore, was to determine the content of artemisinin in dry leaves of *A. annua* grown in different parts of Uganda. The other secondary objective was to document an affordable analytical procedure for routine use in determining the content of artemisinin in the leaves of *A. annua*. The results of the study will give an insight into the suitability of the different geographical regions of Uganda for large scale cultivation of plant.

MATERIALS AND METHODS

Method validation

The principles in the high performance liquid chromatography-ultraviolet (HPLC-UV) methods developed by Guo-Ping et al. (2005) and Nurgün et al. (2007) were combined and modified into one method that was suitable for our purpose. The analytical procedure applied involved conversion of artemisinin into UV-absorbing compounds (Figure 1).

The conversion was then followed by HPLC analysis. The incubation of artemisinin in sodium hydroxide solution (0.2%, w/w) converted it to Q292 which gave maximum UV absorption at 290 nm. Addition of acetic acid (0.08 M) converted the Q292 to Q260 nm which was stable in acid medium after 48 h and gave maximum UV absorption at 260 nm. The conversion of the unstable Q292 to stable Q260 was not rapid as indicated in Figure 2. The graphs show that the peak area of the Q292 compound continued to decrease, while that of the Q260 kept increasing up to 48 h of storage before stabilizing with Q260 at 88% and Q292 at 12% of the total peak area, respectively.

The quantitative determination of artemisinin was therefore based on its Q260 UV-absorbing compound after stabilization. After this period both Q260 and Q292 compounds were stabilized in acid medium with no further conversion of Q292 to Q260.

We therefore recommend that the samples should be processed and then injection into the HPLC system should be done after 48 h when the conversion of compound Q292 to Q260 has been completed. We also observed that the peak for Q292 did not disappear completely but stabilized after 48 h. Figure 3 shows chromatograms with two significant and well resolved peaks corresponding to Q292 and Q260 compounds that were obtained at retention times of 3.7 and 5.1 min, respectively at a wavelength of 260 nm.

Instrumentation

Chromatographic conditions were modified and tested until maximum separation and sensitivity was achieved for the peaks of the artemisinin component. The chromatographic analysis was carried out using an Agilent Technologies ChemStation LC System equipped with a photodiode array detector, a quaternary pump with a degasser and an auto injector. The mobile phase consisted of methanol, acetonitrile and buffer (0.9 mmol Na_2HPO_4, 3.6 mmol NaH_2PO_4 solution) at a pH of 7.76 in the ratios of 45:10:45% (v/v). The absorbance was monitored at 260 nm and elution was carried out at 30°C using a flow rate of 0.7 ml per min. A reverse phase waters spherisorb, C18, ODS1 column (250 × 4.6 mm, 5 µm) was used.

