Development of a chemical tracer and a chromatographic fingerprint for the quality control of finished products based on hydroethanol extract of fresh leaves of Cassia alata (L.) Roxb (Fabaceae.)

Développement d'une empreinte chromatographique et d'un traceur chimique pour le contrôle analytique du savon antimicrobien à base d'extrait hydroéthanolique de feuilles fraîches de Cassia alata (L.) Roxb (Fabaceae.)

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Abstract

Cassia alata is used in the treatment of dermatoses. Quality control of herbal products remains difficult and complex. This study aims to develop a quality control approach for a hydroethanol extract of fresh *Cassia alata* leaves (EHCA), raw material and its antimicrobial soap, the finished product.

The chromatographic fingerprint, phytochemical characterization of EHCA and soap were performed by the method of Wagner and Balt. The spectral pattern of the tracer compound was determined by UV/Visible spectrophotometry. The choice of the tracer compound and its monitoring in the antimicrobial soap was done according to the criteria of the European Medicines Agency.
The chromatographic fingerprint and phytochemical characterization of EHCA revealed anthracenosides, saponosides, tannins and flavonoids. An anthracene compound was characterized in EHCA at an Rf of 0.52 ± 0.01 at 365 nm, with 1.83 ± 0.01 as the resolution factor. Its UV/Visible spectral plot showed an absorption maximum at 223 nm and a detection limit of 2.19 ± 0.01 µg/mL. Its significant presence and formulation suitability was found in the antifungal soap.
The thin layer chromatography and UV-visible spectrophotometry allowed the identification of a tracer compound in the EHCA. The anthracenosides, due to their abundance and their adaptation to the formulation process of the antimicrobial soap, would be the most indicated for the monitoring of the quality of the extract and the finished product.
Key words: Cassia alata; quality control; extract; soap.

Résumé

Cassia alata est utilisée dans le traitement des dermatoses. Le contrôle qualité des produits à base de plantes reste difficile et complexe. Cette étude vise à mettre au point une approche de contrôle qualité d'un extrait hydroéthanolique des feuilles fraîches de *Cassia alata* (EHCA), matière première et de son savon antimicrobien, produit fini.

L'empreinte chromatographique, la caractérisation phytochimique de l'EHCA et du savon ont été réalisés par la méthode de Wagner et Balt. L'allure spectrale du composé traceur a été déterminée par spectrophotométrie UV/Visible. Le choix du composé traceur et son suivi dans le savon antimicrobien a été fait suivant les critères de l'agence européenne du médicament.
L'empreinte chromatographique et la caractérisation phytochimique de l'EHCA a mis en évidence des anthracénosides, saponosides, tanins et flavonoïdes. Un composé anthracénique a été caractérisé dans l'EHCA à un Rf de 0,52 ± 0,01 à 365 nm, avec 1,83 ± 0,01 comme facteur de résolution. Son tracé spectral UV/Visible a présenté un maxima d'absorption à 223 nm et une limite de détection de 2,19 ± 0,01 µg/mL. Sa présence significative et son adaptation à la formulation a été constaté dans le savon antifongique.
La chromatographie sur couche mince et la spectrophotométrie UV-visible ont permis de mettre en évidence un composé traceur dans l'EHCA. Les anthracénosides de par leur abondance et leur adaptation au processus de formulation du savon antimicrobien, seraient les plus indiqués pour le suivi de la qualité de l'extrait et du produit fini.
Mots clés : *Cassia alata* ; contrôle qualité ; extrait ; savon.

