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Microscopic features and phytochemistry of two Congolese medicinal plants: *Aframomum alboviolaceum* (Ridley) K. Schum, and *Aframomum angustifolium* (Sonn.) K. Schum. (*Zingiberaceae*).

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Abstract

The leaves and seeds of *Aframomum alboviolaceum* and *Aframomum angustifolium* are specifically used by traditional healers in the Democratic Republic of Congo (DRC) for the treatment of several pathologies. The aim of present study was to determine the microscopic characteristics and phytochemical composition of these species of the genus *Aframomum*. The microscopic study of these plants revealed the presence of punctate vessels, fiber cluster with calcium oxalate crystals, isolated unicellular hair, fibers, starch grains, spiral vessel fragments, fiber fragments, and indistinct parenchyma fragments. Phytochemical screening revealed several chemical groups such as phenolic acids, flavonoids, coumarins, alkaloids, terpenes, iridoids, saponins etc. The determination of total polyphenols, flavonoids and tannins gave contents ranging from 14.95 ± 0.45 and 63.98 ± 2.04 mgGAE/g, 0.16 ± 0.01 and 10.68 ± 0.32 mgQE/g and between 1.28 ± 0.03 and 28.51 ± 0.56 mg CAE/g respectively. In general, the leaves are richer in secondary metabolites, polyphenols, flavonoids and tannin than the seeds. Both plants also contain iron, magnesium, calcium and sodium. To our knowledge, this is the first time that histological elements have been identified in the leaves and seeds of *A. angustifolium*.

Keywords: Microscopic features; Phytochemical screening; Medicinal plant; *Aframomum*

1. Introduction

Currently, the world and particularly Africa is increasingly faced with the resurgence of certain diseases [1]. To cope with this situation, many researches have been conducted to find new sources of biomolecules in medicinal plants [2-4]. Indeed, these plants serve as remedies against diseases [5, 6] and are used in therapy by about 80% of the population in developing countries [5, 7, 8]. Thus, the biological properties of plant extracts have been the subject of several scientific works [6]. The pharmacological properties of plants are generally attributed to their contents of secondary metabolites including flavonoids, tannins, coumarins, alkaloids, phenolic acids and terpenes [7, 9-12]. The determination of qualitative and quantitative phytochemical composition in plant extracts thus appears as an indicator

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of biological properties such as antioxidant, antibacterial, anti-sickle cell etc. of these plants. The main objective of this study was to determine the microscopic characteristics and phytochemical composition of *A. alboviolaceum* and *A. angustifolium* [8, 11, 13]. As specific objectives the present study aimed to determine the histological elements of the powder of these plants (1), to determine the qualitative phytochemical composition of two plants (2) and to determine the contents of total polyphenols, flavonoids and tannins of these plants (3). To our knowledge, there is no study carried out on the determination of histological elements of *A. angustifolium*.

2. Material and methods

2.1. Material

In this study, two plant species of the genus *Aframomum* (Zingiberaceae) were used: *A. alboviolaceum* (Ridley) K. Schum. and *A. angustifolium* (Sonn.) K. Schum. These plants were selected from our recently obtained research results [8, 11, 13]. The leaves of *A. alboviolaceum* were collected on 07 June 2019 in the savanna of N'djili Brasserie located in the commune of N'djili in Kinshasa/DRC (4°24'18'' south, 15°22'25'' east) and its seeds were purchased from herbalists in the market of Ngaba/Ngaba Township in Kinshasa, while the leaves and seeds of *A. angustifolium* were collected in Mbandaka, Equateur province (0°02'52'' North, 18°15'21'' East) / DRC. These two plants were identified at the Herbarium of the National Institut of Study and Search in Agronomic Sciences (NISRA) located at the Faculty of Sciences of the University of Kinshasa (UNIKIN). After identification, these samples were air-dried (± 27 °C) at the Ethnobiology and Medical Phytochemistry Laboratory (E-PHYMED) (Biology Department) for two weeks and then ground to obtain a fine powder.