Preparation of artemisinin standard stock solutions

Chemical reference standard of artemisinin was purchased from

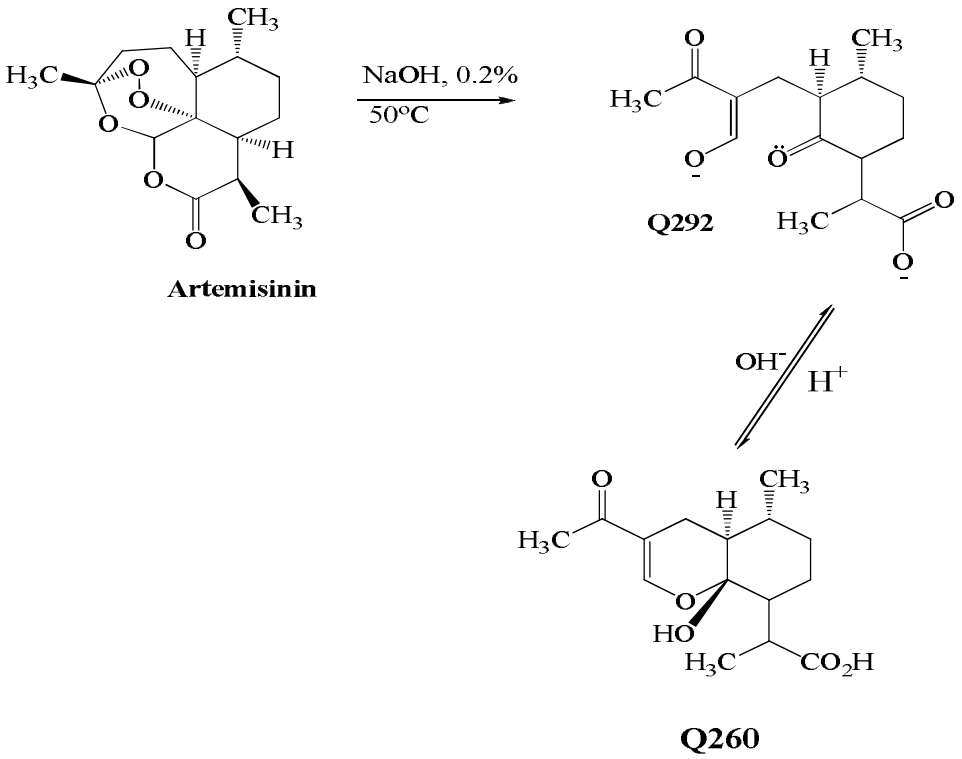

Figure 1. Conversion of artemisinin to UV-absorbing compounds, Q260 and Q292 of artemisinin samples from Uganda.

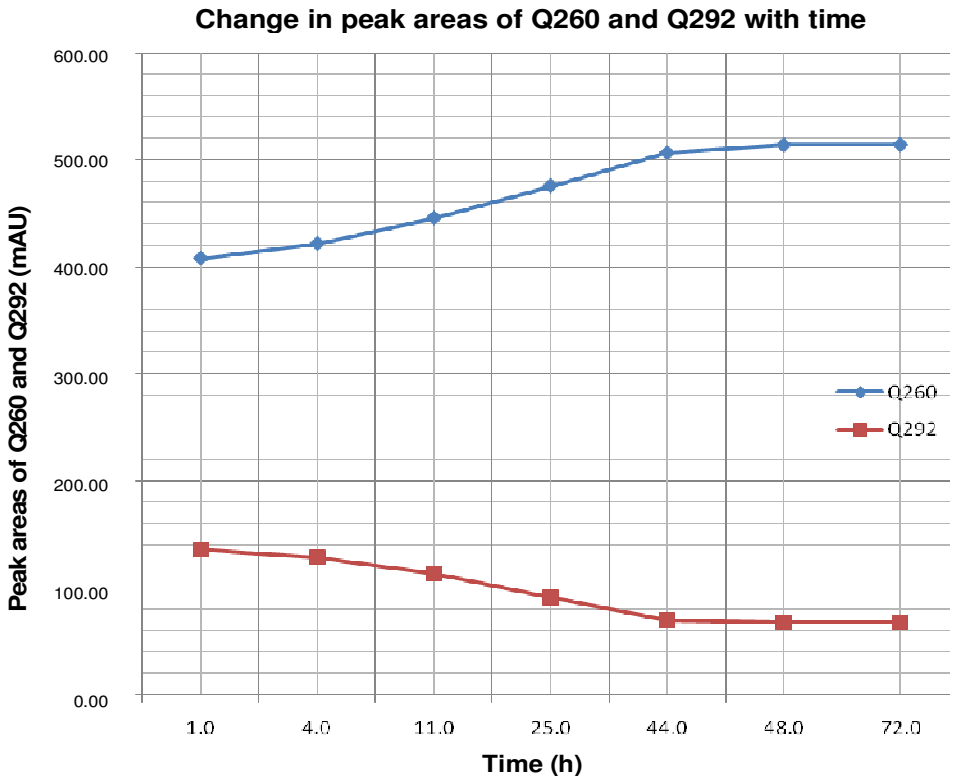

Figure 2. Changes in peak areas of Q292 and Q260 compounds with time of artemisinin samples from Uganda.