1. Introduction

In Africa, more than 80% of the population uses plants for health care [1]. *Cassia alata (L.) Roxb* belonging to the *Fabaceae* family is widely used in the treatment of dermatoses [2, 3]. *Cassia alata (L.) Roxb. (Fabaceae)* has anti-infective properties [3–13] and these properties were used in the formulation of a medical soap for the treatment of dermatosis. To facilitate production, quality controls on the extract and soap must be carried out. However, quality assessment of improved traditional medicines remains difficult as they are often derived from complex mixtures of several herbal drugs or herbal drug preparations to which mineral and/or animal drugs may be added [14]. Various selective and sensitive analytical techniques such as high performance liquid chromatography have already been successfully used in the analysis of improved traditional medicines [15–19]. But it should be noted that these techniques are expensive, and difficult to implement. Considering all these difficulties, and considering the advantages of thin layer chromatography and UV/Visible spectrophotometry, these techniques were used in the present study for the establishment of a chromatographic fingerprint and the search for a chemical tracer that can be used to control the quality of hydroethanol extracts of fresh leaves of *Cassia alata* (EHCA) and preparations based on these extracts, including soap.

2. Methodology

2.1. Material

A UV/Visible spectrophotometer (cary 3500 UV-Vis by agilent technologies; USA), a UV/Visible lamp (254 nm and 365 nm wavelength; CAMAG; France) and TLC silica gel 60 F_{254} plates (5 cm x 10 cm and 20 cm× 20 cm aluminum sheets; Merck; Germany) were used in this study. Hydroethanol extract of fresh *Cassia alata* leaves (EHCA) and soaps were supplied by phytofla laboratories of Banfora (Burkina Faso). Ethyl acetate (Merck, Germany), methanol (VWR CHEMICALS, Germany), ethanol 96% (VWR CHEMICALS, Germany), chloroform (VWR CHEMICALS, Germany) and distilled water (produced by Joseph KI-ZERBO university) were the solvents used.

2.2. Methods

2.2.1. Chromatographic fingerprint

Preparation of EHCA:
Fresh leaves were sampled in the Banfora area. After sampling, the fresh leaves were directly washed and ground with a locally made grinder with a mesh size of 2 mm.

The extraction was carried out by simple maceration by putting 20 g of the fresh leaves of *Cassia alata* crushed in 15 mL of hydroethanolic solution. During the maceration period which lasted 7 days, the mixture was stirred every morning and evening. After the 7 days of maceration, we proceeded to a filtration by taking care to press the material well to empty it. The filtrate obtained was then concentrated.

The concentration of EHCA was determined using a weighted thermodessiccation method. 5 mL of EHCA was placed in four previously tared watch glasses. These samples were placed in a ventilated oven (Memer, France) preset at 105°C for 1H30 min. After cooling for 30 min in a desiccator, the watch glasses and their contents were weighed using an analytical balance (Sartorius, France). The steaming operation was repeated for 30 min until a mass variation of the steamed test specimens of less than 0.05% was obtained. The concentration of the EHCA was calculated according to the following formula:

$$conc. = \frac{m}{Vpe}\ (mg/mL)$$

m: dry weight. Vpe: volume of test sample

Chromatographic conditions, revelation and Rf determinations:
10 µL of EHCA at a concentration of 44.20 ± 2 mg/L, were deposited on a silica gel 60 F_{254} chromatographic plate cast on an aluminum support (5 cm × 10 cm). The extract deposits were eluted over an 8 cm path. The mobile phase was a mixture of ethyl acetate/methanol/distilled water (7,7: 1,3: 1 v/v/v). The method described by Wagner and Balt was used for screening [20].

For spot revelation, the following chemical reagents for revelation were used:

- Coumarins and anthracenosides: 5% alcoholic potassium hydroxide solution,
- Saponosides: sulfuric anisaldehyde,
- Sterols and triterpenes: sulfuric vanillin in 1% ethanol,
- Flavonoids: sodium 2-aminoethyldiphenylborate or Neu's reagent,
- Total polyphenols (tannins): 2% alcoholic solution of iron chloride (FeCl3),

The chromatographic profile was observed in the visible and under a UV/Vis lamp (254 and 365 nm) before and after development.

