



Original Research Article

**Purification and Identification of some Anthocyanins from *Hibiscus sabdariffa*,
a Medicinal Plant of the Ivorian Pharmacopeia**

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| Abstract | Keywords |
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| <p><i>Hibiscus sabdariffa</i> is a medicinal and food plant rich in phytochemical compounds which are the source of its biological properties. The present work was conducted in objective to identify the anthocyanins which are medicinally important compounds by the methods such as CPC and HPLC. The results showed that four <i>Hibiscus</i> anthocyanins were isolated and identified from petals of <i>Hibiscus sabdariffa</i>. It is cyanidin 3-O-sambubioside, delphinidin 3-O-sambubioside cyanidin 3-O-glucoside and delphinidin 3-O-glucoside with cyanidin 3-O-sambubioside and delphinidin 3-O-sambubioside as the major compounds of the petal of this plant. The presence of these phytochemical compounds justifies its uses in folkloric medicines.</p> | <p>Anthocyanins Cyanidin 3-O-glucoside Cyanidin 3-O-sambubioside Delphinidin 3-O-glucoside Delphinidin 3-O-sambubioside <i>Hibiscus sabdariffa</i></p> |

Introduction

In recent times, focus on plant research has increased all over the world and a large body of evidence has collected to show immense potentials of medicinal plants used in various traditional systems. Various medicinal plants have been studied using modern scientific approaches. Ethnobotany and ethnopharmacognosy, the basis of useful knowledge on plants in their relationship with traditional or popular

therapeutic uses, constitute a guide for chemical, pharmacological and physiological studies that allow the establishment of a scientific foundation for supposed therapeutic properties. The results from these plants have revealed the potentials of medicinal plants in the area of pharmacology (Kone et al., 2011 and 2012; Obouayeba et al., 2015a and 2014b).

Hibiscus sabdariffa L., a member of the Malvaceae family, is an annual dicotyledonous herbaceous shrub

popularly known as Roselle (English), l'oiselle (French), karkade (Arabic) and bissap (Wolof). This plant is well known in Asia and Africa and is commonly used to make jellies, jams and beverages. In Cote d'Ivoire, it is a highly source of vegetable food. Indeed, young leaves and stems are eaten raw or cooked in salads, and as a seasoning in curries. The dried petals are used in the preparation of local nonalcoholic cold beverage and as a hot drink highly appreciated in Cote d'Ivoire. This nonalcoholic drink called bissap prepared from the red petals is popular and highly appreciated by population in most of the West African countries (Obouayeba et al., 2014a). In folk medicine, it has been used to treat hypertension (Haji and Haji, 1999), inflammatory disease (Dafallah and Al-Mustafa, 1996) and cancer (Chewonarin et al., 1999). The flowers of *Hibiscus sabdariffa* contain anthocyanins, flavonoids and polyphenols (Lin et al., 2007). Studies have highlighted the role of polyphenolic acid, flavonoids and anthocyanins that may act as antioxidants or have other mechanisms contributing to the cardio protective actions (Rimm et al., 2000).

Anthocyanins are members of the flavonoid group of phytochemicals that are widely distributed in nature, which are responsible for the attractive colors of many flowers, fruits, grains and related products derived from them (Wu and Prior, 2005). Anthocyanins are water-soluble glycosides and acylglycosides of anthocyanidins, and they are found in the form of polyhydroxylated and or methoxylated heterosides which derive from the flavylium ion or 2-phenylbenzopyrylium in nature (Wu et al., 2004). Six anthocyanidins are widespread in fruits and vegetables, which are cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin (Kerio et al., 2012). These compounds are based on the same 2-phenylbenzopyrylium (flavylium) skeleton hydroxylated in 3, 5, and 7 positions and different in the number and position of hydroxyl and methoxyl groups in the B-ring (Escribano-Bailon et al., 2004). Anthocyanins are valuable as kinds of important quality indicators in foods and chemotaxonomic indicators in plants. The roles of anthocyanin pigments as medicinal agents have been well-accepted dogma in folk medicine throughout the world, and, in fact, these pigments are linked to an amazingly broad-based range of health benefits. Recent research has shown that anthocyanins have numerous health beneficial properties, which include antioxidant (Christian and Jackson, 2009; Obouayeba et al., 2014a), anticarcinogenic (Chang et al., 2005; Hou et al., 2005), antimicrobial (Viskeliš et al., 2009), anti-inflammatory

(Obouayeba et al., 2015b), cardioprotective (Ojeda et al., 2010; Obouayeba et al., 2015b) and hepatoprotective (Obouayeba et al., 2014b) properties.

