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RESEARCH ARTICLE

HPLC-MS identification of three major flavonoids in the textile dye extract from dried leaves of *Anogeissus leiocarpus*

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ABSTRACT:

The availability of scientific information useful for the orientation of artisanal and industrial tinctorial practices to ensure the protection of the environment and the health of artisans and consumers remains a major concern for a large number of actors. Qualitative, quantitative analyses and structural identifications works using spectrophotometric, chromatographic and spectral methods were carried out on the total aqueous extract from leaves of *A. leiocarpus*. This extract was used to dye skeins of cotton fibers. With an extraction yield of about 5 %, *A. leiocarpus* leaves gave a total flavonoid content of the order 458.759±27.773 mgEQ/g of dye powder. Hydrolysable and condensed tannins rates are 13.25 % and 12.96 %, respectively. The HPTLC chromatographic profile of the dye showed that the extract of the *A. leiocarpus* leaves contains flavonols. The elongation vibrations ν_{O-H} and ν_{C-O} respectively of alcohols and oxide ethers in infra-Red testify to the presence of flavonic-type dyeing molecules which, by the phenomenon of co-pigmentation, contribute to the final shades in tinctorial practice. High-performance liquid chromatography coupled with mass spectroscopy revealed the presence of major flavonoid molecules such as quercetin 3-O-rhamnoside, quercetin 3-O-glucuronide and kaempferol 3-O-hexoside. Tinctorial practice techniques applied to skeins of cotton fibers have resulted in shades of various colours ranging from *anise* to *chartreuse green*. In addition to being a natural acid dye, the textile dye extracted from *A. leiocarpus* leaves can be classified in the group of metal dyes in terms of the quality of the shades obtained with the use of mordants such as alum, hydrated iron and copper sulphates.

KEYWORDS: Flavonoids, Tannins, HPTLC, HPLC-MS/MS, dyeing.

INTRODUCTION:

Nature has always renewed for our pleasure the multicolored splendours of its adornments, leading us to recognize the richness of the presents offered by the flora[1]. In Europe, in the 19th century, the textile industries acquired huge quantities of easily available dyes. This situation triggered the invention of synthetic dyes, offering a wide range of new colours at a lower cost, and giving better properties to dye materials [2]. West Africa, where there is no legal and regulatory framework for the import and use of dyes, is nowadays a vast

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market for the disposal of even the most toxic synthetic dyes banned in Europe.

To this day, synthetic dyes remain the main option chosen to dye textiles, despite the potential health and environmental risks [3], as some of them would have carcinogenic and cytotoxic effects. However, in some parts of the world, this discovery has led to a resurgence in the use of natural-derived dyes to replace, at least in part, synthetic dyes. Researchers are increasingly interested in natural dyes because of their biodegradability and higher compatibility. This renewed interest in plant dyes is also aligned with a global concern for the development of ecological and sustainable production, including in the textile sector [4].

This diversity of colors offered by the flora has always oriented the choices for the practice of tinctorial able to offer ranges of strong shades appreciated by consumers. The attachment of the man to the colors, allowed him, according to these preferences, to undergo enormous transformations, his costumes, clothes and linens.

Natural dyes for textile use today, through dyeing and its techniques [5], continue to reveal their secrets and are the subject of extensive research to provide a guarantee of the health of users and consumers.

The diversity of these ranges of shades obtained in tinctorial practice derives its deep understanding in the nature of the organic molecules responsible for the pigments of plants in their entirety.

The production of quality cotton fibres and the techniques of processing these fibres in Burkina Faso is a national priority [6] and this is of paramount importance for the accompaniment and mastery of techniques of tinctorial practices especially artisanal.

Knowing the structures of the molecules responsible for pigmentation and co-pigmentation at the plant level remains an issue that can justify or promote the improvement of tinctorial practice protocols [7, 8].

