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Physicochemical Characteristics of *Artemisia annua*, an Antimalarial Plant from the Grass- field Regions of Cameroon

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Abstract

The aim of this study was to characterize the physical and chemical properties of *Artemisia annua*, an anti-malarial plant grown in the Grass-field Regions of Cameroon. Samples have been collected from seven localities with same climatic conditions. The GC-MS analysis of the extracts from the samples showed the presence of artemisinin, scopoletin and 13 volatile compounds including α-pinene, β-pinene, camphene, 3-carene, α-terpinene, limonene, eucalyptol, artemisia ketone, copaene, camphor, caryophylene, menthol and α-terpineol. When compared with *A. annua* grow in other countries, the Grass-field Regions of Cameroon produce seen more concentrated in artemisinin (11.1 mg/kg) than those from temperate regions [Luxemburg (0.4 mg/kg), Germany (0.8 mg/kg) and Belgium (1.1 mg/kg)] and similar to those from tropical countries [Brazil (11.5 mg/kg) and Democratic Republic of Congo (10.3 mg/kg)]. This result also showed that *A. annua* from the Grass-field Regions of Cameroon is highly concentrated in scopoletin, artemisinin and several other volatile compounds. The local production of this plant in Cameroon will be a source of medicinal significance for malaria zones and developing countries to fight against this disease.

Keywords: *Artemisia annua* (Asteraceae), Artemisinin, Scopoletin, Volatile Compounds, Anti-malaria, Grass-field Cameroon

Introduction

Malaria caused by *Plasmodium falciparum*, is one of the diseases responsible for many deaths each year in the world especially in Sub-Sahara Africa. The high mortality (1 to 2 million per year) is due to an increase in drug resistance of the parasite against conventional treatment such as chloroquin and amodiaquin [1]. Nevertheless, the new treatment with artemisinin derivatives has emerged these latest years. Artemisinin was isolated from *A. annua*, a plant of Chinese origin and which culture is in full extension all over the world. Pharmaceutical companies have extracted artemisinin from *A. annua* to manufacture the first-line ACTs (Artemisinin-based Combination Therapeutics) for the treatment of multidrug-resistant malaria [2].

However, these new drugs are very expensive and inaccessible to poor communities. From the malaria prevalence and the effectiveness of these new drugs [3], the WHO has encouraged the local cultivation and the use of *A. annua* in the world [4]. This plant has therefore been grown in many countries such as Brazil, Ethiopia, India, Kenya, Mozambique, Tanzania, Thailand, Uganda, Zambia etc [5]. From this trend, *A. annua* was introduced in Cameroon where their teas are used for the treatment of malaria.

A. annua, belonging to the Asteraceae family, is an aromatic, annual and perennial plant up to 1-3 m high and 1 m wide [6]. It is anti-malarial and has also shown some biological activities as anti-inflammatory, analgesic, antiseptic, antiviral and anticancer [7]. These diverse pharmacological activities, which make it an interesting plant, are due to the presence of certain chemical constituents such as terpenoids, coumarins, flavonoids, polyphenols and volatile compounds [8,9].

In our previous study, we showed that the teas of *A. annua* are clinically comparable to the results obtained by using the association of artesunate/amodiaquine for the treatment of malaria. We therefore acknowledge the cultivation of this plant in many regions in Cameroon (West, North-West and South-West) under the control of some organisms such as CIPCRE (International Circle for the Promotion and Creation) which provides the high quality seeds from MEDIPLANT (Research Center of aromatic plants Conthey-Switzerland).

However, the cultivation and the use of *A. annua* for the production of teas and artemisinin for the treatment of malaria must be tightly controlled because different studies have shown that the concentration of artemisinin is very sensitive to culture conditions, state of maturity, harvesting methods, transportation and extraction. This motivated our effort to continue the study of *A. annua* grown in the Grass-field Regions of Cameroon from which we have evaluated the physical, chemical and climatic properties for the good quality production of this antimalarial plant.