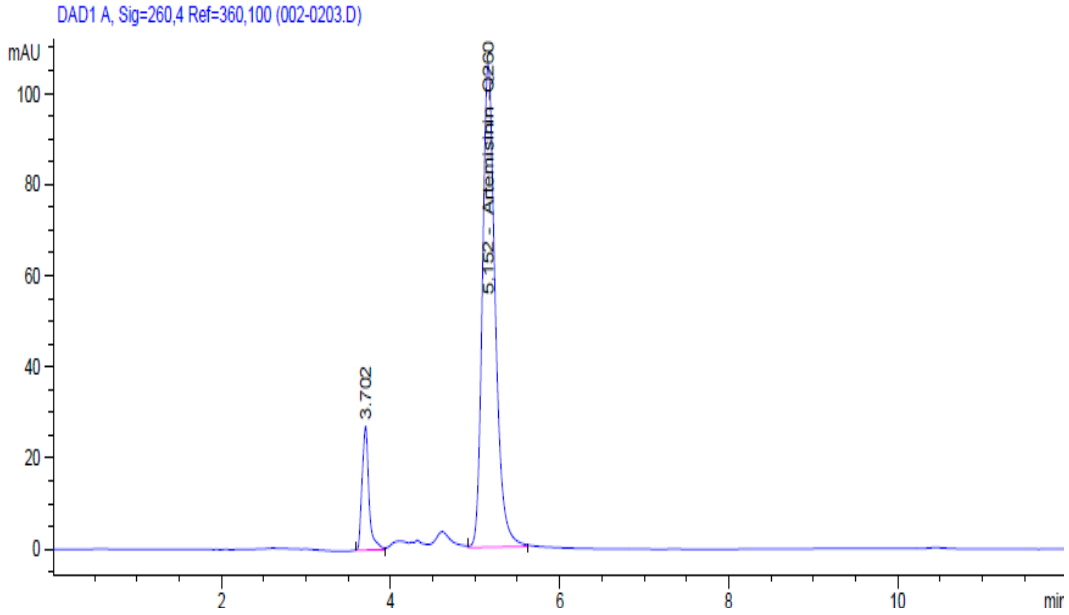

Figure 3. HPLC chromatogram of artemisinin standard at 260 nm and flow rate of 0.7 ml/min using artemisinin samples from Uganda.

WHO chemical reference standards centre in Sweden (Control No. 103222). Standard artemisinin (100.8 mg) was accurately weighed and dissolved by ethanol (95%) in a 100 ml volumetric flask. An aliquot of artemisinin solution (1 ml) and ethanol (9 ml, 95%) were transferred into another 100 ml volumetric flask. This solution was coded as "Solution-1" then sodium hydroxide solution (40 ml, 0.2% w/v) was added and the solution made to react at 50ºC for 30 min. After, acetic acid solution (0.08 mol/L) was added and filled up to the mark of the 100 ml volumetric flask (Nurgün et al., 2007). Four more artemisinin standard solutions namely 2, 3, 4 and 5, respectively were similarly prepared. The five standard solutions covered a concentration range of 10 to 100 mg/L artemisinin. All the solvents and the solid chemicals used were of HPLC grade and analytical grade, respectively.

Preparation of calibration curve

A calibration curve was prepared by using a set of five standard solutions ranging from 10 to 100 mg/L of artemisinin. Triplicate HPLC injections were made for each standard solution. The average peak areas of artemisinin obtained for each standard solution were plotted on the Y-axis against the concentration of artemisinin on the X-axis to obtain the calibration graph. The five concentrations were subjected to regression analysis to obtain the calibration equation and correlation coefficients. The method was revalidated to achieve the recommended value of $R^2 \geq 0.99$ (ICH, 2005).

Quality control standards

At every time of analysis, a control standard solution was also prepared alongside the sample solutions. After every six analyte injections, an injection of neat mobile phase solutions was done followed by artemisinin control standard solution and after the final sample injection of the whole sequence. Both control standard and sample solutions were read against the same calibration curve in mg/L.

Linearity of the method

Standard curves were established for freshly prepared artemisinin standard solutions and the linearity was tested over a range of 10 to 100 mg/L of artemisinin.

Recovery of the HPLC method

This was documented using a sample of ground leaves of *A. annua* obtained from Rukungiri. Five replicate samples (1 g) were spiked with 4.2 mg of pure artemisinin primary reference substance and subjected to the same sample preparation procedure as for the standards. The samples were then analyzed for total artemisinin content and the percentage recovery was calculated (Shuoqian et al., 2008).

Collection, handling and analysis of plant samples

Samples were collected from seven selected parts of Uganda where *A. annua* was being cultivated. These study sites are located in the districts of Kapchorwa, Kabale, Kaberamaido**,** Kabarole, Wakiso, Rakai, and Rukungiri all indicated by shading in the map of Uganda (Figure 4).

In all the districts except Kaberamaido and Rukungiri, two sampling sites were selected. The sites were visited and contact with farmers was established for alert on when the *A. annua* plants would start budding as a sign of beginning to flower. Samples were collected just before the plants began to flower in the periods of August to September, 2010. Each collected sample was labeled with a sample code with respect to the study site in the district, date of sampling as well as the age of plants at flowering stage in months. Leaf samples were harvested immediately as the plants began to flower by plucking leaves from the tops of 50 randomly selected plants on each study site (1 to 2 acres each). A pair of scissors was used to cut-off the fresh leaves from the plants and a polythene bag was used for keeping the samples from each site. All samples were kept in a cold box till they reached the laboratory.