Based on the content of various groups of chemical compounds and the identification of major molecules by chromatographic and spectral methods (HPLC-MS and FT-IR), this work aims to dye cotton threads using *A. Leiocarpus leaves* extract. Through the phenomena of co-pigmentation [9] with many other molecules (tannins [10], phenolic acids...) and the use of non-toxic chemical bitings (alum, iron sulphates and hydrated copper), these flavonoids have contributed to the determination of various shades of color in dye made on skeins of cotton thread.

MATERIALS AND METHODS:

Extraction and yield:

250 g of powder obtained from *Anogeissus leiocarpus* dried leaves, was extracted using water in a soxhlet [11]. Water extraction was justified by the partial preservation of the current practices of dyeing craftsmen in Burkina Faso. The extract was concentrated by evaporation and then frozen and dried with ALPHA 1-2 LDplus brand freeze-dryer.

The yield of the resulting dye powder was assessed by Relationship 1:

$$\text{Yield} = \frac{\text{Mass of Dye extract}}{\text{Mass of plant material}} \times 100 \quad (1)$$

Total flavonoids content:

It consisted of adding 60 µL of the extract, 150 µL of distilled water, 15 µL of a solution of sodium nitrite ($NaNO_2$) to 5 %, 15 L of an aluminum chloride solution ($AlCl_3$) at 10 % and 50 µL of a soda solution (NaOH) at 1 M. Total flavonoid content was determined by reading the absorbances at 510 nm using the microplate reader 96-well quartz (MP96 spectrophotometer SAFAS). Absorbance was related to the standard curve obtained from the quercetin taken as standard (relationship 2) [12, 13].

$$C\ (\text{mg Eq/g}) = \frac{(Abs - b) \times V \times DF}{m \times a} \quad (2)$$

C (mg EQ/g): Quercetin equivalent milligram content per gram of dye powder.

Abs: Absorbance; **b**: Originally ordained; **a**: slope; **V**: Volume; **DF**: Dilution Factor; **m**: Dye mass.

Hydrolyzable and condensed tannins rates:

Hydrolyzable tannins rate:

To 60 µL of the extract, was adding a volume of 220 µL of a mixture of iron chloride III to 10^{-2} M and hydrochloric acid to 10^{-3} M (50 / 50 v/v), the absorption reading was made at 660 nm using the 96-Well quartz microplate (MP96 spectrophotometer, SAFAS). Absorbance read was related to the formula of relationship 3 [14]:

$$\mathbf{R}(\%) = \frac{\mathbf{A \times MW \times V \times DF}}{\boldsymbol{\varepsilon}_{\mathbf{mole}} \times \mathbf{P}} \quad (3)$$

A: Absorbance; ε_mole: 2169 of Gallic acid; MW: Molecular weight of gallic acid; V: Extract volume used;

P: Sample weight; DF: Dilution factor; R (%): Hydrolyzable tannins.

Condensed tannins rate:

To 90 µL of the extract was added 190 µL of a vanillin solution at 0.01% in concentrated sulphuric acid. The absorption is read at 500 nm using a 96-well quartz microplate reader (MP96 spectrophotometer, SAFAS). The rate of condensed tannins is obtained by reporting the absorption read in the formula of relationship (4) [15, 16].

$$\mathbf{R}(\%) = \mathbf{5,2.10^{-2}}\,\frac{\mathbf{A\times V}}{\mathbf{P}} \qquad (4)$$

$5{,}2.10^{-2}$: Constant expressed in cyanidin equivalent; A: Absorbance; V: Extract volume used; P: Sample weight; R (%): Condensed tannins rate.

High Performance Thin Layer Chromatography of dye extract:

The chromatographic profile of the natural dye was achieved by high performance thin layer chromatography (HPTLC) [17-19]. The principle was to deposit 3 µL extract with standard in the form of thin bands 8 mm long, on a 20x10 cm F_{254} HP silica gel plate using a CAMAG Automatic TLC AutoSampler 4 device.