Materials and methods

1) Plant material collection

The leaves of *Artemisia annua* were collected before the flowering period by farmer's from the Grassfield (North-West and West) Regions of Cameroon in 2009 between June and December from 9 a.m. to 3 p.m.[2;10]. The main localities of collection were Bangang-Fokam, Bangangte, Bandjoun, Banengo-Bafoussam, Mbouda and Dschang (West); and Bafut (North-West). Identification of the plant sample was done by Mr. Nana, a botanist from Cameroon National Herbarium-Yaounde, where a specimen was deposited under a voucher number 65 647 HNC.

2) Culture environment

In these localities, the variation of the altitude is between 1544 and 2600 m with an average rainfall of 1800 mm spread over 175-220 days or nine months from late March to late November and more concentrated from July to September. Their temperature range is between 15-27 °C with peaks in some areas up to 37 °C. Their soils are all ferralitics and sometimes clayey, siliceous and volcanic derivatives.

3) Extraction procedure

The leaves were dried and ground into powder with a blender.

Four extraction methods were evaluated including the Accelerated Solvent Extraction (ASE), soxhlet, infusion with water and extraction with a mixture of water/hexane (1/1). Using 100 mg of the dried and powdered leaves with 100 ml of solvent tested each of these methods and finally the ASE was selected as the easiest method. The ASE (Dionex) was therefore, used for extracting 24 samples per run for a running time of 20 min with an extraction volume of 20 ml for each sample. The mixture of hexane/acetone (90/10v/v) was used as extracting solvent while the extraction temperature and pressure were 160°C and 1600 psi (*ca*. 110bar.) respectively.

4) Analytical methods

The identification and quantification of constituents from samples were carried out using the gas chromatography (6890 Network GC System ND of Agilent Technologies, 5973 Inert Mass Selective Detector of Agilent Technologies with Auer™ Toximeter II Pump). Samples were weighed with a precision balance Sartorius BP221S® and ground using a grinder Automatic Electric Retsch MM400®. Standard compounds such as artemisinin (purity > 98 %) from ROTH-Germany NurFür Laborzweckegeprüft, scopoletin (purity >99 %) from Sigma-Aldrich, alpha-pinene (purity >97 %), beta-pinene (purity >90 %), 3-carene (purity >98 %), limonene (purity >99 %),camphene (purity >95 %), camphor (purity >99 %), (trans-) caryophyllene (purity >98.5 %), copaene (purity >90 %), eucalyptol (purity >99 %), menthol (purity >98 %), α-terpinene (purity >90 %), α-terpineol (purity>97%) and artemisia ketone (purity>97%) from the laboratories of Sigma were used as reference standards during the analysis. These standard compounds and extracts were dissolved in analytical ethyl acetate in the concentration of 1 µg/µL and injected to the GC-MS to afford the total chromatogram ions (TIC) from which we were able to list the retention time and mass spectrum of each constituent. The retention times of the reference compounds were used to identify the constituents present in *A. annua* and to establish the calibration lines. Various data were considered with a view to validating one procedure for the determination of constituents of *A. annua*. The rate of the volatile compounds were determined by analyzing a mixture of its standards (MIX-STD) with the method of absorption/thermal desorption on GC and GC-MS while the artemisinin and scopoletin were evaluated by GC-MS accordingly to previous work in the domain [11; 12; 13]. The concentration of the constituentsin dried and ground leaves of our samples were given in mg/kg. The experimental conditions we used were developed by the National Health Laboratory, Luxembourg, following the procedures of thermal desorption technical support Note 67 [14; 15].

Results

Physical parameters

The sizes of the plants of our samples were 72 cm for the shorter and 310 cm for larger, with an average of 186 cm (**Table 1**, **Figure 1**). The collar diameter showed a variation between 6 and 63 mm with an average of 32 mm (**Table 2**, **Figure 2**). The best yield of artemisinin is shown in the samples harvested at the 8th months of age that corresponds to the appearance of the first flower bud (**Table 3**, **Figure 3**). This result is in accordance with those published by Delabays and Coworkers [16]. The morphological aspect of harvested samples showed that these plants had a bushy appearance with a lot of leaves and twigs (**Figure 4**).