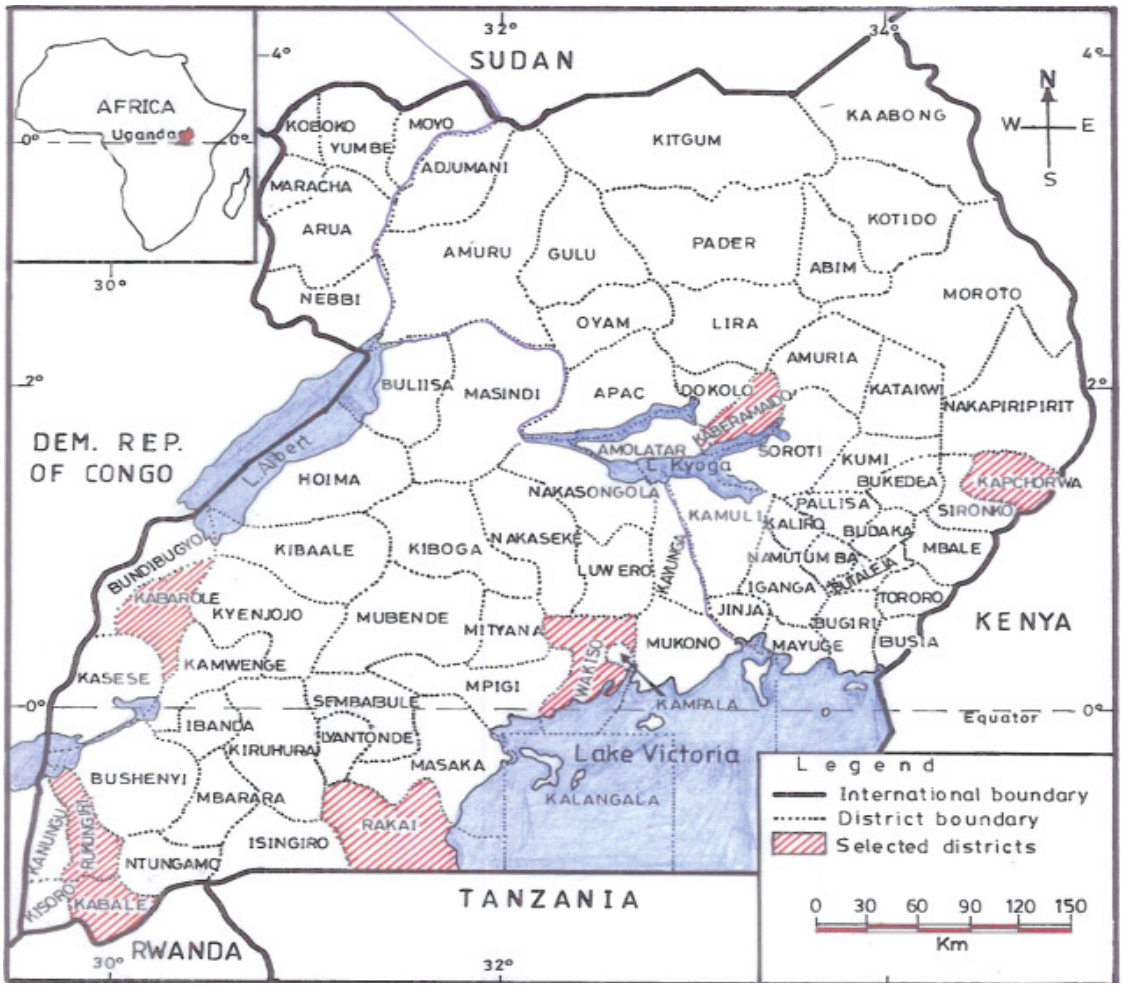

Figure 4. Map of Uganda showing selected districts where *A. annua* is cultivated.

Replicate measurements for a sample from each site were made and after analysis, the results of samples collected from sites within the same district were averaged and given as the artemisinin content for the district under study.