After a saturation stage for 20 minutes, plaque development was achieved using the mobile phase acetate of ethyl-formic acid-acetic-water (100:11: 11:26 v/v).

To observe polyphenols in general and flavonoids in particular, two specific revelators, successively 2-aminodiphenylborate (1 g in 100 mL of methanol) and polyethylene glycol (5 g in 100 mL of ethanol at 96 %), were used to spray the plate which is then heated to 110 °C for 3 minutes.

Analysis of dye by infra-red to transformed Fourrier (IR-TF):

The direct use of raw natural dyes is justified in part by the difficulty of isolating sufficient quantities of dyes for textile dyeing, given the molecular complexity of mixtures at the level of natural substances. For example, infra-red spectra (IR) are often recorded on raw extracts to provide a brief erict of the chemical functions present in the total extracts, which are responsible for observing various final colour shades of dyed textile material [20, 21]. Thus, the total dye extract containing tannins, flavonoids, xanthonoids and other compounds is subjected to an infra-Red spectrometry analysis to Fourrier Transformed (IR-TF) type TENSOR 27 for identification of functional groups present in constituents structures.

HPLC-MS of flavonoids from *A. leiocarpus* dye extract:

The dye extract from the *A. leiocarpus* leaves containing flavonoids, previously filtered using a syringe (type cellulose acetate membrane 25 mm and 0.45 µm) is characterized by high-performance liquid chromatography (HPLC) coupled with mass spectrometry (MS) at the National Laboratory of Public Health (LNSP) in Ouagadougou (Burkina Faso).

The ions are controlled in MS/MS in positive ionization mode (ESI). The HPLC separation is performed on a Zorbax sb C18 column (250 x 4.6 mm; 5 µm) [22, 23, 24].

The mobile phase consists of two solvents A and B in order to achieve an elity gradient with a flow rate of 0.6 mL/min. Solvent A is 5% formic water-acid (v/v), solvent B is made up of 5% formic acetonitrile-acid (v/v). The elion consisted of a 95 % to 90 % stage of solvent A for 5 minutes and then the gradient was maintained at 90 % of A for 10 minutes. Then 90 % to 88 % A in 10 minutes, to 85 % A in 10 minutes, 82 % A in 15 minutes, to 75 % A in 10 minutes, 70% A in 20 minutes and finally returned to 95 % A in 10 minutes (the total run was 90 min). The injection volume was 50 µL. The mobile phase was then injected into the electrospray ionization source.

The data were provided by Agilent Technologies, Inc. 2012 LC/MS Data Acquisition for 6400 Series Triple Quadripole (version B.06.00 Build 6.0.6025.0). The temperature of the gas (N_2) was 200°C, the gas flow of 10 L/min and the pressure of the nebulizer 15 psis. In the experience, Nebulizer and collision gas was nitrogen. The mass spectrometer scans all ions with a m/z value between 100 and 1100. Comparison of retention times and molecular weights of the observed fragments thus allowed the identification of flavonoid molecules. Indeed, the principle was to inject the sample in solution at a constant flow of about 0.6 µL/min into a conductive capillary. A desolvatation gas (N_2 sec) with an approximate flow of 500 L/h, surrounds the capillary and promotes the vaporization of the sample. When the potential of the capillary is positively charged, the positive ions generated will be analyzed and vice versa. The ions generated inside the device are drawn to the extraction cone where they will be fragmented. These fragmentations will continue in the collision cell during tandem mass spectrometry analyses [25, 26].

This characterization of flavonoids in ESI-MS/MS was based on generation in the source of ionization of the aglycone patterns of each flavonoid compound. As a result, the tandem MS analysis of molecular ions resulted in different fragmented ions specific to each type of aglycone.

Tinctorial practice:

Unwinding:

It consisted of ridding the textile material (Cotton wire swaths) of wax, pectin and other dirt that could influence the fixation of the dye [27, 28, 29].

