Standardisation of *Artemisia annua* using Reversed Phase High Performance Liquid Chromatography (RP-HPLC).

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Abstract

Artemisia annua L is a traditional Chinese medicine for the treatment of many disorders including drug resistant malaria. The active constituent against malaria, artemisinin, has been isolated and characterized by Chinese scientists. *Artemisia annua* is now being grown in malaria endemic countries like Nigeria. The aim of the present study was to quantify artemisinin content of Nigerian Grown *Artemisia annua* using a fast and reliable Reversed Phase-High Performance Liquid Chromatography (RP-HPLC) analytical technique since artemisinin content is affected by geographical and seasonal variations. HPLC conditions used for determination of the artemisinin content were established as follows; phenomenex luna column (5 µm; 250 mm × 4.6 mm) was employed with the mobile phase of acetic acid (% 0.1 v/v): acetonitrile: H_2O (70:30) mixture at the flow rate of 1 ml/min. The good linearity of artemisinin was observed with y = 11714 × (r^2 = 0.9989). Artemisinin was detected in our sample and was calculated to be 1.0975 %.

Keywords: *Artemisia annua* L., Artemisinin, HPLC, Standardisation

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INTRODUCTION

Approximately two billion people live in malaria endemic areas. The incidence of this disease is increasing dramatically because malaria parasite strains have become resistant to the available drugs. [1]

Artemisinin has been used in traditional Chinese herbal fever remedies for more than 1,500 years.[2] It is isolated from sweet wormwood (*Artemisia annua*), and its skeleton is used as the basis for the synthesis of several other modern antimalarial drugs, such as artesunate and artemether. These artemisinin derivatives are important in the treatment of drug-resistant malaria. They are presently the most potent antimalarials available, rapidly killing all blood stages of the malaria parasite *Plasmodium falciparum*.[3]

Nurgun, *et al*. 2007,[4] reported a number of analytical methods that have been developed for detection and quantification of artemisinin, e.g., thin layer chromatography (TLC), TLC with visible light densitometric detection, high-performance liquid chromatography with UV detection (HPLC–UV), HPLC with electrochemical detection (HPLC–ECD), HPLC with evaporative light scattering detection (HPLC–ELSD), HPLC with peroxyoxalate chemiluminescence detection (HPLC-PO-CL), HPLC/tandem mass spectrometric (LC/MS/MS) method, liquid chromatography/mass spectrometry (LC/MS), high performance capillary electrophoresis using self-designed conductivity detection system, gas chromatography with mass spectrometric detection (GC–MS), GC with flame ionization detection (GC–FID), enzyme-linked immunosorbant assay

(ELISA) and Reversed Phase High Performance Liquid Chromatography.

An international agreement that will give traditional medicines a foothold in health systems have been endorsed by the World Health Organization (WHO). Member states of the United Nations have been called to formulate policies to ensure the safe and effective use of traditional medicines. The current challenge of researchers around the world is the difficulty in standardizing the active ingredients as these are often present in plant extracts that have regional or seasonal variations. Because of this, drug regulatory agencies around the world have hesitated in approving their use.[5] There is need therefore to begin to standardize the active constituents in medicinal plants grown in different parts of the world.

Artemisinin levels vary in amounts in naturally growing *Artemisia annua*. Artemisinin level in the leaves and flowering parts of the plant is affected by growth conditions, seasonal and geographical variations as well as breeding. [6] *Artemisia annua* in natural habitats contain 0.06-0.5 % artemisinin. Breeding has however made it possible to achieve artemisinin content of up to 2 %.[7]

The demand for Artemisinin is presently very high, and this has made producers to push up the price.[8] This global demand has made different countries of the world like Nigeria, for example to begin to cultivate the plant in large quantity for the local production of artemisinin in order to make the artemisinin based *combination* therapy affordable. Although the synthesis of artemisinin has been achieved, the cultivation of the plant is considered more economical than the synthetic alternative.[9–10]

In this present study, we examined the level of artemisinin in *A. annua* grown in Nigeria. This attempt was to standardise Nigerian grown *A. annua* with respect to artemisinin using reversed phase-high performance liquid chromatography (RP-HPLC) analytical technique.

