

Evaluation of the artemisinin content of *Artemisia annua* L. grown in different Agro ecological zones of Cameroon

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Abstract

Artemisinin and its derivatives are potent antimalarials that have become essential components of Artemisinin Based Combination Therapies (ACTs) for malaria treatment. Production of artemisinin is less compared to its world demand and access to ACTs is still limited in worst hit countries. Secondary metabolite production in plants depends on a number of factors including agro ecology. This work sought to evaluate the growth characteristics and artemisinin content of *Artemisia annua* grown in four agro ecological zones of Cameroon. Experimental fields comprising of *A annua* were set up in the different agro-ecological zones, for growth and artemisinin assessments. Artemisinin detection and quantification was compared in leaves/flowers and stems of *Artemisia annua* from these agro-ecological zones, using High-Performance Liquid Chromatography. Soil provenance and other agro ecological factors significantly affected the growth parameters of *A. annua* as there were significant differences ($p < 0.05$) in plant height, leaf area, number of branches, stem girth and number of leaves across the different study areas. There was significant variation ($P < 0.05$) in the content of artemisinin from the plant in the different agro ecological zones. The mean levels of artemisinin in the samples was 0.34-0.80% in stem samples, and 0.18-0.61% for leaves/flowers. The highest artemisinin yield in leaf/flowers samples (0.61%) was recorded in samples from Bamenda, while that of stem samples (0.80%) was recorded in samples from Ngaoundere. The study confirms the effect of agro ecological factors on artemisinin content of *A. annua* in Cameroon and reveal that *A. annua* grown in most regions of the country contain commercially viable levels of artemisinin which can further be exploited for pharmaceutical purposes.

Keywords: Artemisinin; Content; *Artemisia annua*; Agro ecological zones; Cameroon

1. Introduction

Plants continue to be a major source of medicines in the maintenance of human health throughout the world and notably in the tropics. Over 50% of prescription drugs are derived from chemicals first identified in plants [1]. Interest in medicinal plants is becoming more recognized in health care delivery particularly in developing countries because they are affordable, readily accepted by consumers and locally available [2; 3]. It is estimated that 80% of the people worldwide depend on traditional medicine to meet their primary health care needs [4].

Malaria epidemic remains one of the most common diseases plaguing low socioeconomic empowered regions with a great morbidity and mortality than any other infectious disease in the world [5]. Global trends in burden of malaria cases and deaths according to report published by World Malaria in 2021, showed that there were 241 million cases of malaria in 2020 with an estimated dead toll of 627000. African regions continue to carry a disproportionately high share of the global malaria burden [6]. According to WHO [7], Cameroon is among the 15 highest burden malaria countries and had 3% of all global malaria cases in 2018, representing, 3rd highest number of malaria cases in Central Africa (12.7% of cases). Close to one million clinical cases of malaria occur annually in Cameroon, accounting for more than

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50% morbidity among children under age five, 40 to 45% of medical consultations, and 30 to 47% of hospitalizations [8].

A number of control measures employed against malaria have their weaknesses. For instance, some strains of the malaria parasite have developed resistance to traditional treatments using quinine and chloroquine, which were previously effective [9]. The World Health Organization (WHO) has recommended the use of Artemisinin Based Combination treatments (ACT) such as artemether-lumefantrine, Sulfadoxine/pyrimethamine as the first line treatments for multidrug – resistance strains of malaria (artesunate-mefloquine, artesunate-amodiaquine, and artesunate-sulfadoxine) [7]. Artemisinin has also been demonstrated to be effective against some other parasites including *Leishmania*, *Schistosoma*, *Toxoplasma* and *Trypanosoma* [10]. It also has antiviral activities [11] and can be used in treatment of hepatitis B [12] and a range of cancer cell lines, including breast cancer, human leukaemia, colon, and small-cell lung carcinomas [13]

The world market for products including artemisinin derivatives is now growing rapidly, and the demand for artemisinin is increasing. *Artemisia annua* (L.) (Asteraceae), is the major source of artemisinin, a sesquiterpene lactone, synthesized in the glandular trichomes of the aerial parts of the plant and used as the raw material for the production of semi-synthetic derivatives that are more stable, bioavailable, and effective against chloroquine-resistant strains of *Plasmodium falciparum* [14]. These diverse pharmacological activities, are due to the presence of certain chemical constituents such as terpenoids, coumarins, flavonoids, polyphenols and volatile compounds [15]. The plant is currently processed by pharmaceutical firms for the production of artemisinin and its derivatives for Artemisinin-Based Combination therapies (ACTs) in the treatment of malaria [15]. They have the advantage over other drugs in having an ability to kill faster and kill all the life cycle stages of the parasites. The high efficacy, fast-action and low-toxicity of artemisinin-based combination therapy (ACT) is recommended by World Health Organization (WHO) as the best choice for acute malaria [16]. Research has shown that ACT has a cure rate of up to 97% against severe falciparum malaria [17; 18]. WHO has encouraged the local cultivation and the use of *A. annua* in the world [19]. This plant has therefore been grown in many countries such as Brazil, Ethiopia, India, Kenya, Mozambique, Tanzania, Thailand, Uganda, Zambia etc. [20].