Approximately 70 g of skein cotton thread was immersed in 700 mL of water brought almost to a boil (about 90ºC), to which are added 700 mg of neutral soap (SN CITEC soap or Marseille soap) and 3.5 g of sodium carbonate.

After 1 hour of boiling, 0.7 mL of acetic acid is added to the set for the neutralization of the base. The skein was then rinsed thoroughly with water and washed with neutral soap and dried in a room.

Mordancing:

In natural tinctorial practice, several kinds of biting chemical and plant origins (tannins) are used. Three types of biting [30, 31, 32] were used: alum [$KAl(SO_4)_2$, $12H_2O$], hydrated iron sulphate ($FeSO_4$, $7H_2O$) and hydrated copper sulphate (CuSO4, 5H2O).

140 mg of each type of bite is dissolved in 30 mL of hot distilled water. Then, sodium hydroxide (14 mg) is added for the revelation of different types of ions and their colors (Al^{3+} white, Fe^{2+} pale green, Cu^{2+} blue).

A precise mass of small skeins of yarn (700 mg each) was immersed in each lukewarm mixture and the whole has been kept for 12 hours.

The skeins are then removed and strongly wrung out and placed in lockdown in plastic bags for 72 hours before dyeing.

Dyeing with *Anogeissus leiocarpus* leaves extract:

At this stage, two different dye baths (acid and basic) were prepared in acid and basic baths.

The principle was to immerse the unst chlorinated and bitten yarn tangles (700 mg each) in acidic and basic baths of each type of dye.

The dye rate was set at 4 % and so each bath was obtained by dissolving 28 mg of each type of dye in 14 mL of distilled water.

Thus, after adjusting the pH to 4 for the acid bath and 10 for the basic bath using the mobile pH (826 pH mobile / 827 pH lab, Ω Metrohm), each bath was brought to a temperature of about 70 °C before immersion of the skeins.

These skeins were constantly stirred in the bath to facilitate the homogenization of the dye.

After 45 minutes, the skeins were removed from the bathing area and spread out in the open air in dirty, and then 10 minutes later they were rinsed thoroughly and spread out again. Then, 1 hour later, they were washed with soap (Marseille soap) and then dried permanently in the room [33, 34, 35]. Soap washing is one of the first tests of strength carried out on dyed fibres and fabrics. Other tests such as light, chlorinated water or sweat tests are corroboration tests of wash test results.

For the assessment of the homogeneity of the dye, a magnifying glass (classical-black, Magnification: 3.5x, 5x 50 mm, 60 mm, 75 mm) was used for careful observation and this allowed the development of a scale of assessment of the homogeneity of the fixation, ranging from 1 to 4 (1: non-homogeneous; 2: medium homogeneity; 3: fairly good homogeneity; 4: very good homogeneity).

RESULTS:

Yield, Total flavonoids content (TFC), hydrolyzable tannins rate (HTR) et condensed tannins rate (CTR):

Quantitative results (Table 1) showed that the *A. leiocarpus*, with an extraction yield of 4.94 %, contain total flavonoids content of 458.759 ± 27.773 mgEQ/g of dye powder, hydrolysable and condensed tannins rates of 13.25 % and 12.96 % respectively.

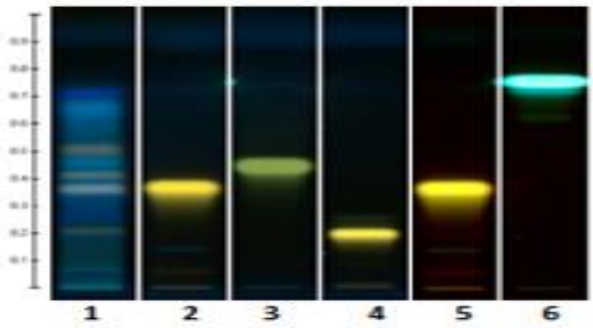
Table 1: Yield, Total flavonoids Content (TFC), hydrolyzable tannins rate (HTR) et condensed Tannins rate (CTR) of *A. leiocarpus* leaves extract

Extrait	Yield (%)	TFC (mg / g)	HTR (%)	CTR (%)
A. leiocarpus	4.94	458.759 ± 27.773	13.255 ± 0.011	12.965 ± 0.245

HPTLC flavonoids profile and IR-TF spectrum:

Chromatographic profiles, in accordance with other works found in literature, indicated the presence of flavonol derivatives (quercetin, kaempferol...) [36, 37].