2.2. Micrography

The various histological elements of the plant material (powder) were identified using the method previously described by Inkoto et *al.* [7]. Using dropper, place 2-3 drops of the distilled water on an object slide, then add a small amount of the pulverized powder and cover it with the slide. Proceed to the observation of the starch grains in the organs used (seeds and leaves). The operation was carried out in the same way to highlight the different histological elements by adding a selected reagent instead of distilled water. From the beginning, it is important to operate very carefully: the external surface of the slide must be free of any trace of reagent or powder to be examined. It is also important to make very light preparations, in order to distribute the tissues well and to avoid superimpositions.

2.3. Phytochemical study

2.3.1. Standard chemical screening

The different groups of secondary metabolites were identified by standard screening as per the method previously described by Ngbolua et *al.* [14]

2.4. Thin layer chromatography (TLC)

2.4.1. Search for Flavonoids and phenolic acids

1 g of pulverized drug was extracted with shaking by 5 mL of methanol for 10 minutes. 10 mL of filtrate was used for TLC analysis. The stationary phase was constituted by silica gel F 254, while the mobile phase 1 was constituted by ethyl acetate-formic acid-glacial acetic acid-water (100:11:11:26). Dichloromethane-formic acid-acetone (80:10:20) served as mobile phase 2. Rutin, hyperoside, isoquercitrin and chlorogenic acid: 1 mg/mL (methanol) deposit: 10 µL served as controls. Once chromatogram developed, observation is done under UV at 254 and 366 nm and then sprayed with DPBAE/PEG reagent and observed under UV at 366 nm. The presence of flavonoids is marked by the presence of fluorescent spots of various colors (yellow-orange-green) varying according to the structure of the highlighted compounds. Blue fluorescence is often due to phenolic acids.

2.4.2. Research of Iridoids

We used the solution prepared for the flavonoid test (10 µL deposits). Stationary phase is Silicagel F254 while the mobile phase is ethyl acetate-methanol-water mixture (100:13.5:10). Revelation with 5% sulfuric acid in ethanol. Heating for 10 minutes at 100°C. True iridoids give staining. The other terpenes stain black.

2.4.3. Research of Terpenes

1g of pulverized drug is extracted under agitation by 10 mL of dichloromethane during 15 minutes. The filtrate is evaporated to dryness and the residue is dissolved in 0.5 mL of toluene: 10 µL deposit. Stationary phase is silica gel F254 and mobile phase by toluene-ethyl acetate (93:7). As controls we used thymol, menthol, oleanic acid: 1 mg/mL (methanol) deposit: 10 µL. These compounds are revealed by sulfuric vanillin. Heating 10 minutes at 100°C. Terpenes give various colors with this reagent.

2.4.4. Research of Coumarins

We use the solution prepared for the terpenes test. Mobile phase is toluene-ether (1:1, saturated with 10% acetic acid). Mix 10 mL toluene, 10 mL ether and 10 mL 10% acetic acid in a separatory funnel. The lower phase is removed; the upper phase is used as mobile phase. Observation under UV at 254 and 366 nm. Spraying with 10% ethanolic KOH. Coumarins give a blue fluorescence.

2.4.5. Research of Alkaloids

0.3 g of drug powder is introduced into an Erlenmeyer flask and 3 mL of 5% hydrochloric acid is added. It is sealed and left under agitation for 30 minutes. The filtrate is filtered and collected. On 1mL of filtrate introduced in a test tube, 5 drops of Mayer's reagent are added. The presence of alkaloids is marked by the appearance of a white precipitate or cloudiness. 1g of drug powder is macerated with about 1mL of 10% ammonia in an Erlenmeyer flask, 5mL of ethyl acetate (or methanol to extract the quaternaries) is added and left under stirring for 30minutes 20µL and 50µL of the filtrate are used for TLC analysis. The mobile phase is formed from the 25% (8:2:0.5) dichloromethane-methanol-ammonia mixture. Caffeine (5 mg/mL) was used as a control. The chromatogram, once developed, was observed under UV at 254 and 366nm and then sprayed with Draggendorff's reagent and observed under visible light. The presence of alkaloids is marked by the presence of spots ranging from yellow-orange to yellow-brown.