Cultivation of *Artemisia annua* in Cameroon, though still on a small-scale cuts across several regions (Northwest, Centre, littoral, Adamawa, Southwest and West) under the control of some organizations such as CIPCRE (International Circle for the Promotion and Creation) which provide the high-quality seeds from MEDIPLANT (Research Center of aromatic plants Conthey-Switzerland). *Artemisia annua* is gradually becoming a garden plant, as many households now cultivate it as a cost-effective, handy, acceptable, and scientifically proven treatment for malaria in Cameroon.

However, environmental conditions, play a key role in the synthesis of artemisinin in *Artemisia annua* [21]. Comparative assessment through GC-MS analysis showed that *A. annua* collected from different localities with similar climatic conditions of the Grass-field Regions of Cameroon produce more concentrated artemisinin than those from temperate regions and similar to those from other tropical countries [22]. Factors such as soil nutrient content and humidity [23], salinity stress [24], irradiation, water stress, elicitation and the age of the plant [10] highly influence the artemisinin content of the plant. This study sought to evaluate the artemisinin content of *Artemisia annua* grown in four agro ecological zones of Cameroon.

2. Methods

Seeds sourced from 'Artemisia Cameroun' were raised in the nursery and in the field established in each of the four agro ecological zones (**Table 1**); High Guinea Savannah, Western highlands, Humid forest with monomodal rainfall, and Humid forest with bimodal rainfall. Seeds were broadcasted in two plastic pots of sizes 40 by 20 cm. Each pot contained 10 kg medium composed of sieved fine soil and coarse sand. Upon sowing, they were set under semi shaded areas as described by Ferreira *et al.* [25]. At a height of 2-3cm (4-5 true leaves and 30 days of germination), forty seedlings were picked out and each transplanted into 10 cm × 12cm polythene bags filled with 5kg of fine soil mixed with 10 g of sand. These seedlings were then maintained until they were 8 weeks (10 true leaves), then hardened off.

An area of 5 m by 3 m (15 m²) was slashed and ploughed to fine tilth in each selected site. 200g of composite soil from each site was collected and prepared following scientific procedures [28], then sent for physical and chemical analysis at the Soil Science laboratory of the University of Dschang- Cameroon. Flat topped beds of sizes 4 m long, 2.5m wide and 20 cm high were established, one in each study area. Seedlings from the nursery were transplanted at an inter and intra row spacing of 60 cm × 50cm respectively, 15cm from the edge to give a seedling density of 35 plants per bed.

Table 1 Precipitation, Elevation, Temperature Range and Soil Types of Agro ecological Zones of Cameroon

Agroecological Zones	Rain fall (mm)	Elevation (m.a.s.l.)	Mean annual temperature (range)	Soil types
Sudano-Guinean High savannah zone (Zone II)	1500-1800	500-1500	23°C (6.4)	Nitisols, Ferralsols, Acrisols, Luvisols, Leptosols, and Cambisols.
Western Highlands zone (Zone III)	1800-2400	1500-2500	21°C (2.2)	Ferralsols, Nitisols, Gleysols, Andosols, Cambisols, and Leptosols.
Monomodal rainfall Forest zone (Zone IV)	2000-5000	0-500	26°C (2.8)	Ferralsols, Lateritic soils and Volcanic soils.
Bimodal rainfall forest zone (Zone V)	1500-2000	400-1000	25°C (2.4)	Ferralsols, Nitisols, Acrisols, Gleysols, Fluvisols, and Andosols.