MATERIAL AND METHODS

Chemicals and reagents

Standard artemisinin was purchased from Sigma-Aldrich Co. (USA). All the solvents used were of HPLC analytical-grade.

Preparation of Standard Solutions

100 mg of standard artemisinin was placed in a measuring flask and solubilised with 95 % ethanol which was added to the 100 ml mark. Serial dilutions of the stock solution were prepared as follows: An aliquot (2 ml) of artemisinin solution was transferred into 100 ml measuring flask and 8 ml of 95 % ethanol was added. This was labeled as "Solution-1". Similarly, "Solution-2" was made with 5 ml of artemisinin solution and 5 ml of 95 % ethanol and "Solution-3" was prepared with 10 ml of artemisinin solution alone without ethanol. This was followed by the addition of 40 ml of 0.2 % NaOH solution to the three flasks respectively, and then, allowed to react at 50 °C for 30 min.

After that, 0.08 mol/L acetic acid solution was filled up to the mark. Three standard solutions were prepared and applied to HPLC.[4]

Sample preparation and calibration curves
Plant Materials

Artemisia annua plant was obtained from the Molecular Biosciences Ltd, Calabar Cross River State, Nigeria where the plant was cultivated in 2008. The plant was identified at the Department of Botany of the University of Calabar, Cross River State, Nigeria.

Extraction procedure

Five grams of *Artemisia annua* plant was weighed accurately in triplicates and macerated in 250 ml of *n*-hexane at room temperature for 2 days using a laboratory-scale shaker. The *n*-hexane phase was filtrated and evaporated under vacuum until dryness. The residue was dissolved again in 100 ml of *n*-hexane and the *n*-hexane phase was washed in a separatory funnel with 2 % NaOH solution to get rid of impurities soluble in NaOH. The alkali solution present in the lower layer was discarded and the upper layer solution was washed several times with distilled water until it was neutralized. This solution was evaporated to dryness under vacuum at 45 °C using a rotary evaporator.

The extract, obtained after distillation was dissolved in 250 ml of 95 % ethanol and then filtered into a measuring flask. Then, 10 ml of filtered liquor was transferred into a 100 ml measuring flask, and 40 ml of 0.2 % NaOH solution was added to the flask and allowed to react at 50 °C for 30 min after which 0.08 mol/L acetic acid solution was added to fill up to the mark. [4]

Chromatography Equipment

The analysis was carried out with an LC system consisting of an HPLC Waters series quaternary pump with degasser and a photodiode array detector. Samples were injected with an HPLC Waters Autosamplers with thermostatted column compartment on an a phenomenex column (5 µm; 250 mm × 4.6 mm), at 30 °C. The system was controlled and data analyses were performed with Waters Empower 2 software. All the calculations concerning

the quantitative analysis were performed with external standardization by measurement of peak areas.

Calibration Solutions

In order to establish the linear detection range for each compound, individual standard stock solutions was prepared in mobile phase in 100 ml-measuring flasks. Aliquots of these solutions were diluted and analyzed to determine method linearity. Calibration ranges for artemisinin 5 – 100 ppm were prepared. Triplicate 10 µL injections were made for each standard solution to see the reproducibility of the detector response at each concentration level. The peak area was plotted against the concentration to obtain the calibration graph. The five concentrations were subjected to regression analysis to calculate calibration equation.

Procedure for HPLC Analysis

A mobile phase consisting of acetic acid (% 0.1 v/v): acetonitrile: H_2O (70:30) by isocratic elution was chosen to achieve maximum separation and sensitivity. Flow rate was 1.0 ml/min. Column temperature was set at 30 °C. The samples were detected at 254 nm using photodiode array detector. Result of artemisinin level in *Artemisia* sample was expressed as the mean of three determinations.