In addition, a review of infrared spectral data revealed the presence of essential chemical functional groups characterized by elongation vibrations.



1: *A. leiocarpus*, 2: Hyperoside (3-O-galactoside of quercetin);
3: Isorhamnetin 3-O-glucoside; 4: Rutin;
5: Hyperoside (Quercetin 3-O-galactoside); 6: Kaempferol

Figure 1: HPTLC flavonoids profile of *A. leiocarpus* extract at 366 nm

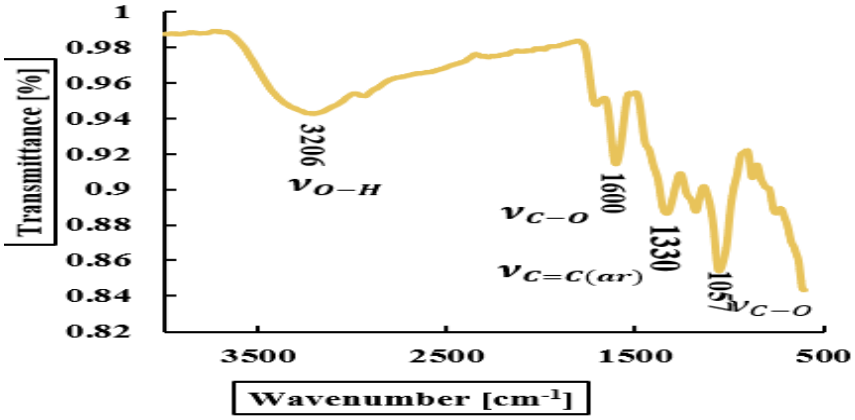


Figure 2: IR-TF spectrum of *A. leiocarpus* extract

HPLC-MS/MS of major flavonoids:

The aqueous extract of *A. leiocarpus* produced three major flavonoids, two of which are isomers at 449 m / z and one at 479 m / z.

Table 2: HPLC-MS/MS of major flavonoids

Peaks	Retention Time (min)	Formula	molecular Ions $[M+H]^+$ (m/z)	Secondary Ions (m/z)	Proposed compound
1	42.4	$C_{21}H_{20}O_{11}$	449	303	Quercetin 3-O-rhamnose
2	45.4	$C_{21}H_{18}O_{13}$	479	303	Quercetin 3-O-glucuronide
3	56.7	$C_{21}H_{20}O_{11}$	449	287	Kaempferol 3-O-hexose