Source: [26; 27]

Four weeks after transplanting, ten plants were randomly selected and tagged in each study area. Plant height, leaf area, number of branches per plant, stem girth and the number of leaves per plant were recorded and continued at a 1 month interval until the onset of flowering (120 days after transplanting to the field). At this time, all the ten tagged plants from each study site were harvested and partitioned into different parts: leaves, flowers and stems. A total of 24 samples were prepared, four samples each comprising of a mixture of leaves and flowers from each study area, and four samples each of stem materials only taken from two of the study sites (Nkwen-Bamenda and Wakwa-Ngaoundere). This was done in order to confirm or deny the results of the study carried out by Ferreira [29] and Mannan *et al.* [10] which showed that there was little or no artemisinin found in the stems of artemisia. The fresh samples were then dried under the sun for two weeks, ground to fine powder using a blender and sieved. The different samples were then taken for artemisinin determination at the Institut de Recherche Medicales et d'études des Plantes Medicinales (IMPM) Yaoundé. Standard artemisinin with purity of 98% was purchased from Sigma-Aldrich Chemical Company. It was used as an external standard to draw the calibration curve. Solvents used for extraction and HPLC analysis (Naoh, Hexane, Dichloromethane and acetronitrile) were of HPLC grade, purchased from MERCK Company. Formic acid was gotten from Sharlar Company. Ultrapure double-distilled water was used throughout the experiment. All other chemicals were analytical reagent grade.

100g each of ground leaves/flower and stem samples from the different study sites were lyophilized and their dry matter measured. The samples were then extensively extracted at room temperature by maceration with 100 ml of hexane, for 72 h. The eluates were subsequently evaporated under vacuum to obtain the crude extracts. Five milligrams of the hexane extracts of each sample were accurately weighed and suspended in acetonitrile (1.0 ml) in a volumetric flask. The suspensions were sonicated for 20 min. for HPLC analysis.

HPLC analysis was performed using a water 1525 pump with a Water 2995 Photodiode Array Detector, and a manual injector. Separations were performed on a SIL C.30 column, with particle size of 5 mm. The solvent system contained: water adjusted to pH 3.2 by formic acid (A), and acetonitrile (B). The mobile phase was isocratic, using 50% A and 50% B for 20 min at a flow rate of 1.3 ml/ min. The system was operated with oven temperature at 26 C and the injection volume was 20 ml. Before HPLC analysis, each sample was filtered through a cartridge-type sample filtration unit with a polytetrafluoroethylene (PTFE) membrane (d = 13 mm, porosity 0.45 mm) and injected immediately. Chromatograms were recorded at 210 nm, 270 nm, and 350nm to detect artemisinin and any other constituents. The artemisinin percent was calculated on the basis of the peak area using the calibration curve of authentic samples of artemisinin using the same conditions of samples analyses [30].

All data collected were entered into SPSS (2010) Statistical Software and subjected to one-way analysis of variance (One-way ANOVA). Means were separated using the Least Significant Difference (LSD), at 5%.

3. Results

The results of mean physico-chemical properties of soils at the various experimental sites of some essential soil elements are presented in **Table 2**. The pH of the soil samples from the four study sites ranged from moderately acidic

to very strongly acidic. The pH values were highest in soil samples from Yaounde (5.9) and least in Ngaoundere (4.9). The mean phosphate ion concentration in the soil was highest in soil samples from Bamenda with a value of 153.40mg/kg and least in soil samples from Buea (30.98mg/kg). Nitrogen content in the soils from the different experimental sites was highest in Bamenda (0.39%) and least in Ngaoundere (0.14%).

Table 2 Physico-chemical properties of soils at the experimental sites of four agro-ecological zones in Cameroon

Parameter	Buea	Bamenda	Ngaoundéré	Yaoundé
clay%	27	28	20	36
Silt%	19	24	28	14
Sand%	54	48	52	50
pH water	5.2	5.6	4.7	5.9
Nitrogen%	0.34	0.39	0.14	0.175
Organic carbon%	5.91	1.53	1.60	4.66
Organic matter %	10.19	2.64	2.76	8.04
C/N	17.60	3.98	11.43	26.63
Available phosphorus(mg/kg)	30.98	153.40	43.19	36.37
Calcium méq%	2.41	1.025	1.045	2.85
Magnesium méq%	1.24	0.89	0.75	1.55
Potassium méq%	1.84	0.87	0.98	1.54
Sodium méq%	0.01	0.02	0.01	0.03
Sum of exchangeable base méq%	5.5	2.81	2.79	5.97
Capacity exchange cation méq%	17.6	24.16	13.6	23.84
Base saturation (%)	31	12	20	25
Exchange acidity (méq%)	0.4	0.01	0.98	0.01

General increase in all vegetative growth parameters was observed, from 4WAP to the last week (16WAP) across the four study sites (**Table 3**). Records at 16WAP showed that plant height had significant differences ($P=0.013$) across the study sites with the highest obtained for plants in Buea (182.73 cm) while Ngaoundere recorded the least (137.21cm). The mean number of branches was equally significant ($P = 0.000$) across all study sites with the highest gotten in Buea (74) and the least in Yaounde (44). The highest number of leaves was obtained in Buea (317) while the least was obtained in Yaoundé (115). In terms of stem girth, Buea recorded the highest (1.69) while Ngaoundere recorded the list (0.88).