Extraction of artemisinin from leaf samples

The samples of *A. annua* leaves collected from the field were dried on clean and dry laboratory benches using indoor air at ambient temperature for 3 weeks (Laughlin, 2002). The dried leaves were manually separated from the branches, ground into fine powder using a motor and a pestle and then sieved through a 1.4 mm USA stainless steel test sieve. Artemisinin was extracted from the sieved powder by heating under reflux with hexane at a temperature of 75°C for 1 h (Peng et al., 2006).

Accurately weighed samples (1 g) in duplicate of ground and sieved dry leaves were refluxed with hexane (100 ml) at 75°C for 1 h. In all extractions, the plant material to solvent ratio of 1:100 was maintained to prevent solvent saturation (Shuoqian et al., 2008).

The *n*-hexane extract was filtered through a Whatman filter paper No.1 and the filtrate was evaporated under vacuum by a rotor evaporator until dryness under a fume hood. The residue was again dissolved in 50 ml of *n*-hexane and the *n*-hexane phase washed in a separating funnel with 2% NaOH solution to get rid of the impurities, which are soluble in aqueous sodium hydroxide. The lower layer which contained the alkali solution was abandoned, while the upper solution (hexane extract) was washed with distilled water several times until it was neutralized. This hexane extract was filtered through anhydrous sodium sulphate on the Whatman filter paper No.1 and the filtrate was evaporated to dryness by distillation under vacuum at 45ºC by a rotary evaporator. The dried extract was dissolved with ethanol (95%), and then topped to the mark in a 50 ml volumetric flask (final Aliquot). The solution was then ready for use with both the HPLC and spectrophotometric techniques. Thus, the filtered liquor of final aliquot (10 ml) was transferred into a 100 ml volumetric flask. The artemisinin ingredient in solution was converted into compound Q260 as follows: 40 ml of 0.2% NaOH solution was added into the flask containing artemisinin solution and then, the solution was made to react at 50ºC for 30 min. After that, 0.08 mol/L acetic acid solution was added up to the mark (Guo-Ping et al., 2005; Nurgün et al., 2007). This sample solution was ready for injection into the HPLC system. These solutions were kept in the fridge at 4°C in the dark until they were injected into the HPLC system.

Determination of the percentage moisture content

In order to standardize the measurements of the artemisinin content,

Table 1. Percentage recovery of artemisinin in crushed leaves of *A. annua* Linn collected from Rukungiri.

Sample codes	Art added (mg)	Art recovered (mg)	Recovery (%)
Replicate 1	4.50	4.45	99.0
Replicate 2	4.40	4.35	98.8
Replicate 3	4.30	4.56	106.0
Replicate 4	4.40	4.27	97.0
Replicate 5	4.10	4.18	102.0
Mean			101.3
% RSD			3.58

content, it was necessary to determine the moisture content of the dried samples. This was done at the time of weighing samples for solvent extraction. Samples (5 g) were weighed in duplicate and then dried to constant weight for 3 h in an oven set at 105°C. They were cooled in a desiccator before being weighed again for moisture loss. Percentage moisture content was calculated as loss on drying by using Equation 1 adopted from Siau (1984), Walker et al. (1993), Mukherjee (2002) and IP (2010).

$$M = \frac{(A-B)}{5} \times 100 \quad (1)$$

Where, M = percentage moisture content, A = total weight of watch glass and artemisinin powder before oven drying B = total weight of watch glass and artemisinin powder after drying and cooling.

HPLC determination of artemisinin content in dry leaves

The identification and quantification of artemisinin was made by comparing its retention time and peak area found in plant samples with that of the artemisinin peak from a standard reference solution injected with each batch of samples. All the calculations concerning the quantitative analysis were performed with external standardization by measurement of the peak area values against the concentrations of artemisinin standard solutions. Results of artemisinin quantities in *A. annua* samples were expressed as the mean of three determinations. The percentage of artemisinin content in the absolutely dry leaves of *A. annua* was calculated using Equation 2 below adopted from WHO (2006b) with modification because of the different analytical method used.

$$P = \left(\frac{A \times V \times d.f}{1000 \times Wt}\right) \times \left(\frac{100}{100-B}\right) \times 100 \quad (2)$$

Where P = percentage of artemisinin, A = concentration of artemisinin in sample (mg/L), V = initial aliquot volume of reconstituted extract = 50 ml, $d.f$ = dilution factor, Wt = mass of dry leaves of *A. annua* (mg), B = percentage moisture of the dry leaves of *A. annua* as determined using Equation 1 above.