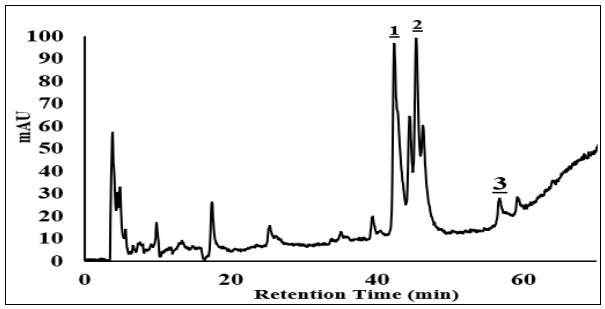


Figure 3: Chromatogram of flavonoids *A. leiocarpus* extract

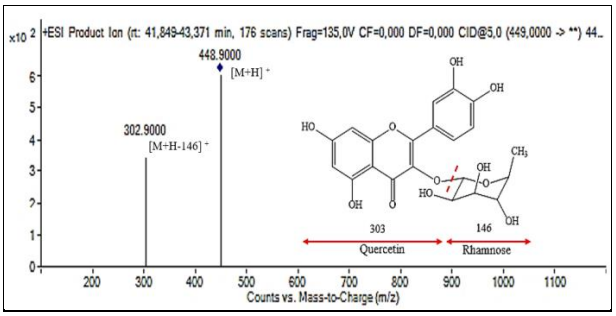


Figure 5: ESI$^+$ MS/MS spectrum of compound 1

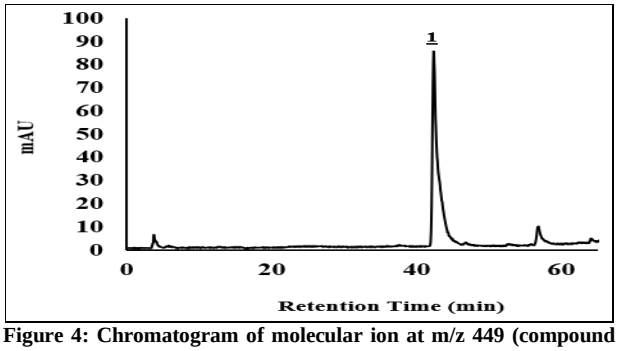


Figure 4: Chromatogram of molecular ion at m/z 449 (compound 1)

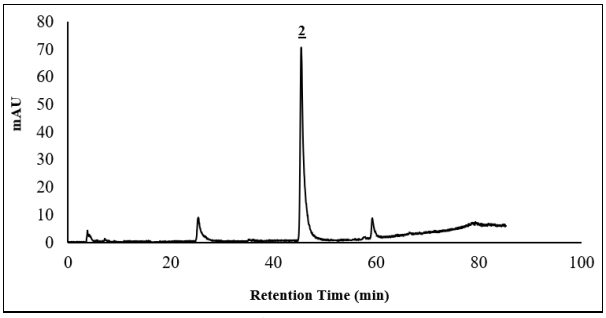


Figure 6: Chromatogram of molecular ion at m/z 479 (compound 2)

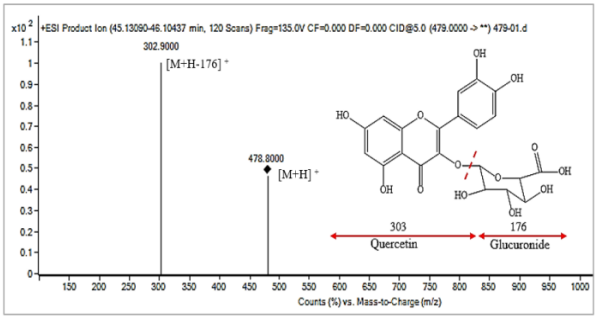


Figure 7: ESI$^+$ MS/MS spectrum of compound 2

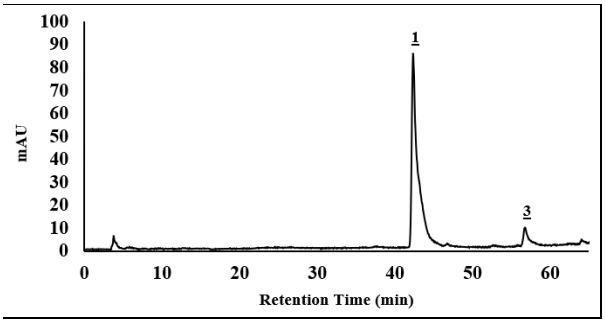


Figure 8: Chromatograms of compounds 1 et 3 at retention times RT = 42.4 and 45.4 min