Validation of the method of extraction and assay

The best extraction method was validated by performing various tests with samples from Bangangte (**Table 4**). For each of the extracts obtained, we analyzed the concentrations of artemisinin and scopoletin. The qualitative analysis allowed us to note that scopoletin has a retention time of 12.44 min with m/z = 192 and artemisinin

was represented by two major peaks with retention times 13.31 min (peak artemisinin 1) and of 14.04 min (peak artemisinin 2) with m/z = 166 and 222, respectively. In **Table 4**, are shown the content of artemisinin and scopoletin expressed in terms of area under the peak. It shows that the extraction with soxlhet led to the best yields but due to practicability ASE was chosen [8; 17; 18].

Chemical parameters

The TIC obtained from *A. annua* from Mbouda (**Figure 4**) showed several peaks reflecting a large number of constituents in the extract such as artemisinin derivatives, scopoletin and volatile components.

1) Artemisinin

Artemisinin a sesquiterpene, was identified on the TIC of our extracts (**Figure 5**) by 2 peaks at the retention times 13.83 and 14.06 min corresponding to its degradation derivatives. Its concentration has been evaluated in each extract and the values ranged from 0.4 to 19.2 mg/kg with an average of 11.1 mg/kg and a standard deviation of 3.9. The lowest value (0.4 mg/kg) was obtained for samples from Bafut (North-West) that was grown under shade and leafless at 5 months of maturity while the highest were obtained from samples from Bangang-Fokam (16.5 mg/kg), Dschang (17.8 mg/kg), Bafoussam (CIPCRE) (18.2 mg/kg) and Bandjoun (19.2 mg/kg). These high concentrations were obtained with plants having a collar diameter greater than 30 mm, many branches at maturity and the onset of the first flower bud during the collection.

2) Scopoletin

The scopoletina coumarin aglycon, was identified at the retention time 12.45 min (**Figure 5**). Their concentration in the samples was between 0.24 and 10.38 mg/kg with an average of 1.90 mg/kg and a standard deviation of 1.73. The highest value was obtained with the samples from Bandjoun (10.38mg/kg) with an average of 3.9 mg/kg.

3) Volatile compounds

The AFNOR and ISO norms were observed to assay the volatile components in the extract of our samples. From the TIC obtained (**Figure 6**) we were able to identify 13 volatile components including α-pinene (8.81 min), camphene (10.01 min), ß-pinene (11.12 min), 3-carene (12.14 min), α-terpinene (12.82 min), limonene (13.40 min), eucalyptol (13.68 min), artemisia ketone (16.73 min), copaene (19.97 min), camphor (20.72 min), caryophylene (22.08 min), menthol (22.36 min) and α-terpineol (23.34 min).The amount of these compounds in our samples was evaluated and compared to that of sample from Luxembourg.

The most volatile component (α-pinene) was identified with concentrations between 0.6 to 21.2 mg/kg and an average of 7.5 mg/kg, and a standard deviation of 6.82. Luxembourg that gave 6.23 mg/kg.

Camphene concentrations were between 0.68 and 26.46 with an average of 13.3 mg/kg and a standard deviation of 9.49.

For 3-carene, concentrations varied from 0.01 to 3.86 with an average of 0.51 mg/kg and a standard deviation of 1.14.

The amounts of α-terpinene in extracts of *A. annua* from our localities were between 0.18 and 2.33 mg/kg with an average of 0.88 mg/kg and a standard deviation of 0.62. 1.08 mg/kg obtained from extract of samples from Luxembourg.

The amount of limonene present in samples from Grass-field Regions of Cameroon was identified with concentrations between 0.17 and 21.25 mg/kg.

The concentrations of eucalyptol in our samples were between 2.9 and 102 mg/kg with an average of 33.9 mg/kg and a standard deviation of 29.66.

Amounts of artemisia ketone in the extracts of samples from our localities were very low. Concentrations ranged from 0.02 to 103 mg/kg, with the value of 103 mg/kg obtained for once and the rest below 0.1 mg/kg. Luxembourg gave an average of 37.41 mg/kg.