2.5. Secondary metabolite contents

2.5.1. Total polyphenols content

We determined the total polyphenol content of our extracts using the Folin-Ciocalteu method [7, 15]. 10mg/mL of each extract was diluted in 80% methanol to obtain a 1mg/ml solution for each extract. Then we prepared a reaction mixture for each extract consisting of 0.5mL of extract; 5.0 mL of distilled water and 0.5mL of Folin-Ciocalteu reagent. After 3 minutes, add 1.0 mL of a saturated 20% Na_2CO_3 solution. The prepared mixtures are stirred and incubated at laboratory temperature in the dark for 1 hour. The absorbances were read with a spectrophotometer at 725 nm. Each determination was repeated 3 times. The amount of total polyphenols is expressed as mg gallic acid equivalents (GAE)/g dry extract using the following equation from the calibration line: $y= 1.7097 \ln(x)+ 5.2062$ and $R^2=0.965$, where x is the absorbance and y is the gallic acid equivalent (mg/g).

2.5.2. Total flavonoids content

We estimated the total flavonoid content of our extracts by following a spectrophotometric method. Aluminum trichloride forms a yellow complex with flavonoids that absorbs at 415 nm [7]. The reaction mixture contained 1mL of the methanolic solution (80%) of each of the extracts of concentration 10mg/mL and 1mL of 2% $AlCl_3$ (dissolved in methanol) and the whole was shaken well. After 1 hour of incubation at laboratory temperature and protected from light, measure the absorbances with a spectrophotometer at 415 nm. The mixtures were prepared in duplicate for each analysis and the average value was retained. For the preparation of the blank, we operated in the same way except that instead of the extract, 1 mL of methanol was put. The flavonoid content of the extracts is expressed in mg quercetin equivalent (QE)/g of corresponding dry extract using the equation from the calibration line: $y= 0.5001 \ln(x) + 3.442$ $R^2 =0.944$ where x is the absorbance and y the quercetin equivalent (mg/g).

2.5.3. Tannins content

We estimated the total flavonoid content of our extracts by following a spectrophotometric method used by Ali-Rachedi et *al.* [16]. The reaction mixture contained 1mL of the methanolic solution (80%) of each of the extracts of concentration 10mg/mL and 1mL Na_2CO_3 3.5% (dissolved in methanol) and the whole was shaken well. After 1 hour of incubation at laboratory temperature and protected from light, measure the absorbances with a spectrophotometer at 725 nm. The mixtures were prepared in duplicate for each analysis and the average value was retained. For the preparation of the blank, we operated in the same way except that instead of the extract, 1 mL of methanol was put. The tannin content of the extracts is expressed in mg tannic acid equivalent (TEA)/g of corresponding dry extract using the equation from the calibration line: $y= 0.0011 \ln(x)+ 3.442$ $R^2 = 0.2236$ where x is the absorbance and y the tannic acid equivalent (mg/g).

2.6. Mineral elements content

The mineral elements studied were determined in the residue of the product calcined at 500 °C for 5 hours, with a test sample of 15 g for the leaves. The determination of the studied minerals (Ca, K, Mg, Fe) was carried out by the aqua regia method and analysis by UV-visible spectrometry (brand LGS 53). The aqua regia method is based on the principle of dissolving the sample in a mixture of hydrochloric acid (5N) and nitric acid (0.1N) according to the following procedure: 0.15 g of mineral material is weighed, 2 to 3 mL of aqua regia are added, the sample is placed on a hot plate and allowed to evaporate to dryness. Then 25 to 30 mL of hydrochloric acid (2M) are added until total dissolution and then the UV-visible spectrophotometry reading is taken at 525 nm (Fe), 472 nm (Na), 610nm (Ca), and 510 nm (Mg).

