The artemisin potential of leaves from cultivars and wildlings of *Artemisia annua* L. grown in Western Kenya

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ABSTRACT

Half of the world's population and especially those in Africa are most vulnerable to malaria. In Western region of Kenya, malaria prevalence levels are above 40%. The disease is preventable and treatable with currently recommended interventions one being the use of Artemisinin-based combination therapy. A hybrid plant *Artemisia annua* anamed ('A3'), a clone of artemisinin annua is being embraced in western Kenya. We report the levels of artemisinin in leaves of 'A3' grown in regions of Western Kenya and of soil nutrients Zinc (Zn), Boron (B), Nitrate (NO_3^-) and Ammonium (NH_4^+). High performance Liquid Chromatography, Atomic Absorption Spectrometry and Ion Selective Electrodes were employed. In comparison to the expected levels in soils for artemisinin accumulation; Zn was above the minimum tolerable levels; B was very low in the top-soil but high at in-depth; nitrogen NH_4^+ and NO_3^- ions were found sufficient and the ratio of NO_3^- : NH_4^+ was high. Artemisinin in leaves of cultivars ranged between 0.04-0.88% dry matter. The levels of artemisinin in 'A3' grown in Western region of Kenya can be improved if nutrient levels are well managed. These findings showcase the need to expand cultivation of A. *annua* in Western Kenya and consequently produce artemisinin that would be useful in addressing malaria.

KEY WORDS

Artemisia annua leaves; artemisinin; cultivars and wildlings, Western Kenya

INTRODUCTION

Half of the world's population is at risk of malaria with people living in the poorest countries being the most vulnerable. In 2010, 90% of all malaria deaths occurred in the WHO African Region, mostly among children under five years of age. Increased prevention and control measures however have led to its reduction in mortality rates by more than 25% globally and by 33% in the WHO African Region (WHO, 2012). In Kenya, malaria is the leading cause of morbidity and mortality, with 25 million out of a population of 34 million Kenyans at risk (Ministry of Health, 2009). Other associated effects of the disease include; loss of productivity and income associated with illness and death; loss of working day and/or absenteeism from school and formal employment; and in case of

death of a family member, the loss of future lifetime earnings. Malaria is an entirely preventable and treatable disease, provided that currently recommended interventions are properly implemented.

The WHO recommends the use of Artemisinin-based combination therapy (ACT), an effective drug based on artemisinin, a sesquiterpene lactone peroxide found in the plant *Artemisia annua L (A. annua)* (Graham *et al.*, 2010). The Kenya government allocates about KShs. 69.5 billion annually in acquiring ACTs since chemical synthesis of artemisinin is complex and uneconomical (Health Sector Strategic Plan HSSP, 2005-2015). These figures are projected to increase for expenditure on malaria treatment, prevention and administration. Malaria has significant measurable direct and indirect costs that place major constrains in economic development in Kenya thus retarding efforts on wealth creation and poverty eradication. These costs could however be reduced by more production of artemisinin from *A. annua.*

Among the species of Artemisia; *Artemisia absinthium, Artemisia tridenta, Artemisia vulgaries* and *Artemisia aboratum*, only *A. annua* has been shown to produce artemisinin (Duke *et al.*, 2005). Additionally, the species is widely studied for its numerous purposes including being used as artemisia tea, anti-cancer, mosquito repellant, a source of essential oils, crafting of aromatic wreaths and as a natural herbicide with its flowers having antiperiodic, antiseptic, digestive, antiprotozoal, antibacterial and supposedly anti-yeast properties (Efferth *et al*., 2001; Singh and Lai, 2001; Li *et al*., 2005; Sen *et al*., 2007). *A. annua* has been undergoing genetic improvement to develop high yielding strains. Its hybrid plant named *A. annua* anamed or 'A3' is now widely available for cultivation in Central and East Africa (Yeung *et al*., 2004). Latitudes closer to the equator such as the Western region of Kenya have ecological features that favor stable malaria parasite transmission and as well would benefit the A3 crop establishment. In Kenya, A3, was introduced in early 1990's and is cultivated in large acreage in parts of Central and Rift valley provinces. There is current effort to embrace its cultivation in the Western region (Western and Nyanza provinces) where malaria prevalence levels are above 40%. The plant is grown in small plots by community based organizations in Kajulu, Nyakach, Asembo in Kisumu county, Maseno, Rang'ala in Siaya county and Ingidi in Vihiga county. In the region, it is majorly used as artemisia tea and as mosquito repellant.