The concentrations of copaene and camphor were ranged from 1.90 - 124 to 55 - 541 mg/kg with averages of 28.2 and 327 mg/kg and standard deviations of 28 and 152, respectively. Luxembourg (62.86 mg/kg)

The concentrations of caryophyllene and α-terpineol obtained were between 5 – 217 and 3 - 108 mg/kg with averages of 85 and 36 mg/kg, and standard deviations of 69 and 25, respectively, Luxembourg (30.94 mg/kg).

The amounts of menthol in our samples ranged from 0.09 to 9.13 mg/kg with an average of 1.7 mg/kg and a standard deviation of 3, Luxembourg (0.69 mg/kg).

Discussions

From this study, we noted that the soils in our localities need organic fertilizers such as compost and a mixture of nitrogen/phosphorus/potassium (60/30/30) to give good yields (**Table 1**). In effect, to obtain optimal production of artemisinin in *A. annua*, the soil needs a high concentration of iron and boron; and a mixture of nitrogen/phosphorus/potassium (60/30/30) fertilizer [19]. The *A. annua* from the Grass-field Regions of Cameroon had a very important vegetal yield and it can be explained by the fact that climatic conditions of these localities were much the same. Nevertheless, the sample from Bamenda was grown under shade and that explains its low collar diameter (**Table 2**) at maturity. Thus, climatic conditions affect the physical parameters (height, collar diameter, appearance of the first flower bud) and consequently the yield of constituents.

The identification and the quantification of artemisinin, scopoletin and 13 volatile components were carried out by GC-MS and the TIC of each extract of our samples showed characteristic peaks at the same retention time with standard compounds. The artemisinin was identified from the TIC by the presence of two peaks (13.83 and 14.06 min) respectively characteristic of its degradation derivatives arteanniun B [20] and dihydroartemisinin (**Figure 5**) [21] [22]. Christen and Veuthey analyzed extract of *A. annua* and obtained three peaks for artemisinin [17], this difference could be explained by the type of injector, the column and the apparatus, keeping in mind that at a temperature above 150°C artemisinin is decomposable [21].

Constituents of *A. annua* from localities of the Grass-field of Cameroon and their yield were compared graphically with samples from Luxembourg (**Figure 7**). It is obvious and interesting to observe that the scopoletin identified in samples from Cameroon is approximately 1/5

of artemisinin and quite less compared to those from Luxembourg. The average yield of artemisinin in samples from Cameroon (11.1 mg/kg) was higher than that of samples from Luxembourg (0.8 mg/kg).We have also compared the yield of artemisinin of *A. annua* from Cameroon and that of samples from other countries different from Luxembourg such as Germany, Belgium, Brazil, Democratic Republic of Congo (DRC) and Central Africa Republic (CAR) (**Table 5**). We observed that the concentration of artemisinin in *A. annua* from tropical countries (Brazil, DRC) was similar to that from Cameroon (**Table 5**). When comparing these results with those obtained on samples from temperate countries (Belgium, Luxembourg) (**Table 5**) were is a marked difference. In effect, the strain of *A. annua* from Brazil loses 50 % of its content in artemisinin when it was grown in Luxembourg while that from Luxembourg grown in Katanga (DRC) showed a concentration of the same constituent 8 times better (**Table 5**). This shows that the yield of artemisinin in *A. annua*isbio-ecological dependence. It goes from 13mg/kg in Chinato 4 mg/kg in Europe and is almost nonexistent in other parts [23; 24]. The average concentration of this component in samples from Cameroon was not significantly different to that from China.

A sample of *A. annua* from China collected in German pharmacy, had a concentration in artemisinin of 0.8 mg/kg instead of 13 mg/kg (**Table 5**). This difference could be explained by the conservation conditions. So in addition to the ecological environment of culture, the preservation conditions namely away from light and moisture also affect the extraction yield of artemisinin [17].

It is also increasingly recognized that artemisinin is not acting alone but in synergy with other compounds such as flavonoids, coumarins and volatile components [25]. This suggests that if treatment against malaria is made with infusions or crude extracts of *A. annua*, the amount of artemisinin is not essential. This has been verified in studies conducted at the hospital of the Cite Verte in Yaounde (Cameroon) from which tea of *A. annua* from Luxembourg have been used satisfactory for the treatment of malaria, despite their low content of artemisinin [7].