3. Results and discussion

3.1. Botanical microscopic characters

The various histological features of *A. alboviolaceum* leaves such as paracytic stomata, fragment of spiral vessels, fragments of suber and crystal fiber and tector hair were reported in our previous study [7]. Microscopic examination (Figure 1) revealed several characteristic histological features in *A. angustifolium* leaves including punctate vessels, fiber cluster with calcium oxalate crystals, isolated unicellular hair, fibers and starch grains. Calcium oxalate crystals can play several roles including protection against herbivores, calcium storage, maintenance of ionic balance in cells [17] but also detoxification [18]. Then Figure 2 shows the presence of spiral vessel fragments, fiber fragments, and indistinct parenchyma fragments (D) identified in the seeds of two species of the genus *Aframomum.* It should be noted that this is the first time that histological elements have been detected in the leaves and seeds of *A. angustifolium*. These results would constitute a database for the characterization of powder of these two medicinal plants used in traditional medicine in order to avoid adulteration, a serious problem involving the mixing of the powder of these plants with other materials that are toxic at times [19, 20]. The results of the micrograph performed on the powders of our plants are presented in the following figures 1 and 2:

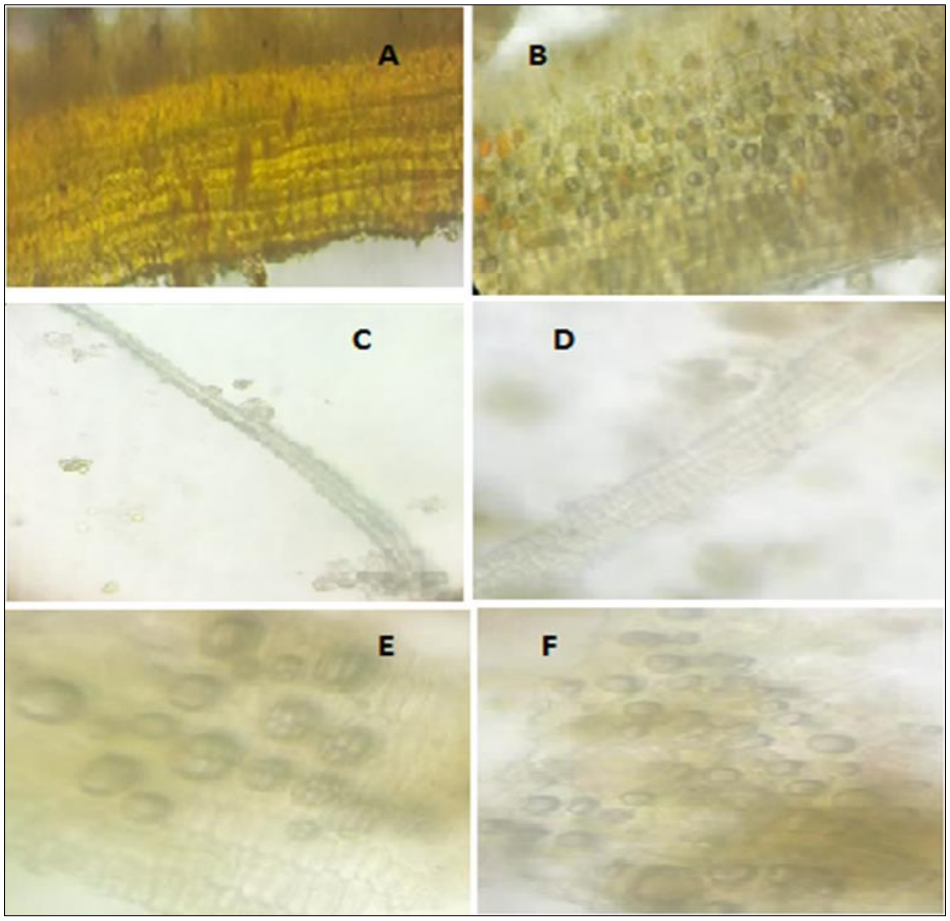

Figure 1 Micrographic features of *A. angustifolium* leaves: punctate vessels (A), fiber cluster with calcium oxalate crystals (B), isolated unicellular hair (C), fibers (D), and starch grains above punctate vessels (E) and starch grains (F)