The frontal ratios (Rf) were calculated according to the following formula:

$$Rf = \frac{\text{Distance traveled by the compound}}{\text{Solvent migration distance}}$$

2.2.2. Choice of chemical tracer

Choice of tracer:

1 mL of EHCA at the concentration of 44.20 ± 2 mg/L was deposited on a silica gel 60 F_{254} chromatographic plate (dimensions: 20 cm × 20 cm). Elution of the extract deposits was performed under the same mobile phase conditions as the chromatographic fingerprint and over an 18 cm run. After elution, the chromatography plate was removed and dried at room temperature, approximately 25°C. The developed chromatogram was observed under ultraviolet radiation at 254 nm and 365 nm. The majority spots; quite distant from the others were circled and scraped with a spatula. The extract compounds adsorbed by silica gel were solvated with 96% ethanol (2 x 25 mL). The obtained solutions were filtered on Wattman paper No. 5. The extracting solvents of the compounds were then evaporated under reduced pressure in the rotary evaporator (BÜCHI, Germany). The dry extracts of the observed compounds were dissolved in 1 mL of 96% ethanol for phytochemical characterization using chemical reagents specific to the different classes of compounds investigated. The choice of the tracer compounds was guided by the criteria of biological activity and physicochemical characteristics to allow its tracing in the finished product. For this purpose, the criteria established were those recommended by the European Medicines Agency [21].

Determining the evaluation parameters of the chemical marker:
Chemical characterization: The tracer compound was characterized by chemical tests specific to the different classes of secondary metabolites after elution by TLC in a mobile phase composed of a mixture of ethyl acetate/methanol/water in the proportions: 7.7/1.3/1: v/v/v. For the development of the spots, the specific development reagents used previously were used.

Resolution factor: The TLC resolution factor of the tracer compound was calculated using the difference between the frontal ratio of the tracer compound (Rf_2) and its nearest neighbor (Rf_1) in relation to the sum of the diameters of both compounds. The calculation of the resolution factor was done using the following formula:

$$R = \frac{2(Rf2 - Rf1)}{(W2 + W1)}$$

W2: Spot diameter 2; W1: Spot diameter 1; Rf_1: Spot front ratio 1; Rf_2: Spot front ratio 2.

Spectral pattern: The spectral pattern followed by absorbances of EHCA solutions at different concentrations (69 ± 0.01 µg/mL to 2.19 ± 0.01 µg/mL) were determined by scanning from 200 nm to 800 nm. The tracer compound was determined at a concentration of 4.31 ± 0.01 µg/mL. The absorption maximum of the spectral pattern of the tracer was used for its quantification in the total EHCA used to manufacture the antifungal soap.
2.2.3. Application to the analytical control of antimicrobial soaps

Preparation of the soap sample:
A mass of 25g of antimicrobial soap was dispersed in 100 mL of 96% hydroethanol solution. After complete dissolution, the sample was centrifuged at a speed of 9000 rpm for 10 min. The supernatant was collected in a glass jar for analysis. The genins of the soap extract were also extracted by acid hydrolysis according to the procedure described:

A 25 mL volume of the hydroethanol soap extract was placed in a ground-necked Erlenmeyer flask. To the test portion, 15 mL of a 10% hydrochloric acid solution was added. The acidic mixture was boiled under reflux for 30 min. After cooling, the alcohol was removed by distillation under reduced pressure with a rotavapor.

The residual acidic aqueous solution was transferred to a 100 mL separatory funnel. After addition of 10 mL of distilled water and cooling, it was extracted twice with 10 mL of chloroform. The organic phases were combined, returned to the separatory funnel and rinsed with distilled water until neutral pH was reached. The organic phase was recovered, dehydrated with anhydrous sodium sulfate and then filtered on Wattman paper. The filtrate obtained (total genins) was concentrated under reduced pressure in the rotary evaporator and dried in a ventilated oven at 40°C.

Characterization by chromatographic fingerprinting in the soap sample:
10 µL of hydrolysate with a concentration of 69 ± 0.01 µg/mL was used by thin layer chromatography to study the tracer compound in the finished soap product. Thin layer chromatographic analysis of the soap extract was performed under the same experimental conditions as those used for the EHCA analysis.

Determination of the chemical marker in the soap sample:
The tracer compound was characterized after elution by TLC in a mobile phase composed of a mixture of ethyl acetate/methanol/water in the proportions: 7.7/1.3/1: v/v/v. For the tracer revelation, the specific 5% alcoholic solution of potassium hydroxide used previously was used.