The viable levels of artemisinin in *A. annua* are usually above 0.6% although most collections of artemisia derive from natural stands have highly variable content, some as low of 0.01% while others have ranged between 0.3 to 1.5% dry weight (Mueller *et al*., 2000; Delabays *et al*., 2001; Abdin *et al*., 2003; EABL, 2005). Factors that will determine these levels include variation in soil nutrient (Zinc (Z), Boron (B), Nitrate ion (NO_3^-), Ammonium ion (NH_4^+)), plant part (leaves, flowers, stem and roots), climatic conditions (temperature, water and light), age of growth, species and method of cultivation (Dhingra *et al*., 2000; Laughlin *et al*., 2002; Liu *et al*., 2003; Zhang *et al*., 2004; Khudsar *et al*., 2004; Kumar *et al*., 2004; Covello, 2008). Zn is needed by plants to activate several important enzymes while its deficiency has several negative effects (Khudsar *et al*., 2004). At extremely low levels of 0.05mg/L (50µg/g) in soil, zinc apparently increased the artemisinin

yield while levels higher than this are considered toxic for *A. annua* (Yekuan *et al*, 2010). The role of B in plant nutrition is still not very well known but some postulations have been made (Tanaka and Fujiwara 2007). Levels of 0.05 mg/L and 2.50 mg/L are considered very low and very high respectively and there is evidence of a linear relationship between B and artemisinin content (Zhang *et al*., 2004; Yekuan *et al*, 2010). NH_4^+ is tolerated by plants in small amounts, and can be toxic at higher levels while increased NO_3^- is not toxic and has been associated with increased artemisinin production (Liu *et al*., 2003). A ratio of higher NO_3^- : NH_4^+ has been found to increase artemisinin content while increases in NH_4^+ resulted in decreased artemisinin content (Wang and Tan, 2002; Liu *et al*., 2003).

Artemisinin compounds have been predominately found in the upper parts (flowers and leaves) of the *A. annua* plant, with higher concentrations found just before or during full flowering (Laughlin, 1995; Delabays *et al.*, 2001). Glandular trichomes are more abundant in the flowers and there is strong evidence that artemisinin is produced in them (Mehrotra *et al*., 1990; Duke *et al*. 1994). Duke and co-workers, (1994) showed neither artemisinin nor its derivatives were detected from a glandless plant and that artemisinin content was shown to be 4 to 11% times higher in the flowers as compared to leaves. With the assumption that leaves of A3 grown in Western region of Kenya will have viable levels of Artemisin, we report the levels and correlate them with levels of Zn, B, NO_3^- and NH_4^+ soils growing them.

MATERIALS AND METHODS

Chemicals

Acetonitrile (ACN) and petroleum ether were of HPLC grade and were sourced from Sciencescope Ltd, Nairobi, Kenya. All the other reagents and solvents used including pure zinc metal granules (Zn), 99.8% Hydrochloric acid (HCl), Boric acid (H_3BO_3), Ammonium chloride (NH_4Cl), Hydrated Aluminum sulphate ($Al_2(SO_4)_3.18H_2O$), Silver sulphate (Ag_2SO_4), Sulphamic acid (H_2NSO_3) and Sodium hydroxide pellets (NaOH) were of analar grade sourced from Merck. Artemisinin (98.9% pure) standard was sourced from Sigma-Aldrich Chemical Company, St. Louis, U.S.A. Buffer solution for the analysis of nitrate ions was prepared by dissolving $Al_2(SO_4)_3$, Ag_2SO_4, H_3BO_4 and H_2NSO_4 in about 400ml distilled water, adjusting pH to 3.0 by slowly adding 0.1N NaOH and the solution made to 1 liter using distilled water.