Table 3 Means of Vegetative Growth Parameters of *A. annua* Collected from Four Agro-ecological zones in Cameroon.

Parameters	Location	Weeks			
		4WAP	8WAP	12WAP	16WAP
Plant height (cm)	Yaoundé	49.63 ^g	69.40 ^g	93.62 ^g	144.55 ^g
	Bamenda	60.88 ^{gh}	81.18 ^{gh}	143.54 ^h	182.73 ^h
	Buea	63.38 ^h	90.94 ^h	138.65 ^h	175.10 ^{gh}
	Ngaoundéré	46.83 ^g	67.94 ^g	95.28 ^g	137.21 ^g
	F values	3.323	3.149	9.166	4.097

	Sig.	0.030	0.037	0	0.013
Stem girth (cm)	Yaoundé	0.55 ^d	0.80 ^{ef}	0.98 ^{ef}	1.07 ^d
	Bamenda	0.52 ^d	0.73 ^{de}	0.97 ^e	1.24 ^d
	Buea	0.62 ^d	0.97 ^f	1.32 ^f	1.69 ^e
	Ngaoundéré	0.36 ^e	0.52 ^d	0.71 ^d	0.88 ^d
	F values	4.026	5.843	4.378	6.900
	Sig.	0.014	0.002	0.010	0.001
N° of branches	Yaoundé	17.0 ^a	23.0 ^a	34.0 ^a	44.0 ^a
	Bamenda	24.0 ^b	39.0 ^c	55.0 ^b	71.0 ^c
	Buea	27.0 ^b	35.0 ^{bc}	44.0 ^a	74.0 ^c
	Ngaoundéré	20.0 ^{ab}	31.0 ^b	42.0 ^a	51.0 ^b
	F values	11.46	19.23	39.49	57.71
	Sig.	0	0	0	0
N° of leaves	Yaoundé	30.0 ^a	58.0 ^a	84.0 ^a	115.0 ^a
	Bamenda	50.0 ^b	95.0 ^{bc}	188.0 ^a	276.0 ^a
	Buea	57.0 ^{cb}	106.0 ^c	216.0 ^a	317.0 ^a
	Ngaoundéré	39.0 ^b	82.0 ^{abc}	131.0 ^a	170.0 ^a
	F values	11.63	10.65	0.83	0.87
	Sig.	0	0	0.48	0.46

The relative leaf area (cm²) was highest in Buea (160) and least in Bamenda (68) (**Fig 1**).

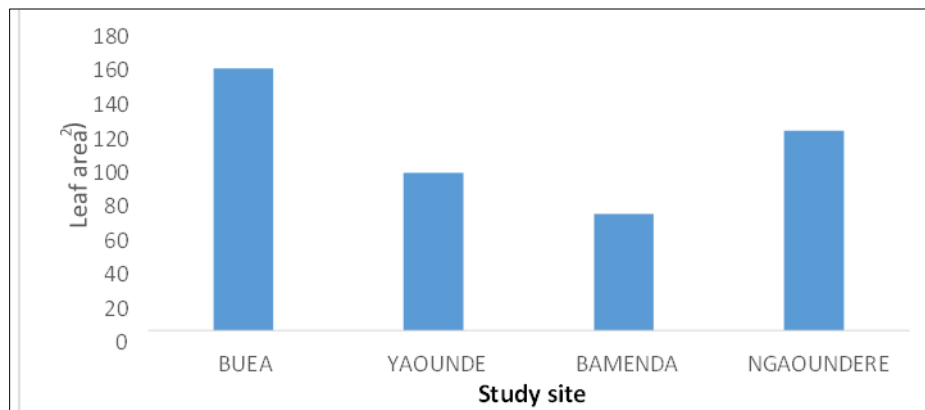


Figure 1 Variation in Leaf area (cm²) of *Artemisia annua* per study area

Overall, artemisinin detected in the plant was between 0.18% and 0.80%. The highest artemisinin from leaves/flowers was gotten in samples from Bamenda (0.61%) while the least was in Ngaoundere (0.18%). In the stems, the highest was in Ngaoundere while the least was in Bamenda (0.34%). No artemisinin was detected in plant samples from Buea. The mean levels of artemisinin in samples from the three sites where it was detected showed significant differences ($p < 0.05$, **Table 2**). Identification of artemisinin (sesquiterpene) on the TIC of the four extracts by peaks followed the following retention times: 7.213 min for samples from Bamenda, 7.214 min for samples from Ngaoundere and 7.211 min for samples from Yaounde.