3. Results and discussion

3.1. Chromatographic fingerprint

EHCA had a mean concentration of 44.2 ± 2 mg/mL.

The chromatographic fingerprint of EHCA showed ten distinct spots at 365 nm and eight spots at 254 nm (Figure 1 and Table 1). For Nicaise François BONY, the chromatographic fingerprinting of phytochemical metabolites constitutes an important tool for the evaluation of the quality of extracts and its end products [14].

The phytochemical characterization performed on TLC plate with specific chemical reagents revealed the presence of various chemical groups including tannins, flavonoids, anthracenosides, leuco-anthocyanins, sterols/triterpenes and saponosides (Table 2, Figure 2). The presence of these phytochemical groups has been reported in previous studies [22–26]. Indeed, their presence justifies the use of *C. alata* in the treatment of skin infections (infectious dermatitis, eczema and scabies) and other infections [27]. The anthracenosides appeared abundant in EHCA. Muhammad et al, 2020 reported their high content in extracts of the genus *Cassia* [28]. Anthraquinones are quinone derivatives (oxygenated aromatic compounds) known to have antifungal and antibacterial activities [12, 14]. One of the characteristic anthracenoside spots of EHCA with orange-yellow coloration by day-light observation and reddish after application of Bornsträger's reagent was spotted at an Rf of 0.52 ± 0.01.

Henceforth, the following conditions should be used for chromatographic fingerprinting of EHCA: 10 µL of EHCA with a concentration of 44.20 ± 2 mg/L with a silica gel 60 F_{254} chromatographic plate cast on an aluminum support (5 cm × 10 cm) as stationary phase. The EHCA deposits were eluted over a path of 8 cm. The mobile phase should be a mixture of ethyl acetate/methanol/distilled water (7.7: 1.3: 1 v/v/v)

Table 1: Frontal ratio (Rf) of the main visible spots at 365 nm and 254 nm of the EHCA.

Spots	Frontal Ratio	Color	
		254 nm	365 nm
1	0,91 ± 0,01	Black	Red
2	0,90 ± 0,01	Black	Green
3	0,80 ± 0,01	Black	Blue
4	0,73 ± 0,01	Black	Red
5	0,71 ± 0,01	Black	Blue
6	0,64 ± 0,01	Black	Green
7	0,52 ± 0,01	Black	Yellow-orange
8	0,42 ± 0,01	Black	Blue
9	0,35 ± 0,01	-	Green
10	0,17 ± 0,01	-	Purple

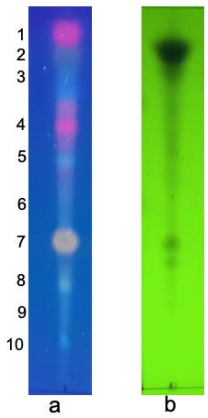
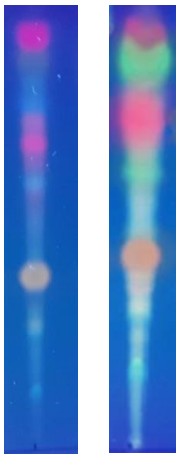


Figure 1: Chromatographic fingerprint of EHCA: a: 365 nm; b: 254 nm

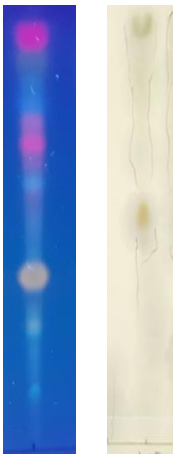
Table 2 : Phytochemical characterization of EHCA

Chemical groups	Specific reactions	observations
Flavonoids	Neu reaction	+
Anthracenosides	Bornsträger reaction	+
Leuco-anthocyanins	Bate smith reaction	+
Tannins	$FeCl_3$ reaction	+
Sterols/triterpenes	Sulfuric vanillin reaction	+
Saponosides	Sulfuric anisaldehyde reaction	+
Alkaloids	Dragendorff reaction	-
Coumarins	Reaction with KOH 5%	-



before after

Flavonoids (brown and green spots 366 nm)