Soil sampling and analysis of nutrients

Sampling and analysis of soil was done according to the procedure described in Alloway, (2008). Soil was sampled before transplanting of seeds from the nurseries. About 200g of soil was randomly obtained from different spots within the plot using a 1" diameter stainless steel metallic hollow pipe at two depths; 10-15cm (top soil) and 30-45cm (in-depth). The soil samples were then transported to the laboratory in labeled bags and stored at low temperatures to minimize microbial activity. Before digestion, soil was dried at 105 C, stone particles and plant debris were removed and the soil pulverized to a fine powder (pulverizer- Siebtechnic Model TS 250 made in Hague,

Switzerland). The pulverized fine powder was apportioned into four for analysis of Zn, B, NH_4^+ and NO_3^-.

Analysis of Zn and B was done according to Alloway, (2008). Briefly, the pulverized soil sample (2.5g and 10 g for Zn and for B measurement respectively) was accurately weighed into a 250ml glass flask. 10ml distilled water was added and left to stand for about an hour. 25ml aqua regia (1:3 HNO_3 and HCl) was added and the mixture heated at 450^{o}C for approximately 2-3 hours. A few drops of the aqua regia were added continuously into the flasks in order to avoid dryness. The contents were then filtered using a filter paper No. 541 into a 50 mL volumetric flask and the residue washed before topping up with warm distilled water. Calibration curves were obtained by calibration the instrument using five standard solutions. Measurement of Zn and B was done using computerized Varian Atomic Absorption Spectrometer (Model: AA-10). The operating parameters were set according to the specification given by the manufacturer including lamp current of 1.2 amperes, fuel system of N_2O-Acetylene and oxidant flow rate of 4.5 L/min. The equation of each generated calibration curve was used in calculating Zn and B in the soil sample.

Soil samples were analyzed for NH_4^+ and NO_3^- according to Lachat instruments (1995). Briefly, the pulverized soil sample (1.0g and 0.5g for NH_4^+ and NO_3^-analysis respectively) was accurately weighed into a 100ml glass beaker. 50ml of distilled water was added and thoroughly stirred using a magnetic stirrer for 2 minutes. To 25 ml of the solution was added 25 ml of buffer and the solution was stirred before taking millivolts readings on an Ion Selective Meter (ISE-Model 290A, made in U.S.A) that was connected to either NH_4^+ or NO_3^-ISE.

Leaf samples

A. annua anamed (A3) seeds were sourced from the International Center for Research in Agroforestry (ICRAF-Nairobi, Kenya) and grown in five nurseries (cultivars) one each at the study areas; Kajulu, Nyakach, Asembo, Maseno and Ingidi in Western region, Kenya. Seeds were also broadcasted on soils with minimal tillage to serve as control. After germination and growth in a period of 3 months, the seedlings from the nurseries were transplanted into to 50 m^2 plots and allowed to grow for six months a period when flowering began. While no manure or fertilizer was used on either the cultivars or controls, the former were watered and weeded regularly during their growth. Approximately 250g of the leaves from each of the five plots were randomly picked at onset of flowering (6 months old). These were immediately transported to the laboratory in aerated bags, weighed and dried in an oven (40^{o}C) for about 72 hours to achieve moisture content of less than 10%.

Artemisinin extraction and HPLC analysis

The procedure of extraction was adapted from Aditi and Sarin (2010). Dry leave samples were ground to powder using a pestle and mortar and sieved through 0.5 mm pore size sieve. 2.0 g of the powder was soaked in 25 ml of petroleum ether for 48hrs to extract artemisinin. The extract was filtered, solvent evaporated by a rotatory evaporator and the residue re-dissolved in 25 ml

acetonitrile for injection into an HPLC (Model Agilent 1100 Shimadzu LC-20AT). A calibration curve within the range of 500-2500 ppm was prepared. 20 µL sample extract was injected onto the column head (Hyper Clone BDS C_{18} column; 5 µm, 250 mm x 4.6 mm) that was maintained at 30 °C for separation of artemisinin for a 12 minutes elution time. A mobile phase consisting of water: acetonitrile (25:75 v/v) was isocratically eluted at a flow rate of 1.0 mL/min and detection was achieved at 260 nm using a photodiode array detector. Artemisinin was identified by comparing the retention times with that of standard solutions while peak areas were used for quantification.