Table 4 Mean levels of artemisinin in flowers/ leaves and stems/branches of *Artemisia annua* samples cultivated in four agro-ecological zones in Cameroon.

Study Site	Plant samples	Content of artemisinin on extract
Buea	Leaves/flower samples	Not detected
Yaoundé	Leaves/flower samples	0.52 ± 0.01 ^c
Ngaoundere	Leaves/flower samples	0.18 ± 0.01 ^d
Ngaoundéré	Stem samples only	0.80 ± 0.01 ^a
Bamenda	Leaves/flower samples	0.61 ± 0.01 ^b
Bamenda	Stem samples only	0.34 ± 0.02 ^e

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

4. Discussion

Across the study sites, the artemisinin levels in the plant samples were seen to vary significantly ($P < 0.05$). This was in accordance with studies carried out by Laughlin *et al.* [31] and Abdin *et al.* [32], who postulated that the artemisinin content in *A. annua* varies according to growth conditions, geographical orientation and climatic conditions. Production of some secondary metabolites can be induced by low soil or low plant tissue concentrations of nutrients [33] and this seems to be the case with this study. Studies carried out by Widiyastuti *et al.* [34] showed that high soil nitrogen content will increase above ground biomass production of *A. annua*. However, a similar study by Ferreira [29], showed that reduced nitrogen, as well as phosphorus and lime deficiency, caused an increase in artemisinin production while potassium deficiency or low soil potassium content caused a 75 % increase in artemisinin production (per biomass). Soils from Ngaoundere and Bamenda had the lowest soil potassium content, while soils of Ngaoundere had not only the least soil nitrogen content, but also very low phosphorus content. This could be associated to the high artemisinin content from plant samples grown in these two areas. Abiotic stresses like nutrient deficiencies cause oxidative stress, which in *A. annua* might cause rapid conversion of the precursors; artemisinic acid and dihydroartemisinic acid to artemisinin by activated oxygen species [24]. Moderate water deficiency over a short period of time (38 h) has also been shown to increase artemisinin production [35].

Findings of Thu *et al.* [21], showed that temperate weather induced significant levels of artemisinin in *A. annua* and correlated the high levels of artemisinin with the robustness of plants which grew in cold weather, which had heights and branch lengths that were 2-4 times taller or longer to those cultivated in areas with higher temperatures. This finding held true for plants grown in Bamenda, which were tall and had relatively high artemisinin content.

However, contrary to the above findings, no artemisinin was detected in plant samples from Buea. This result also contradicts the findings of Kumar *et al.* [36], which showed that the artemisinin content of the *Artemisia annua* plant, is positively related to plant height, stem base diameter, crown diameter, inter node length, and leaf length and further postulated that plant height, stem diameter, and primary branch angle are the main characteristics that contribute to the identification of high-value *A. annua*. Plants grown in Buea recorded the highest values for all the above growth parameters and its soils also recorded high values for most soil physico chemical properties, yet its plant samples lacked artemisinin. According to Mahan and Wanjura [37], Atkinson and Urwin [38], and Davies *et al.* [39], environmental stress propagates the production of secondary metabolites in plants. Environmental factors in Buea, permitted the healthy growth of *Artemisia annua* with no provision for environmental stress, possible reason why no artemisinin was produced.

Stem samples from Ngaoundere had the highest artemisinin content, while its leaves/flower samples had the overall lowest artemisinin content. This is contrary to studies carried out by Ferreira *et al.* [40] and Mannan *et al.* [10]; which showed that artemisinin is mostly found in the leaves, inflorescence and the seeds due to the presence of higher concentrations of glandular trichomes wherein artemisinin accumulates as the glands reach physiological maturity [41], with low levels in the stem and branches, and none in the pollen and the roots. However, this does not dispute the fact that the leaves and flowers do contain very high levels of artemisinin as there is strong evidence [42]. Nevertheless, this study has also shown that the stems of *Artemisia annua* are very valuable plant material with potentially high artemisinin content and should not be discarded during harvest.

5. Conclusion

Results from this study strongly indicate that the artemisinin content of *Artemisia annua*, is very much affected by agro ecological factors. Plant samples grown in the Sudano-Guinean high savanna and the Western Highland agro ecological zones contained relatively higher mean levels of artemisinin compared to the other zones. *Artemisia annua* grown in Cameroon contains artemisinin with a percentage above 0.6%, therefore the levels of artemisinin in plants grown in these areas are commercially viable and exploitable.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

No conflict of interest to be disclosed.

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