The regular and intensive use of the juice obtained from the petals of *Hibiscus sabdariffa* as beverage in various ceremonies in West Africa in general and particularly in Cote d'Ivoire led us to initiate this study. The aim of this work was to carry out the phytochemical study of the petals extract of *Hibiscus sabdariffa*. This will generate more knowledgeable information on their potentiality for a wider utilization.

Materials and methods

Plant material

The petals of *Hibiscus sabdariffa* were used as plant material in the present study. The material was purchased from a local market in Adjame (Abidjan, Cote d'Ivoire). The petals were cut, cleaned, washed thoroughly under running tap water, drained and oven-dried at 55 °C for 12 hrs. The samples were packed in polyethylene bags and stored at 4 °C for laboratory analysis.

Drugs and chemicals

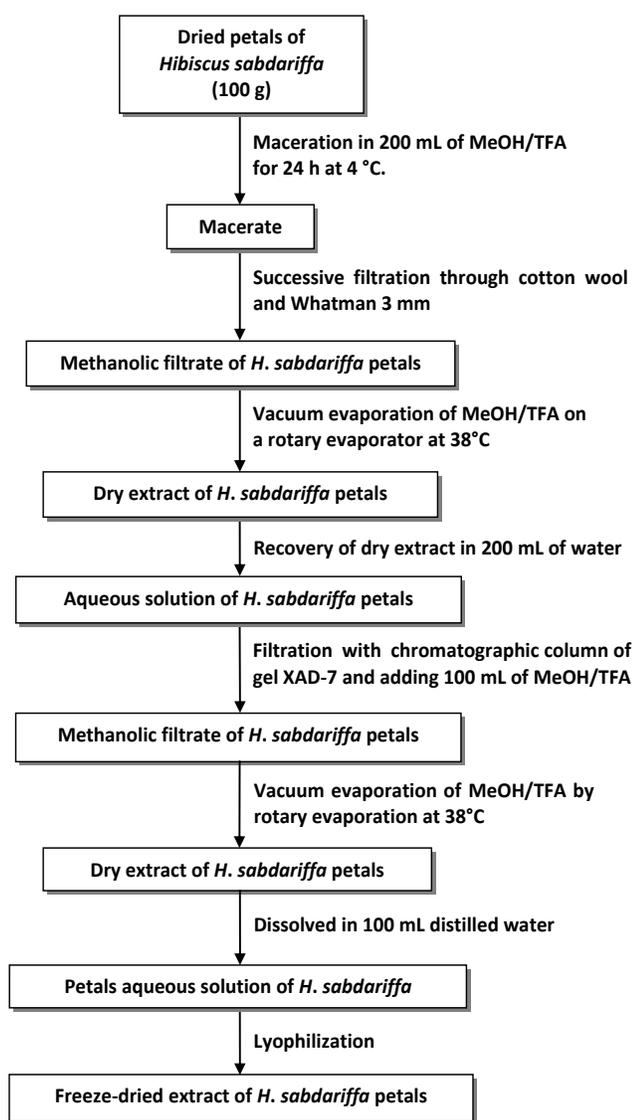
All reagents, solvents and chemical compounds used for analysis met the quality criteria in accordance with international standards. Anthocyanins standards (cyanidin, delphinidin, malvidin, cyanidin 3-O-glucoside, delphinidin 3-O-glucoside and malvidin 3-O-glucoside) were purchased from Sigma-Aldrich (Steinheim, Germany). The trifluoroacetic acid (TFA), methanol (MeOH), n-butanol (n-BuOH), acetic acid and ethyl acetate (EtOAc) were obtained from Merck (Darmstadt, Germany).