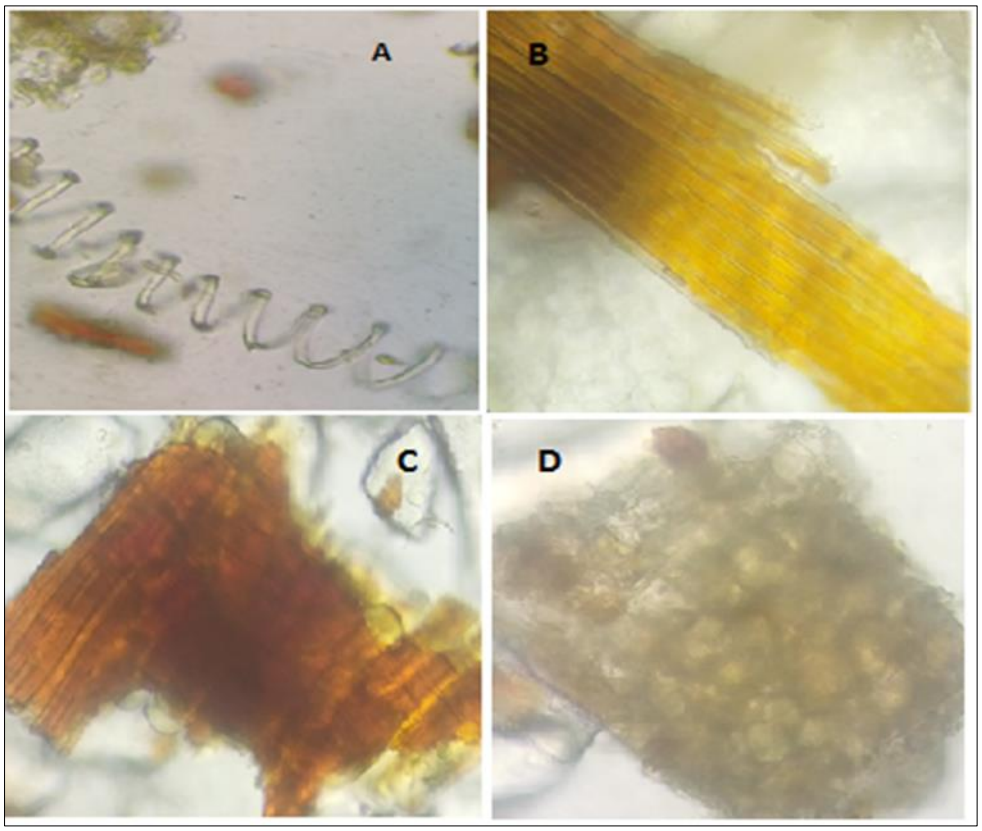

Figure 2 Micrographic characteristics of *A. angustifolium* and *A. alboviolaceum* seeds: fragment of spiral vessels (A), fiber fragments (B) and (C), indistinct parenchyma fragments (D)

3.2. Phytochemical analysis

It results from this table (Table 1) that the studied plants are rich in secondary metabolites. The leaves are richer in secondary metabolites than the seeds. Among the eleven metabolites studied, free quinones were found to be absent in the leaves and seeds of two plants. We also note the absence of bound quinones, terpenes and steroids in the seeds of these two plants. These results corroborate with the work of Djeussi et *al.* [21] who report the presence of anthocyanins, flavonoids, alkaloids, polyphenols and tri terpenes in the extracts of *A. alboviolaceum.* It is the same with Rakotonirina [22] who reported the presence of flavonoids, leucoanthocyanins and triterpenes in *A. angustifolium*. The present results will also be confirmed by thin layer chromatography screening in the next section. The results of the TLC chromatographic analysis revealed the richness of phenolic compounds and terpenoids in the different extracts of the two plants studied. The chromatogram of figure 3 corresponding to that of the research of flavonoids and phenolic acids, showed the presence of yellow-orange spots corresponding to flavonoids in the seeds of two plants and in the leaves of *A. alboviolaceum*, and blue corresponding to phenolic acids in the leaves of *A. angustifolium.* These results confirm those we obtained in the ordinary screening which showed that these two plants are rich in phenolic compounds. These results are similar with de Inkoto et *al.* [7], Djeussi et *al.* [21] in *A. alboviolaceum* and Rakotonirina [22] in *A. angustifolium*. The analysis of Figure 4 shows us that the various colorations observed in the chromatographic profiles of each sample (purple, orange color) mark the presence of terpenes including irridoids in all three plants. These results are similar to those obtained previously by Djeussi et *al.* [21] in *A. alboviolaceum*. The chromatograms in Figures 5 and 6 corresponding to that of the search for alkaloids and coumarins. The analysis of figure 5 showed the presence of the orange-black spots in the leaves of two plants, corresponding to alkaloids, and the blue fluorescent spots corresponding to coumarins (Fig. 6). These compounds were confirmed by our previous work [7]. Table 1 gives the results of the standard chemical screening of the aqueous and organic extracts of the leaves and seeds of *A. alboviolaceum* and *A. angustifolium*