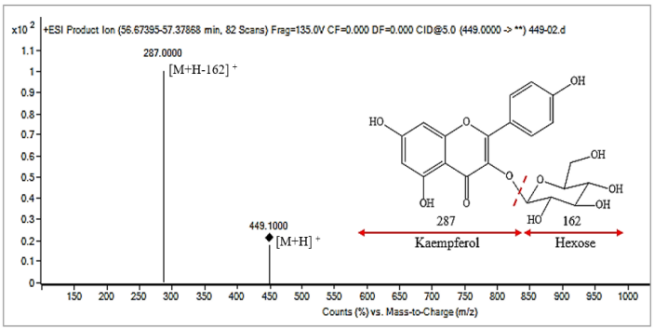


Figure 9: ESI$^+$ MS/MS spectrum of compound 3 (m/z 449)

Table 3: Results of tinctorial practice with *A. leiocarpus* dye extract

	**Without mordant**		**Alum ($KAl(SO_4)_2$, $12H_2O$)**		**Hydrated iron sulphate ($FeSO_4$, $7H_2O$)**		**Hydrated copper sulphate ($CuSO_4$, $5H_2O$)**	
Dye bath	Acid	Basic	Acid	Basic	Acid	Basic	Acid	Basic
Fixation	2	2	3	3	3	3	3	3
Homogeneity	1	1	4	2	4	4	3	4
FQ = CFxCH	**2**	**2**	**12**	**6**	**12**	**12**	**9**	**12**
Quality	***Poor***	***Poor***	***Excellent***	***Good***	***Excellent***	***Excellent***	***Very good***	***Excellent***
Shades of colors	*Anise*	*Anise*	*Lime Anise*	*Anise pistachio*	*Military anise*	*Anise green of gray*	*Olive*	*Chartreuse green*
Not dyed								
Skeins dyed								

Anise = greenish yellow
***Legend**: **Fixation** (Scale from 1 to 3): 1: non fixation; 2: medium fixation; 3: fairly good fixation*
***Homogeneity** (Scale from 1 à 4): 1: non-homogeneous; 2: medium homogeneity; 3: fairly good homogeneity; 4: very good homogeneity.*
***FQ**: quality factor de; **CF**: coefficient of fixation; **CH**: coefficient of homogeneity – **FQ** ≤ 3: bad quality*
***QF** ∈]3**;** 6[: very fair quality; **FQ** = 6: good quality; **FQ** ∈]6; 9]: very good quality; **FQ** ∈]9; 12]: excellent quality*

RESULTS OF TINCTORIAL PRACTICE:

Dyeing practice on cotton fibers allowed for various shades of colors based on the protocols described above

DISCUSSION:

Beyond the quantitative aspect (Table 1), the dye extracted from the leaves of *A. leiocarpus* on the chromatographic (HPTLC) and spectral (IR-TF) level, showed the presence of a subgroup of flavonoids (flavonol) characterized partly by the detection of essential chemical functions in IR-TF (Figures 1 and 2)

Natural dyes, as used in natural dyeing, are complex mixtures of molecules. Each molecule has chemical functions that give it particular properties. The most information-rich and experimentally accessible part is the medium IR (4000 to 400 cm^{-1}). In this area, absorptions corresponding to wavenumbers less than 800 cm^{-1} form a kind of fingerprint of compounds which allows to recognize them and those greater than 800 cm^{-1} were characteristic of the chemical bonds present in the sample, allowing functional and structural analysis[38]. The IR spectrum of the dye extracts has characteristic bands corresponding to specific functional groups (Figure 3). Thus, the observed wavenumber at 3206 cm^{-1}

characterizes an elongation vibration ν_{O-H} corresponding to the functional hydroxyle OH bound alcohol group. Other wavenumbers at 1330 cm^{-1} and 1057 cm^{-1} corresponding to the ν_{C-O} elongation vibration refers to the presence of functional C-O group in the extract, indicating the functions of either esters or oxide ethers (Figure 3). According to the literature, *A. leiocarpus* (ν_{O-H} : 3206 cm^{-1} ; ν_{C-O} : 1330-1057 cm^{-1}) contains flavonoids belonging to the subgroups of flavonols and flavanols [39, 40].