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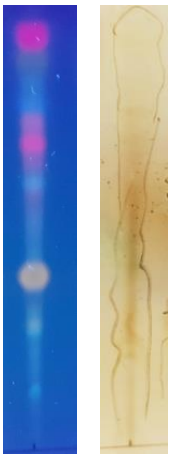
Tannins (revelation in the visible)



before after

Saponosides (revelation in the visible)

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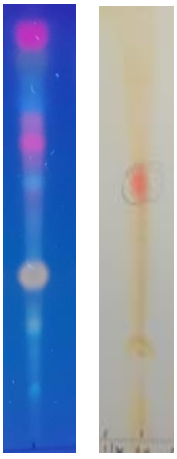
Sterols & triterpenes (revelation in the visible)



beforee after

Anthracenosides (revelation in the visible)

Figure 2: Phytochemical screening of EHCA

3.2. Choice of chemical tracer

the criteria of activity, quantity and stability established by the european medicines agency were considered for the choice of the tracer [21].

The chemical characterization of the EHCA after separation by TLC of the various constituents allowed to choose the anthracenosides which are counted among the major compounds characteristic of the *Cassia* genus [29].

The anthracenosides are secondary metabolites with pharmacological interest, particularly antifungal and antibacterial properties [25, 30–32]. Bioactive molecules such as aloe-emodin, emodin, alatinone belonging to this chemical group have already been isolated from *C. alata* [16, 33]. The anthracenosides in the case of EHCA, meet two of the criteria of the EMA which recommends that a tracer compound must be in sufficient quantity and bioactive [21].

The TLC analysis allowed the determination of the resolution factor (1.83 ± 0.01) to ensure a good separation of the tracer compound from the other components of the EHCA and to indicate its belonging to the group of anthracene compounds.

The UV-Visible spectrophotometry analysis of a hydroethanol solution of the tracer compound gave a spectral pattern that showed an absorption maximum at 223 nm and minima at 274 nm; 284 nm; 292 nm (Figure 3). The absorption maximum was followed by measuring absorbances in a series of dilutions (69 µg/mL to 0.55 µg/mL) of EHCA. A good determination coefficient of 0.99 and a detection limit of 2.19 µg/mL have been obtained with the different dilutions of EHCA (Table 3 and Figure 4).

The monitoring of the stability of EHCA could be done by UV-visible spectrophotometry measurement of the tracer compound at the maximum absorbance wavelength.

The following conditions should be used for the investigation and determination of the tracer in the soap sample: 10 µL of the hydrolysate with a concentration of 69 ± 0.01 µg/mL should be deposited on a silica gel 60 F_{254} chromatographic plate cast on an aluminum support (5 cm × 10 cm). Deposits should be eluted over an 8 cm path. The mobile phase should be a mixture of ethyl acetate/methanol/distilled water (7.7: 1.3: 1 v/v/v). For the tracer revelation, the specific 5% alcoholic solution of potassium hydroxide used previously was used.