Table 1 Results of the standard chemical screening of aqueous and organic extracts of leaves and seeds of *A. alboviolaceum* and *A. angustifolium*

N°	Search groups	Plants			
		AAF	AAG	AAgF	AAgG
01	Total polyphenols	+	+	+	+
02	Flavonoids	+	+	+	+
03	Alkaloids	+	+	+	+
04	Anthocyanins	+	+	+	+
05	Leuco-anthocyanins	+	+	+	+
06	Saponins	+	+	+	-
07	Tanins	+	+	+	+
08	Linked quinones	+	-	+	-
09	Free quinones	-	-	-	-
10	Terpenes	+	-	+	-
11	Steroids	+	-	-	-

Legend: - Presence of search compound, -: Absence of search compound, AAF: A. alboviolaceum leaves, AAG: A. alboviolaceum Seeds AAgF: A. angustifolium leaves, AAgG: A. angustifolium seeds.

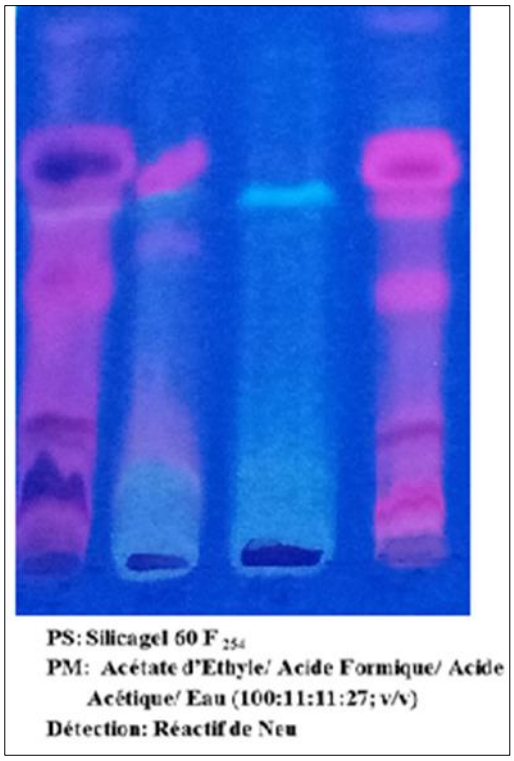

Figure 3 TLC chromatogram of methanolic extracts of the two plants studied at 366 nm

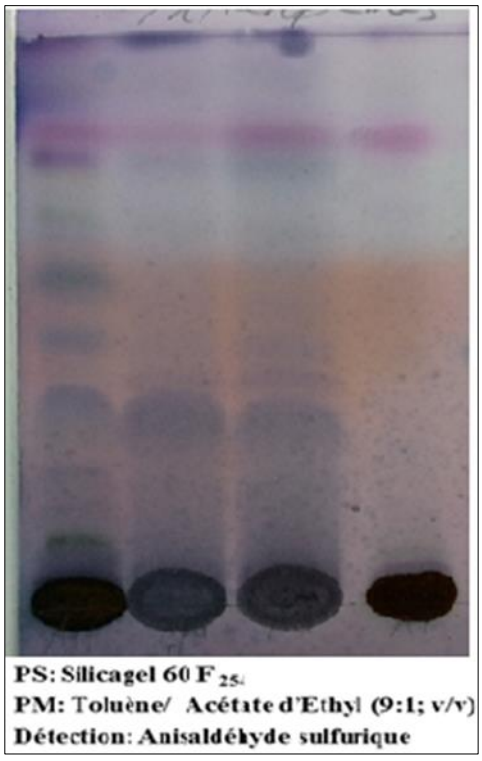

Figure 4 TLC chromatogram of dichloromethane extracts of the two plants studied, in the visible