HPLC of the total extract of *A. leiocarpus* leaves presented three (03) essential peaks in the investigation of flavonoids [**1**: RT = 42.4 min; **2**: RT = 45.4 min; **3**: RT = 56.7 min].

Compounds **1** and **3** are isomers whose molecular ion at m/z $[M+H]^+$ 449 u ($C_{21}H_{20}O_{11}$). MS/MS secondary ions at m/z 303 u and 287 u respectively corresponding to losses of rhamnose $[M+H-146]^+$ and hexose that may be either glucose or galactose $[M+H-162]^+$ respectively.

The molecular ion at m/z 479 u ($C_{21}H_{18}O_{13}$) corresponding to compound **2**, produced in MS/MS a secondary ion at m/z 303 u following to the loss of glucuronide $[M+H-176]^+$.

The peaks at m/z 449 u of compound **1** and **3** would be quercetin 3-O-rhamnose and kaempferol 3-O-hexoside respectively. The peak at m/z 479 of compound **2** would correspond to quercetin 3-O-glucuronide.

These flavonic compounds in association with many other phenolic compounds (tannins, phenolic acids...) present in *A. leiocarpus* leaves extract such as 3,4,'-tri-O-methylflavellagic acids and its glucosylated derivative (3,4,3'-tri-O-methylflavellagic-4'-β-D-glucose), leiocarpanes A and B glucuronomannanes, castalagin [41-44] would explain the different colors obtained in tinctorial practice. These hydroxylated compounds, beyond the intermolecular association, often pass through chélator metals (Al^{3+}, Fe^{2+}, Cu^{2+}) for the creation of complexes favorable to the fixation on the monomer unit (D-glucopyranose) of the cellulose of cotton fibers. These complexes are preferred to the hydroxylated sites 2,3, and 6 of D-glucopyranose [45, 46]. In addition, the affinity of the dye molecules with the cellulosic fiber and their fixation on the cotton fiber, depends on the acid or basic nature of the dye bath.

The tinctorial practice has allowed the obtaining of very different shades of color with a fairly good fixation and a fairly good homogeneity.

Dyeing (Table 3) realized with *A. leiocarpus* leaves extract resulted in a range of characteristic shades of color (anise lime, military anise, grey green anise, chartreuse green...). The quality factor (QF) based on the product of the degree of fixation by the degree of homogeneity allowed to obtain significant strong color shades using alum in acidic medium, hydrated iron sulphate in acid and basic environments and copper sulphate in a basic medium. The fixation of molecules of the coloring material from *A. leiocarpus* leaves requires the intervention of mordants making it a mordanting or metal-bearing dye.

CONCLUSION:

This study allow to understand that *A. leiocarpus* leaves contain flavonoids, which, through intra- or intermolecular associations, contribute to obtain shades of colors in tinctorial practice.

Total flavonoids content from *A. leiocarpus* leaves was about 458.759 mg of EQ/g of dye powder and total hydrolyzable and condensed tannins rates were respectively, of 13.255 % and 12.965 %. The natural dye from the dry leaves of *A. Leiocarpus*, in a dyeing process with alum, iron and copper sulphates as mordants allow the obtaining of various shades of colors. These shades would explain the affinity of flavonols such as kaempferol 3-O-hexoside and quercetin 3-O-glucuronide with the mordants used (alum, iron sulphates and copper). This affinity therefore justifies the chelation of these compounds with the metal salts (Al^{3+}, Fe^{2+}, Cu^{2+}) already fixed by mordanting on cotton fiber (cellulose) whose monomer unit is D-glucopyranose where hydroxyl groups in positions 2, 3 and 6 (active sites) allowed chelation. Dye from the *A. leiocarpus* leaves, can be classified in groups of natural mordanting dyes, so-called metal dyes.

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