Spectrophotometric determination of artemisinin content in dry leaves

The UV spectrophotometric method specified for artemisinin determination by Zhong (2007) was adopted. The procedure described herein was applied to all the samples. 10 ml of the final aliquot after extraction was pipetted into a 100 ml volumetric flask; diluted to the mark with sodium hydroxide (0.2%), mixed thoroughly, and warmed to 50°C in a water-bath for 30 min. The solution was then cooled to room temperature. The absorbance of a 1 cm layer was measured against a solvent cell containing a blank prepared with 10 ml of ethanol (95%) diluted with sufficient sodium hydroxide (0.2%) to produce 100 ml. The solutions were scanned in the UV range of 200 to 350 nm. Artemisinin identification and quantification was made by comparing the spectrum shape and absorbance of artemisinin Q290 peak at a wavelength of 290 nm for the samples and the reference standard of artemisinin. The standard stock solutions were prepared as described above for HPLC.

RESULTS AND DISCUSSION

The content of artemisinin in *A. annua* leaves from different parts of Uganda was investigated using two analytical methods. With the HPLC method some changes were made in the working chromatographic conditions and this necessitated revalidation of the method before it could be used. The method was validated over a range of 10 to 100 mg/L of artemisinin and was found to be linear over the entire range with a mean R^2 value of 0.9965 ± 0.0033) (n = 5). This was indicative of its accuracy. The recovery of the method was also very good as indicated in Table 1. The results obtained using the two analytical techniques are also shown in Table 2.

The content of artemisinin ranged from 0.36% (w/w) (HPLC) for *A. annua* grown in Wakiso district to 1.4% (w/w) (HPLC) in Kabale district. Both methods showed that Kabale and Kapchorwa districts had the highest yields of artemisinin in *A. annua*. This was attributed to the fact that the districts are located at high altitudes, cold climates and the late flowering periods of 5 to 6 months. Rukungiri, Kabarole and Rakai also had relatively good yields above 0.5% (w/w) and this was due to slightly longer plant flowering periods of 4 to 5 months. Wakiso and Kaberamaido showed relatively lower yields and this was attributed to the early flowering periods of 2 to 3 months, low altitude and warm climate as compared to Kabale and Kapchorwa.

Globally, a large variation in artemisinin content has been observed in the leaves of *A. annua* (Delabays et al., 2002). In fact, artemisinin content ranging from 0.02 to 1.38% has been reported (Table 2). It has also been documented that *A. annua* plants with artemisinin content greater than 0.5% (w/w) are regarded as high yielding

Table 2. Artemisinin content determined using both HPLC and UV spectrophotometric techniques from Ugandan *A. annua* Linn plants and the global variation of artemisinin content.

Source (Uganda)	Mean % assay by HPLC (n = 4)	Mean % assay by UV spec. (n = 4)	Other sources**	Artemisinin content (%)	Year
Kapchorwa	1.20	1.05	Europe	0.02	1998
Kabale	1.40	1.33	Argentina	0.10	1986
Kaberamaido	0.47	0.54	Dakota (USA)	0.21	1990
Kabarole	0.93	0.96	Spain	0.24	1993
Wakiso	0.36	0.40	Vietnam	0.86	1994
Rakai	0.73	0.77	China	1.07	1993
Rukungiri	0.57	0.47	Switzerland (hybrid)	1.38	1996

**Adapted from Delabays (2002).

and fit for commercial growing (Billia et al., 2006). This suggests that five out of the seven districts investigated in Uganda, were fit for commercial growing of *A. annua*. Even the lowest value of 0.36% (w/w) of artemisinin content found in one part of Uganda compared well with the values reported in other parts of the world as indicated in Table 2 (Delabays et al., 2002; Wright, 2002). Uganda therefore has a very high potential for commercial production of artemisinin.

Although both methods yielded comparable results for the content of artemisinin, the spectrophotometric method would be more cost effective than HPLC, which requires elaborate technical specifications and user expertise. In addition, the costs incurred in both purchase and maintenance and that of consumables for HPLC work are substantially higher than those for spectrophotometric analysis.

Conclusions

The study showed that it was possible to determine the content of artmisinin using both HPLC and spectrophotometric techniques. However, the spectrophotometric technique is more cost effective because it is cheaper than the HPLC technique. In both cases, the results revealed that the content of *A. annua* grown in different geographical regions of Uganda varies. Despite the observed variation, all regions were found to be suitable for commercial production of *A. annua*.

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