Figure 5 TLC chromatogram of methanolic extracts of the two plants studied at 366 nm

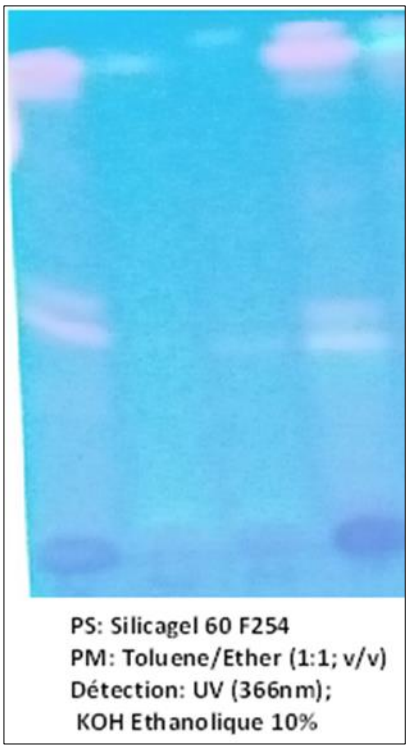

Figure 6 TLC chromatogram of methanolic extracts of the two plants studied at 366 nm

The results of the secondary metabolites content are reported in Table 2 below.

Table 2 Total polyphenols, flavonoids and tannins content

Plants	Total polyphenols (mgGAE/g)	Flavonoids (mgQE/g) (%R)	Tanins (mg CAE/g)
AAF	63.98 ± 2.04	2.48 ± 0.06 (3.87)	28,51 ± 0.56 (44.56)
AAG	24.98 ± 0.77	0.16 ± 0.01 (0.64)	1,28 ± 0.03 (5.12)
AAgF	14.95 ± 0.45	10.68 ± 0.32 (71.43)	9.77 ± 0.52 (65.35)
AAgG	23.51 ± 0.69	0.84 ± 0.02 (3.57)	8.93 ± 0.75 (37.98)

Legend: GAE/g Gallic acid equivalent (GAE)/ g dry extract, QE/g Quercetin equivalent (QE)/g dry extract, CAE/g Catechic acid equivalent (CA)/dry extract. %R= [(flavonoids or tannins/total polyphenols ratio)] x 100.

The results of the assay reveal that the two plants studied are rich in phenolic compounds. The total polyphenol contents varied between 14.95 ± 0.45 and 63.98 ± 2.04 mgGAE/g respectively for the two plants. The highest content was observed in the leaves of *A. alboviolaceum* (63.98 ± 2.04). The flavonoid contents of these plants ranged from 0.16 ± 0.01 to 10.68 ± 0.32 mgEQ/g. The highest content was observed in the leaves of *A. angustifolium* (AAgF: 10.68 ± 0.32). For tannins, the contents varied between 1.28 ± 0.03 and 28.51 ± 0.56 mg CAE/g. The content of *A. alboviolaceum* leaves was the highest (28.51 ± 0.56). Several factors may influence the content of phenolic compounds. Studies have shown that extrinsic factors (such as geographical and climatic factors), genetic factors, but also the degree of maturation of the plant and storage time have a strong influence on polyphenol content [23-25]. The figure 7 gives mineral elements content of the two plants.