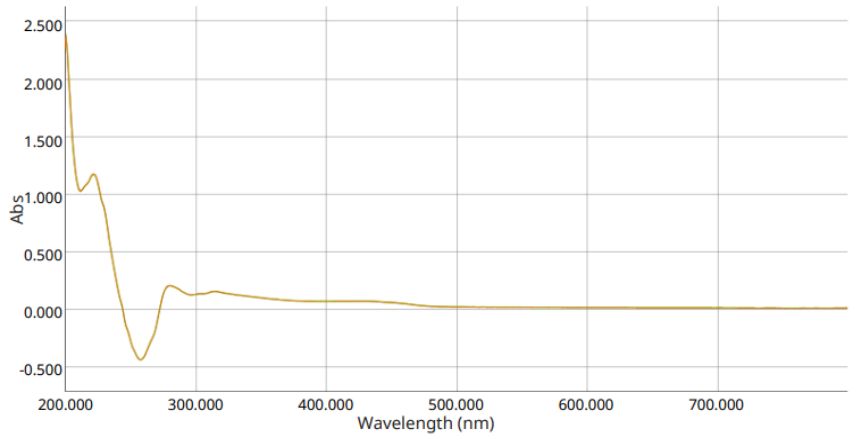
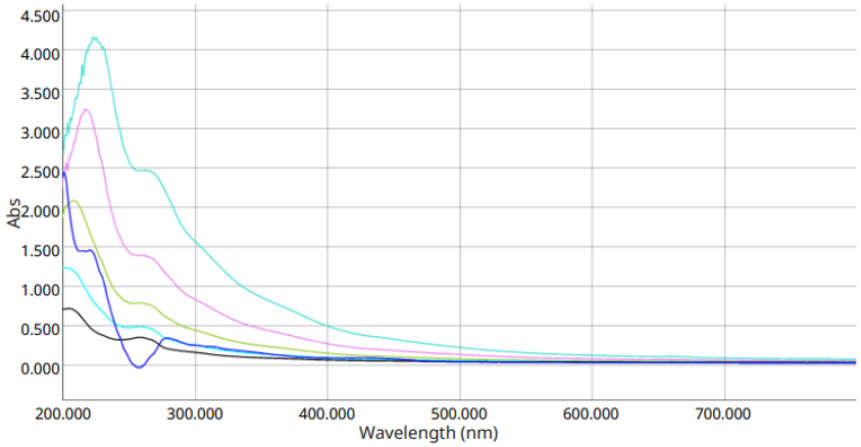


Figure 3: UV-visible spectral pattern of the EHCA tracer compound

Table 3: The detection limit of the tracer compound in the total EHCA

Concentration (µg/ml)	69	35	17,5	8,75	4,38	2,19
Absorbance (nm) at 223 nm	0.993 ± 0.004	0.823 ± 0.003	0.447 ± 0.002	0.221 ± 0.003	0.100 ± 0.004	0.045 ± 0.005



Legend: - EHCA - EHCA 1/2 - EHCA 1/4 - EHCA 1/8 - EHCA 1/16 - Tracer

Figure 4: Spectral pattern of the EHCA dilution series

3.3. *Application to the analytical control of antimicrobial soaps*

The different characteristics of the tracer compound allowed monitoring its stability in the antimicrobial soap. Thin layer chromatographic analysis of the antimicrobial soap extract showed the presence of the tracer compound (Figure 5) at the identical Rf of 0.52 ± 0.01. The tracer compound showed stability against formulation processes and could therefore be used as a reference for the quality control of EHCA based antimicrobial soap. Since the selected tracer compound was found in both the raw material and the finished product (soap), it meets one of the EMA criteria [21].

The following conditions should be used for the investigation and determination of the tracer in the soap sample: 10 µL of the hydrolysate with a concentration of 69 ± 0.01 µg/mL should be deposited on a silica gel 60 F_{254} chromatographic plate cast on an aluminum support (5 cm × 10 cm). Deposits should be eluted over an 8 cm path. The mobile phase should be a mixture of ethyl acetate/methanol/distilled water (7.7: 1.3: 1 v/v/v). For the tracer revelation, the specific 5% alcoholic solution of potassium hydroxide used previously was used.

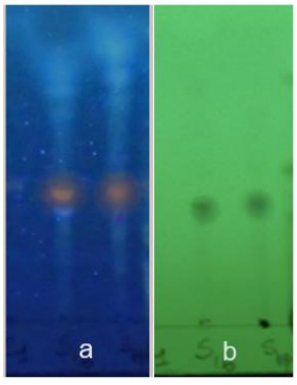


Figure 5: Chromatographic fingerprint of the antimicrobial soap: a: 365 nm; b: 254 nm

Conclusion

The present study showed that the quality of EHCA serving as raw material and the finished product, antimicrobial soap could be monitored by thin layer chromatography and UV-Visible spectrophotometry analysis. The group of anthracenosides would be the most indicated as marker for their biological activities, their abundance and stability in the formulation process, for the quality control of the raw material and the finished product.

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