It is evident to observe from the **Figure 7** that the amount of volatile components in our samples also varies depending on the locality of cultivation. In effect, the dried leaves of *A. annua* from Luxembourg gave more concentration of eucalyptol, artemisia ketone and copaene than that from Cameroon which was most concentrated in menthol and camphor by comparing with each other.

We found that camphor was the major constituent of our samples with a proportion of 50 %, which is similar to the yield obtained from *A. annua* from Iran [26] and both are higher to that of the sample from India (15 %) [27].

Conclusion

The determination of cultivation conditions of *A. annua* grown in the Grass-field Region of Cameroon and the determination of their physicochemical and morphological characteristics showed that *A. annua* adapted well in Cameroon and could provide raw materials for cheaper local antimalarial medicine. The local production of this plant will enhance socio-economic conditions for malaria zones and developing countries to fight against this disease.

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Legends for Figures

Fig. **1:** Histogram of the average size of plants; Average = 185.9 cm, SD = 60.68, N (number of samples) = 37.00.

Fig. **2:** Average of the collar diameter of *A. annua* from the Grassfield Regions of Cameroon

Fig. **3:** Average of the appearance of the first flower bud of *A. annua* in the Grassfield Regions of Cameroon

Fig. **4:** Photograph of the *A. annua* growing in the Grass-field Regions of Cameroon.

Fig. **5:** TIC of the extract of *A. annua* from Mbouda showing artemisinin derivatives and scopoletin.

Fig. **6:** TIC of the extract of *A. annua* from Bangangte showing volatile components.

Fig. **7:** Comparative curves of the variation of constituents of *A. annua* from the Grass-field Regions of Cameroon compared to those from Luxembourg.