Linearity

Equation of the regression line formula was y = 11714x; R^2 = 0.9989 for artemisinin.

RESULTS AND DISCUSSION

Analytical data were obtained when artemisinin standard concentrations of 5, 10, 20, 50 and 100 ppm were injected into the HPLC system. For each of the standard concentrations, triplicate injections were utilized. The mean peak areas for 5, 10, 20, 50 and 100 ppm were 30596, 109160, 242947.7, 583807 and 11772787 respectively Table 1.0. The peak areas were plotted against the concentrations to give the calibration curve in Figure 1.0. The linear regression equation was y=11714 and r^2 = 0.9989 for artemisinin. Excellent linearity was obtained for artemisinin between peak areas and concentrations. The standard curve was used to estimate the artemisinin content of *Artemisia annua* extract.

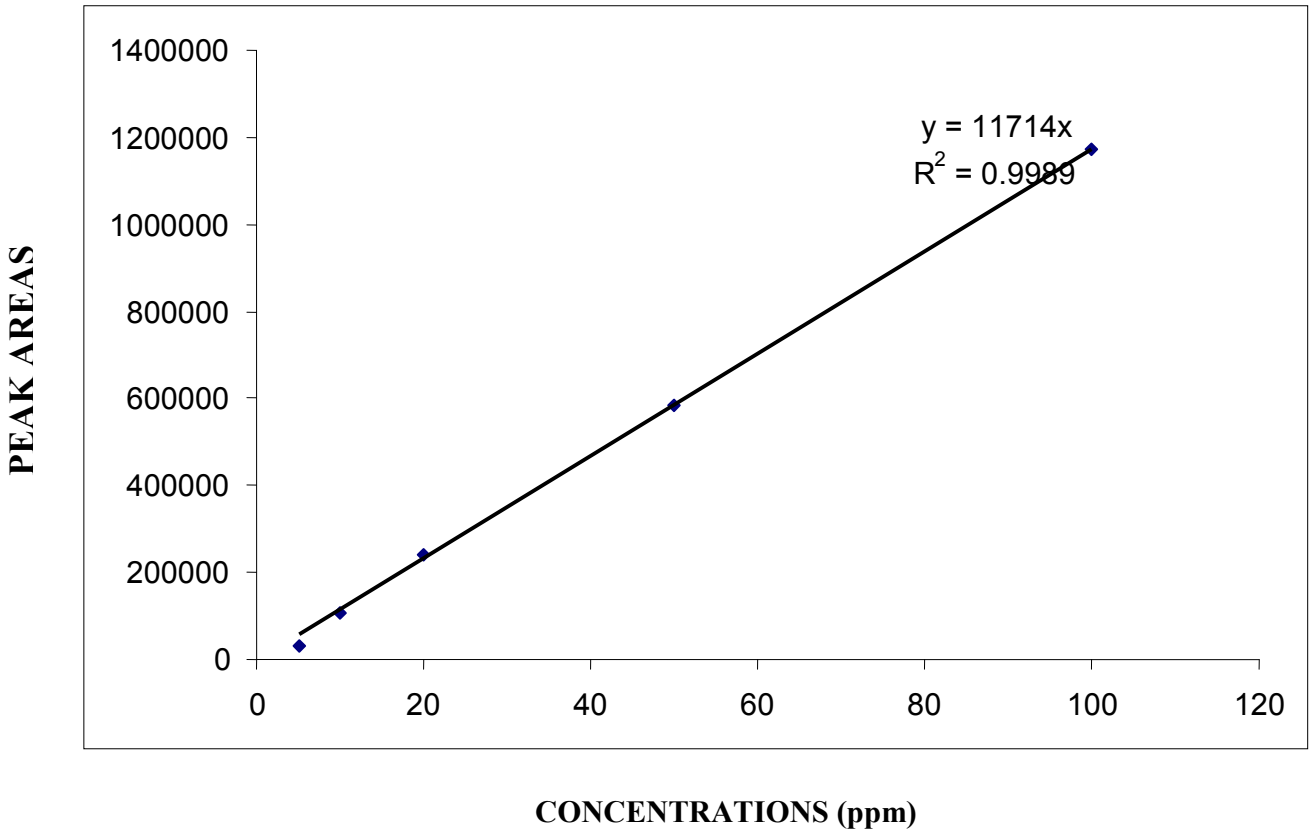


Figure 1: Calibration curve for the determination of artemisinin in Artemisia annua extract solution.