Data analysis

Data was analyzed with SPSS 17.0 for windows. The mean and standard deviation of means were calculated and one-way analysis of variance (ANOVA) was used for statistical differences with Duncan's multiple range tests used to separate means ($P < 0.05$).

RESULTS AND DISCUSSIONS

Nutrient levels in soil growing cultivars of A3

The concentrations of Zn and B in soil at depths of 10-15cm and 40-50 cm from Kajulu, Maseno, Ingidi, Nyakach and Asembo are presented in table 1. Those of NH_4^+ and NO_3^- are given in table 2.

Table 1: Levels of Zn and B (µg/g) at two depths of soils growing A3 cultivars in five regions of Western Kenya

	Zn Mean±SD (n=3)			Boron Mean±SD (n=3)		
Area	10-15cm depth	40-50cm depth	p-values	10-15cm depth	40-50cm depth	p-values
Kajulu	49.13±0.42^a	77.07±1.15^b	0.414	347.3±23.4	135.0±30.4^a	0.201
Maseno	108.80±0.69^c	94.73±0.6^e	<0.001	ND	187.3±41.0^c	-
Ingidi	116.40±0.69^d	79.53±1.01^c	<0.001	ND	192.7±24.4^d	-
Nyakach	79.60±0.72^b	91.60±0.69^d	0.641	ND	163.2±12.4^b	-
Asembo	110.00±0.80^c	72.47±0.42^a	<0.001	ND	196.5±72.8^e	-
p-values	<0.001	<0.001				

Same small letters within the same column are not significantly different (p>0.05, SNK test).

Table 2: Levels of NH_4^+ and NO_3^- (µg/ml) at two depths of soils growing A3 cultivars in five regions of Western Kenya

	NH_4^+ ion Mean±SD (n=3)			NO_3^- ion Mean±SD (n=3)		
Area	10-15cm depth	40-50cm depth	p-values	10-15cm depth	40-50cm depth	p-values
Kajulu	0.13±0.00^b	0.09±0.01^c	<0.001	0.34±0.01^b	0.34±0.09^a	0.243
Maseno	0.11±0.00^a	0.07±0.00^b	0.005	1.37±0.02^d	0.28±0.01^a	<0.001
Ingidi	0.18±0.01^d	0.23±0.00^d	0.876	0.42±0.02^c	0.37±0.02^a	0.665
Nyakach	0.19±0.00^d	0.01±0.00^a	<0.001	0.31±0.02^a	0.36±0.02^a	0.731
Asembo	0.16±0.01^c	0.10±0.00^c	<0.001	1.45±0.01^e	1.15±0.02^b	0.002
p-values	<0.001	<0.001		<0.001	<0.001	

Same small letters within the same column are not significantly different (p>0.05, SNK test).

There was no specific trend in levels of Zn observed for the top (10-15cm depth) and in- depth (40-50cm depth) soils (table 1). However, the levels were all above 50 µg/g, the minimum tolerable levels of Zn in the soil for maximum artemisinin accumulation in *A. annua* (Zhang *et al.*, 2004; Yekuan *et al*, 2010). As well, significant differences were generally noted between the regions as well as between depths ($p<0.05$). While the importance of Zn is known and increased artemisinin yield have been reported for extremely low levels, the concentration of Zn reported in this study could pose potential toxicity for the plant (Hopkins and Hüner, 2004; Khudsar *et al.*, 2004; Zhang *et al.*, 2004; Yekuan *et al*, 2010).