Fig.**1**

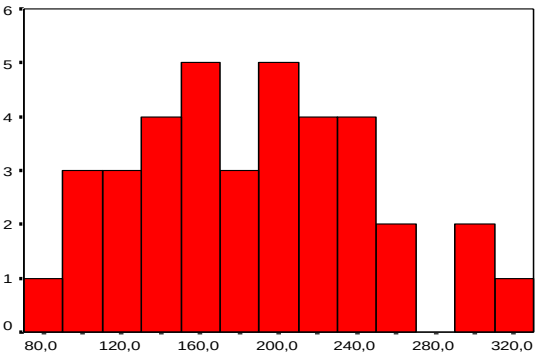


Fig. **2**

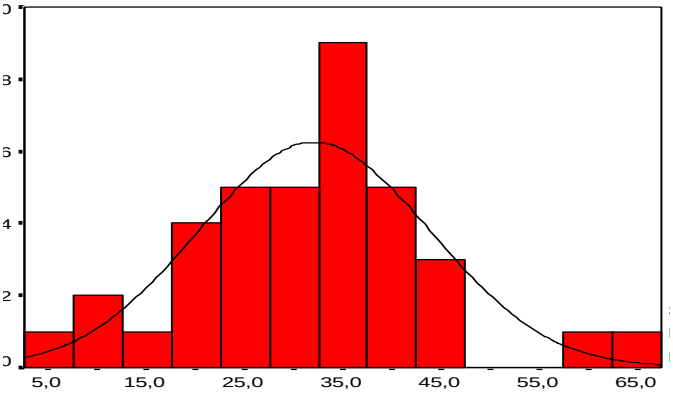


Fig. **3**

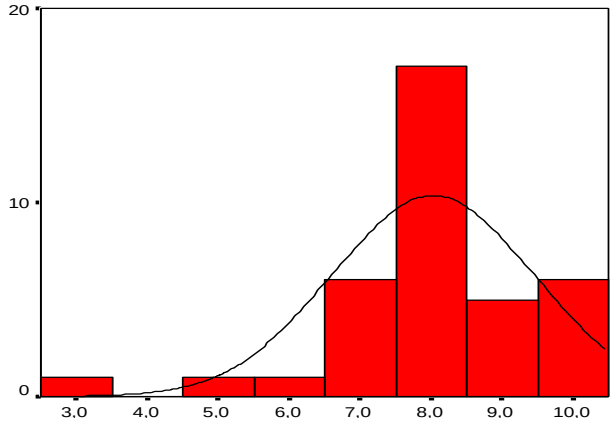


Fig. **4**

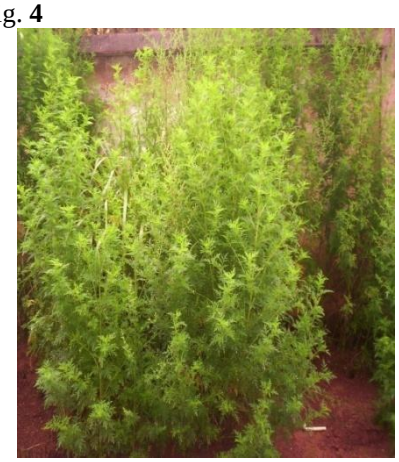
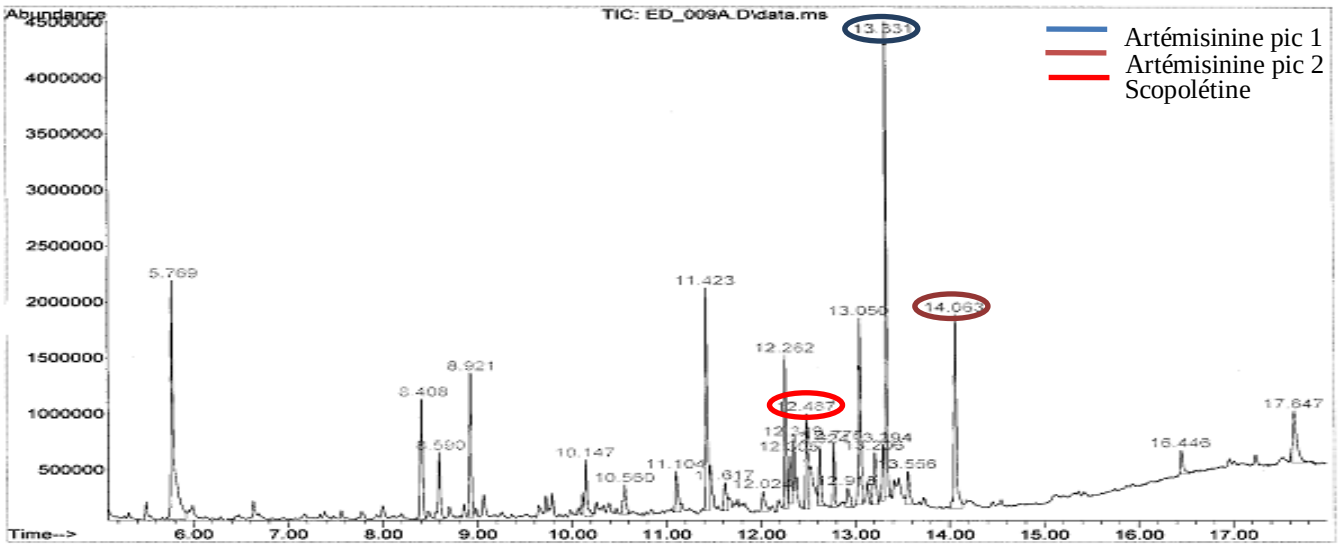