TABLE 1: ANALYSTICAL DATA OBTAINED FROM INJECTION OF ARTEMISININ STANDARDS UTILISED TO PLOT CALIBRATION CURVE

CONCENTRATIONS (ppm>	5	10	20	50	100
PEAK AREAS	30596	109160	243557	577306	1173799
PEAK AREAS	30596	109160	242643	586689	1172806
PEAK AREAS	30596	109160	242643	587426	1171757
PEAK AREAS	30596	109160	242947.7	583807	1172787

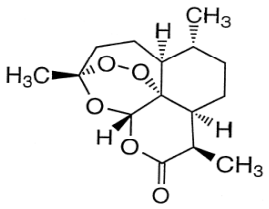
TABLE 2: PEAK AREAS OFARTEMISININ IN *ARTEMISLA ANNUA* EXTRACT

	ARTEMISIA EXTRACT 1	ARTEMISIA EXTRACT 2	ARTEMISIA EXTRACT 3
AREA 1	26350	25236	25578
AREA 2	24449	24512	23683
AREA 3	25409	24526	26337
AREA 4	27342	27368	27614
AREA 5	26837	27014	26737
AREA 6	26681	26142	26920
AREA 7	27931	26617	26841
AREA 8	27806	26762	27163
AREA 9	28737	26462	27973
MEAN	**26838**	**26071**	**26538.44444**

TABLE 3: DETERMINATION OF ARTEMISININ CONCENTRATION IN *ARTEMISIA ANNUA* USING THE CALIBRATION CURVE. (y=11714x; R^2=0.9989)

ARTEMISIA	MEANS OF PEAK	CONCENTRATION
EXTRACT 1	26838	2.29
EXTRACT 2	26071	2.23
EXTRACT 3	26538.44	2.27
MEAN		**2.26**

The peak areas shown in Table 2.0 are the areas under the elution profile of triplicate samples of *Artemisia annua* extract solutions 1, 2 and 3 injected nine times each into the HPLC system. The mean values of these areas were 26838, 26071 and 26538.4 respectively. The