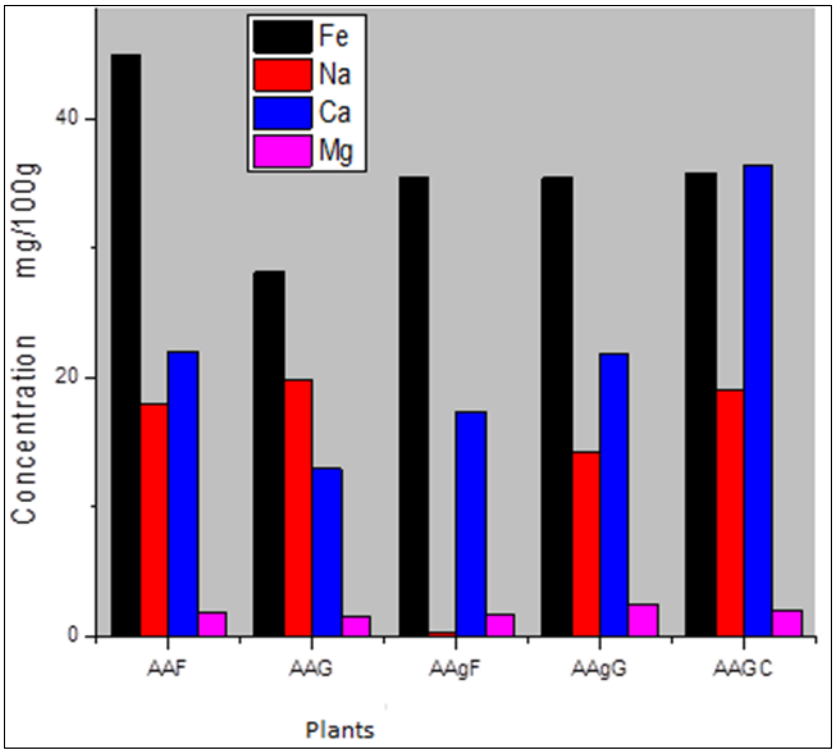

Figure 7 Fe, Ca, Na et Mg content

Present figure shows that four mineral elements are identified in each of the samples. Iron is the most abundant element in our plants, followed respectively by calcium, sodium and magnesium. The highest content of iron was observed in the leaves of *A. alboviolaceum* (AAF: 44, 93 mg/100g), while calcium is in great quantity in the sample consisting of seeds and kernels of *A. Alboviolaceum* seeds and kernels (AAGC), 36.47 mg/100g. The low concentration of these two elements (Fe: 28.16 mg/100g and Ca: 12.93 mg/100g) were observed in the seeds of *A. alboviolaceum* (AAG). Regarding sodium, this element is more concentrated in the leaves of *A. alboviolaceum* (19.76 mg/100g) and it is almost at trace level in the leaves of *A. angustifolium* (AAgF). Magnesium is the less represented element in both plants. We notice that the concentration of iron, sodium and calcium in the seed-almond mixture of *A. alboviolaceum* is higher than that of the seeds alone. This shows a quantity of iron that is also in the kernels of this plant. These four mineral elements play several in the proper functioning of the body [26, 27]. It has been reported that both plants studied contain anti-sickle cell properties and they are used by the local population in the management of sickle cell disease [8, 11, 13]. On this, the high iron content in these plants may prove the use of the extract of these species by traditional practitioners to increase the hemoglobin level in sickle cell patients. Indeed, iron is important in the production of hemoglobin. Iron deficiency causes the body to produce fewer and smaller red blood cells, resulting in anemia [28].

4. Conclusion

The aim of the present study was to determine the microscopic characteristics and the phytochemical composition of the leaves and seeds of *A. alboviolaceum* and *A. alboviolaceum* used expressly in Congolese traditional medicine in the treatment of several diseases including sickle cell disease. The results obtained in this study show that:

- Both plants contain characteristic histological elements including punctate vessels, fiber cluster with calcium oxalate crystals, isolated unicellular hairs, fibers, starch grains, spiral vessel fragments, fiber fragments and indistinct parenchyma fragments;
- The leaves and seeds of these plants are rich in secondary metabolites such as phenolic acids, flavonoids, coumarins, alkaloids, terpenes, irridoids and saponins.
- These plant plants also contain iron, magnesium, calcium and sodium. The presence of these mineral elements which play a very important role in the metabolism of the body would justify the use of these plants in traditional medicine for the management of diseases such as sickle cell disease.

To our knowledge, this is the first time that histological elements have been identified in the leaves and seeds of *A. angustifolium*. In the light of these results, it would be desirable to pursue thorough phytochemical studies in order to isolate the bioactive compounds and elucidate their structures.

Compliance with ethical standards

Acknowledgments

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The all authors have declared that no competing interests exist.

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