Fig.**5**



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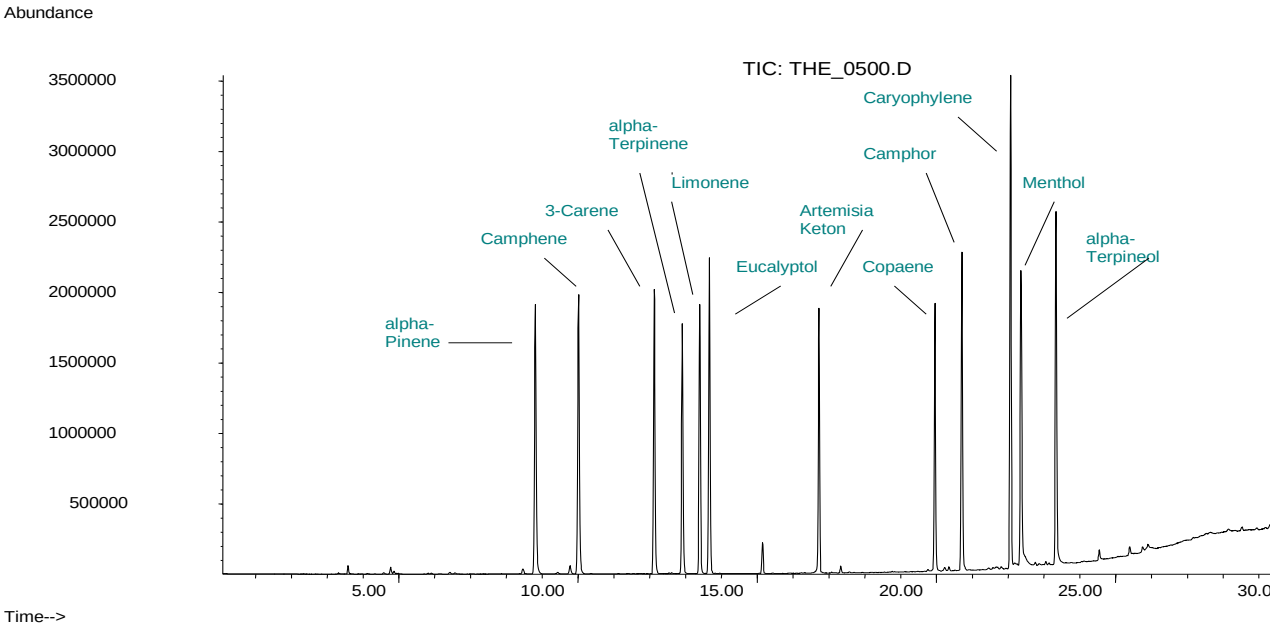
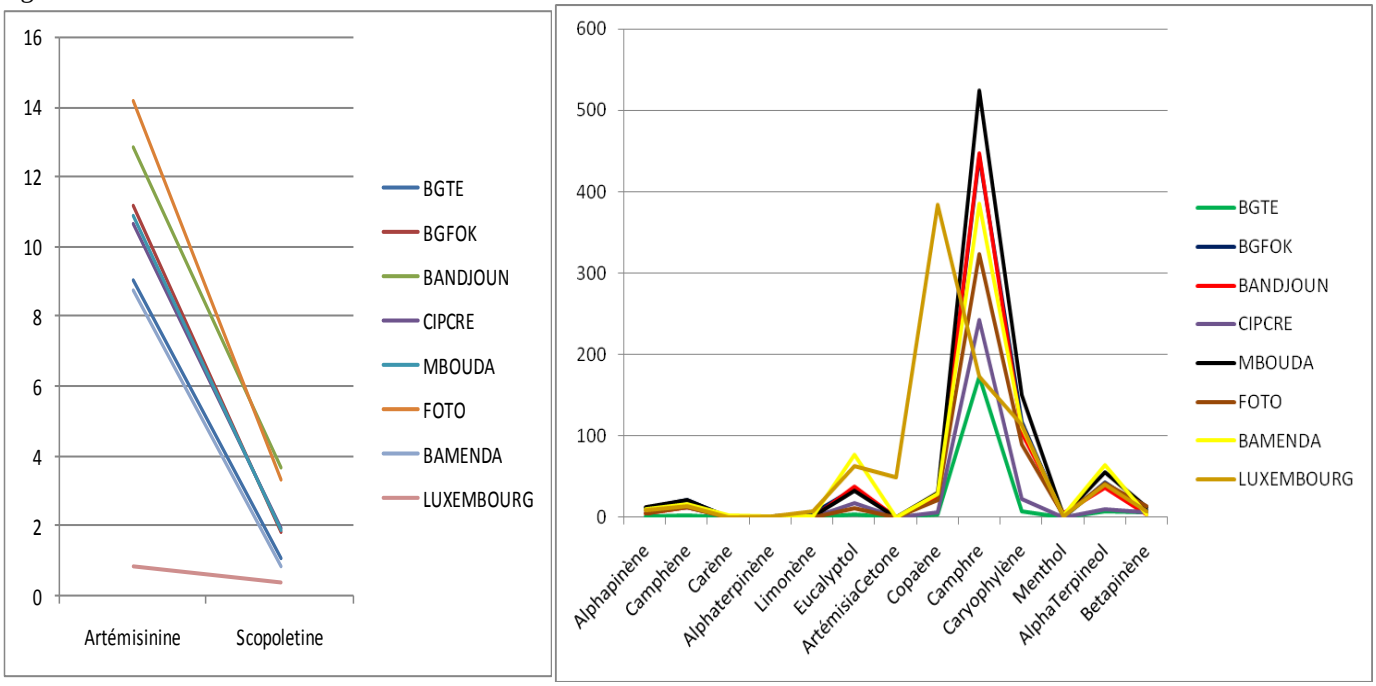