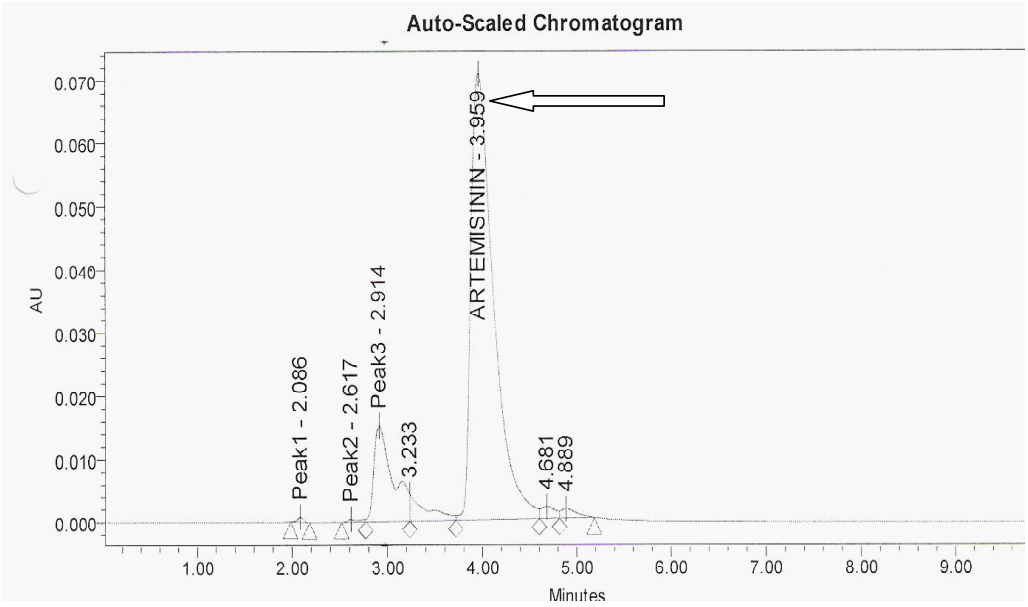


Figure 2: *Representative chromatogram of three determinations of standard solution of atremisinin* (RT=3.959). Peak area: 577306.

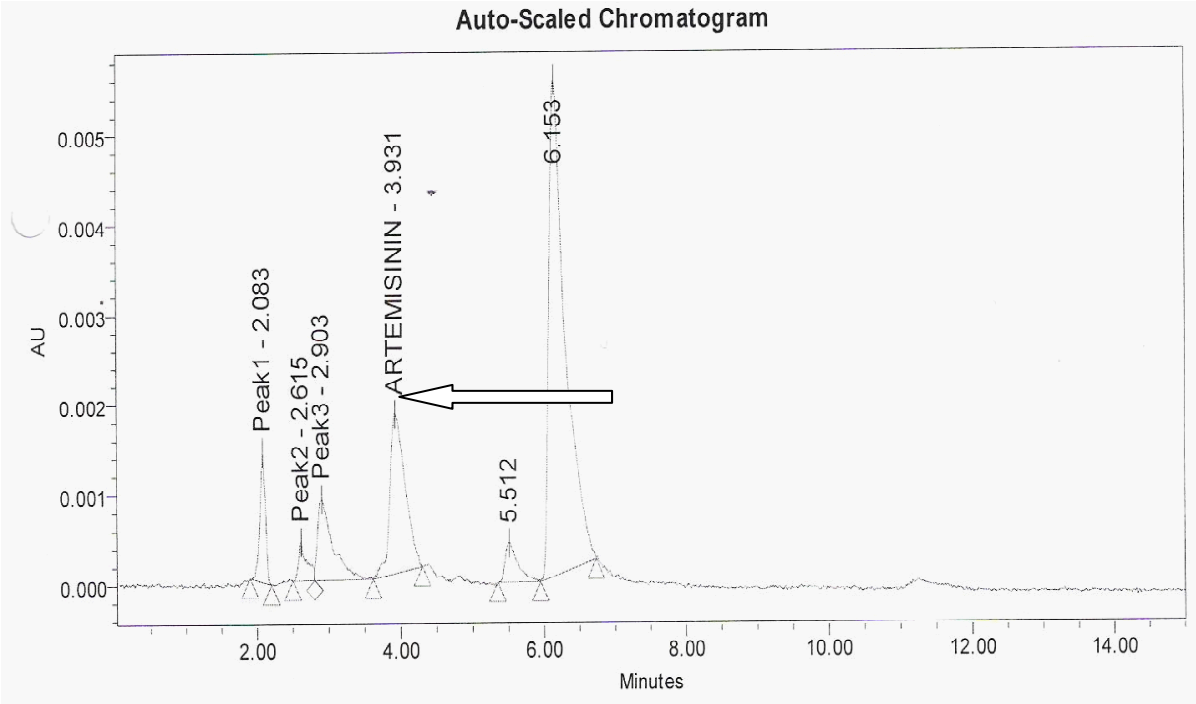


Figure 3: *Representative chromatogram of nine determinations of Artemisia annua triplicate extract solution* (RT=3.931). Peak area: 24526.