Boron in the topsoil (10-15 cm depth) was detected only at Kajulu while in the indepth soils the range was 135-196 µg/g (table 1). However, in all the five regions of study B increased with depth and was found to be above 2.5 µg/g, the minimum for plant requirement. Although its role in plant nutrition is still not very well known evidence of a linear relationship between B and artemisinin content has been reported (Zhang *et al.*, 2004; Yekuan *et al*, 2010). Contrary to the levels being very low in the top-soil, those in the in-depth soil are described as being very high. The explanation may not be deduced from this study but could be attributed to leaching (Khudsar *et al*., 2004; Zhang *et al*., 2004; Yekuan *et al*, 2010; Aftab *et al*, 2010).

The concentration of NH_4^+ ions in the top and in-depth soil was found to range between 0.11-0.19 µg/ml and 0.01-0.23 µg/ml respectively (table 2). Generally, the concentration of NH_4^+ ions in the top soil was higher than that in soils at in-depth. There were significance differences in the levels of NH_4^+ ions in the soil across the regions and also between depths for soils in Kajulu, Nyakach and Asembo ($p<0.05$). The levels found in both top-soil and in-depth are described as sufficient since NH_4^+ is tolerated by plants in small amounts, and can be toxic at higher levels (Liu *et al*., 2003).

There was no specific pattern on the concentrations of NO_3^- ions in the soils. A range of 0.31-1.45 µg/g and 0.28-1.15 µg/g was found for top and in-depth soils respectively. These were found to differ significant across the regions although between depths only soils from Maseno showed significant differences ($p<0.05$). NO_3^- ions however are not toxic and they have been associated with increased artemisinin production (Liu *et al*., 2003). The ratio of NO_3^- : NH_4^+ was high and would therefore lead to an increase in artemisinin content (Liu *et al*., 2003; Wang and Tan, 2002).

Artemisinin in leaves of A. annua cultivars

The mean content of artemisinin in leaves of *A. annua* from Kajulu, Maseno, Ingidi, Nyakach and Asembo are given in table 3.

Table 3: Mean percent content (DM) of artemisinin in leaves of A3 cultivars in five regions of Western Kenya

Region	Cultivar %DW (Mean±SD, n=3)	Wildings %DW (Mean±SD, n=3)	p-values
Kajulu	0.27 ± 0.01^{b}	0.25 ± 0.00^{b}	0.116
Maseno	0.04 ± 0.00^{a}	0.25 ± 0.00^{b}	<0.001
Ingidi	0.46 ± 0.02^{c}	0.08 ± 0.01^{a}	<0.001
Nyakach	0.88 ± 0.02^{d}	0.90 ± 0.03^{c}	0.370
Asembo	0.04 ± 0.00^{a}	0.12 ± 0.02^{d}	0.003
p-values	<0.001	<0.001	

Same small letters within the same column are not significantly different (p>0.05, SNK test).

Artemisinin in leaves of the A3 clone cultivars ranged between 0.04-0.88% DM. There were significant differences between the levels of artemisin between the cultivars and the wildings at maseno and Ingidi ($p<0.05$). However, between the regions, the leaves differed significantly in their artemisin levels ($p<0.05$). This can be attributed to differences in soil nutrient composition of Zn, B, NH_4^+ and NO_3^- (Wang and Tan, 2002). Except in Nyakach, the leaves from the other four regions studied contained levels that were lower than the commercially viable levels of artemisinin, being above 0.6% DW (EABL, 2005; Abdin *et al*., 2003). In our unpublished work, flowers had abundant artemisinin as would be expected since there is strong evidence that artemisinin is produced in the glandular trichomes which are found in flowers (Covello, 2008).

Artemisinin levels had negative correlation with the levels of nutrients in the soils ($r>-0.800$). While these findings agree with some previous studies, others have reported positive correlation of artemisinin with soil nutrients (Khudsar *et al.*, 2004; Zhang *et al*., 2004; Yekuan *et al*. 2010; Aftab *et al*., 2010). For NH_4^+ and NO_3^-, the correlation was found to be positive with artemisinin levels ($r>0.800$) in the top-soil.

CONCLUSIONS

The levels of artemisinin in leaves of *A. annua* grown in Western region of Kenya can be improved if nutrient levels are well managed. These findings showcase the need to expand cultivation of A. *annua* in Western Kenya and consequently production of artemisinin from flowers of *A. annua* in this region.

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