Fig. **7**



Tables

Table 1: Average size of samples collected

Localities	N	Average (cm)	Standard deviation	Average standard error
Bangang-Fokam	5	240	41	18
Bangangte	4	157	99	49
Bandjoun	5	228	50	22
Bafoussam	8	141	37	13
Mbouda	5	225	51	23
Dschang	4	180	8	4
Bamenda	6	165	47	19

Table 2: Collar Diameter of *A. annua* from the Grass-field Regions of Cameroon

Localities of Collection	N	Average (mm)	Standard deviation	standard average Error
Bangang-Fokam	5	33.2	4.8	2.1
Bangangte	4	29.2	9.5	4.7
Bandjoun	5	32	92	4.1
Bafoussam	8	39.6	16.6	5.9
Mbouda	5	34.8	8.9	3.9
Dschang	4	34.2	5.3	2.6
Bamenda	6	19.3	9.3	3.8

Table 3: Appearance of the first flower bud of *A. annua* in the Grass-field Regions of Cameroon

Localities of Collection	N	Average (mm)	Standard deviation	standard average Error
Bangang-Fokam	5	8	0	0
Bangangte	4	8.5	1	0.5
Bandjoun	5	10	0	0
Bafoussam	8	6.6	1.8	0.6
Mbouda	5	8	0	0
Dschang	4	9	0	0
Bamenda	6	7.3	0.8	0.3

Table 4: Peak area of artemisinin and scopoletin according to the method of extraction

Compounds	Methods of Extraction			
	ASE	Soxlhet	Infusion	Water/Hexane
Scopoletin	95997	122505	51502	3188
Artemisinin peak 1	79075	87907	78826	65510
Artemisinin peak 2	38169	42962	28239	41501

Table 5: Yield of artemisinin in *A. annua* from other countries than Cameroon

Country	Localities of Sample	Concentration (mg/kg)
1	ArtChinaPhcyGer (Germany)	0.8
2	BrazilSachetb (Brazil)	11.5
3	Katanga seed Luxb (DRC)	10.3
4	YpresBelgiumb (Belgium)	1.1
5	BrazilGrowLuxb (Luxembourg)	5.0
6	ArtCentrafriqb 2008 (CAR)	2.6
7	ArtVulgarisb (Luxembourg)	0.1
8	Cameroon (Grassfield Regions)	11.1

1ArtChinePhcieAlle (Germany):*A.annua*from Chinafound in pharmacyin Germany;

2BresilSachetb (Brazil): Bagfrom Brazil;

3Katanga semenceLuxb (RDC): seed from Luxembourg and used in Katanga;

4YpresBegiqueb (Belgium): sampleb of Ypresin Belgium;

5 BresilPousséLuxb (Luxembourg): Seed from Brazil and used in Luxembourg;

6ArtCentrafriqb 2008 (RCA): *Artemisia* grown in the Central African Republic;

7ArtVulgarisb (Luxembourg): *Artemisiavulgaris* from Luxembourg;

8Cameroon (West & North-West): *A.annua* from localitiesconsideredin this study.