Table 4: PEAK AREAS AND CONCENTRATIONS DURING SPIKING

	***ARTEMISIA ANNUA* EXTRACT*+STANDARD ARTEMISININ**	***ARTEMISIA ANNUA* EXTRACT**+STANDARD ARTEMISININ**	***ARTEMISIA ANNUA* EXTRACT***+STANDARD ARTEMISININ**
EA1	79974	170766	350711
EA2	80701	169059	352537
EA3	78772	170766	352278
PEAK ***S ***ATIONS	79815.67	170197	351842
6.813698708	1452936657	30.03602527	

* 100µL of PLANT EXTRACT SPIKED WITH 300µl of 20ppm STANDARD ARTEMISININ.
** 100µL of PLANT EXTRACT SPIKED WITH 300µl of 50ppm STANDARD ARTEMISININ.
*** 100µL of PLANT EXTRACT SPIKED WITH 300µl of 1000ppm STANDARD ARTEMISININ.

corresponding concentrations were extrapolated from the calibration curve to give concentrations of 2.29, 2.23 and 2.27 respectively. The mean concentration of the triplicate samples of *Artemisia annua* extract solutions was 2.26 ppm (Table 3.0).

In order to be sure that the analysis was reproducible, accurate and reliable, recovery analysis was carried out using spiking procedure shown in Table 4.0 and recovery analysis data shown in Table 5.0. Recovery of extract 1, 2 and 3 were 99.1, 105.3 and 118.55 % respectively after spiking. Representative portions of the chromatograms of artemisinin standard and *Artemisia annua* extract solutions were as shown in Figures 2 and 3 respectively. The amount of artemisinin present in the Nigerian grown *Artemisia annua* was calculated to be 1.0975 %.

The search for artemisinin, the active antimalaria constituent of *Artemisia annua* is in progress. Synthetic alternative is not economical because it gives low yields. The only economical procedure is the cultivation of the plant. Since the discovery of the plant, different countries of the world have naturalized the plant. The aim is to make the plant available locally so as to drastically increase the amount of artemisinin used in the manufacture of artemisinin based combination therapy and to bring down the cost.[10–11] There is need to standardize the *Artemisia annua* cultivated in different parts of the world

Table 5: ANALYTICAL DATA OBTAINED FROM RECOVERY ANALYSIS

CONCENTRAT ION DETERMINED IN THE PLANT SAMPLE (ppm)	CONCENTRAT ION ADDED TO SAMPLE (SPIKING)(ppm)	EXPECTED CONCENTRAT ION (ppm)	OBTAINED CONCENTRAT ION (ppm)	RECOVERY (%)
2.26	4.615	6.875	6.814	99.10
2.26	11.538	13.798	14.529	105.30
2.26	23.076	25.336	30.036	118.55

because its levels is affected by the plant part collected, breeding methods, seasonal and geographical variations as well as growth conditions.[10] This standardization will give an idea of the amount of artemisinin to be expected before going on a large scale production. *A. annua* grown in the natural habitats contain 0.06–0.5 % artemisinin, but breeding yielded artemisinin content of 2 %. The levels also vary with geographical, seasonal as well as growth conditions as mentioned above. Artemisinin content of sixteen seed-generated lines of the cultivar *A. annua* ranged from 0.2 % to 0.9 % by both GC-FID and HPLC-ELSD.[12] HPLC analysis of artemisinin in *Artemisia annua* herb yielded 0.652 % by RH-HPLC method. [13]

Different solvents such dichloromethane, hexane, toluene, petroleum ether and chloroform can be used for the extraction of artemisinin from *Artemisia annua*. [11–15] In our study, n-Hexane was used because it is more selective in isolating artemisinin.[6] The calculated content of artemisinin in the Nigerian grown sample (1.0975 %) is comparable with the levels in other countries.

CONCLUSION

As Nigeria is embarking on a large scale production of the plant, the local production of artemisinin is a good development because the cost of production of artemisinin based combination therapy will be reduced compared with the past. The synthetic option is not presently feasible in Nigeria because it is not economical. The large vast of land available in Nigeria if well utilized, will make Nigeria to be a good source of artemisinin which is now a global remedy for the treatment of severe malaria.

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