Extract preparation

The extract was prepared according to the method of Kouakou et al. (2009). One hundred grams (100 g) petals of *Hibiscus sabdariffa* were extracted from 200 mL of acidified methanol with trifluoroacetic acid 0.1 % (v/v) for 24 h at 4°C. The macerate was filtered successively on cotton wool and Whatman paper. After low-pressure vacuum evaporation of the methanol in BÜCHI Rotavapor R-114 at 38°C, the dry extract was obtained. Two hundred milliliters (200 mL) of distilled water were added to the dry extract and the aqueous

solution was submitted to a filtration on gel XAD-7, in order to eliminate sugars and chlorophyll pigments. One hundred milliliters (100 mL) of acidified methanol with trifluoroacetic acid 0.1 % (v/v) were poured over the gel XAD-7 and the methanolic filtrate obtained was resubmitted to low-pressure vacuum evaporation in BÜCHI Rotavapor R-114 at 38 °C. The dry extract obtained was dissolved in 100 mL of distilled water. The aqueous solution was lyophilized with the freeze dryer CHRIST ALPHA 1-2. The dried extract obtained represented the petals extract of *Hibiscus sabdariffa* (PEHS) which polyphenols content and compounds were previously determined by Obouayeba et al. (2014a) as presented in Fig. 1.

Fig. 1: Diagram of obtaining from the petals extract of *Hibiscus sabdariffa*.



Centrifugal Partition Chromatography analysis

Analysis by centrifugal partition chromatography (CPC) was performed according to the method described by Bouat-Cottards and Burgaud (2005). The apparatus used to carry out the CPC is the FCPC 200[®] provided by Kromaton Technologies (Angers, France). Quaternary biphasic solvent systems were prepared by mixture of ethyl acetate/n-butanol/water/ trifluoroacetic acid (50/50/900/1, v/v) for the stationary phase and (400/460/140/1, v/v) for the mobile phase at 25°C. Two phases were obtained in each case, an aqueous phase and an organic phase. The solvents were pumped by a Gilson 321 binary pump-H1, two-way high pressure gradient. The FCPC 200[®] column was filled with the stationary phase (aqueous phase) to 300 rpm in ascending mode. Two grams (2 g) of the calyces extract of *Hibiscus sabdariffa* were dissolved in 8 mL of a mixture of stationary phase and mobile phase (1/1, v/v) and were then introduced into the column CPC through a high pressure injection valve (3725 (i) 038 Rheodyne) equipped with a sampling loop 10 mL. The effluent was monitored with a UV-1010 detector Lambda equipped with a preparative flow cell. The rotor speed was increased to 1000 rpm. The organic phase from the mobile phase was then pumped into the column in ascending mode at a flow rate of 3 mL/min. Fractions of 9 mL were collected every minute by a fraction collector Gilson FC 204. The back pressure was 25 bars. The stationary phase retention at the end of the separation represented 75 % of the column volume. The experiments were conducted at room temperature.

Thin Layer Chromatography analysis

All the fractions were checked by thin layer chromatography (TLC) cellulose plates (Merck) and developed with n- butanol/acetic acid/water (4/1/5, v/v) upper phase.

High Performance Liquid Chromatography analysis

High performance liquid chromatography (HPLC) analysis was conducted using the method described by Drust and Wrolstad (2001). The analyses were carried out on a HPLC (Agilent), model-LC 1100 series, equipped with a degasser, an autosampler automatic injector, a high pressure pump and a UV/Visible detector at multiple wavelengths wave, and running on Windows XP Workstation. HPLC experiments were conducted using a ProntoSil C-18 column (5 µm particle size, 250 x 4 mm I.D.) with a flow

rate of 1 mL/min at room temperature. The mobile phase used was a binary gradient eluent (solvent A, 0.1 % trifluoroacetic acid in water; solvent B, 0.1 % trifluoroacetic acid in acetonitrile). Acetonitrile (MeCN) used was of HPLC grade (Sigma/Aldrich) and was degassed in an ultrasonic bath before using. The water was distilled using a Milli-Q system (Millipore).

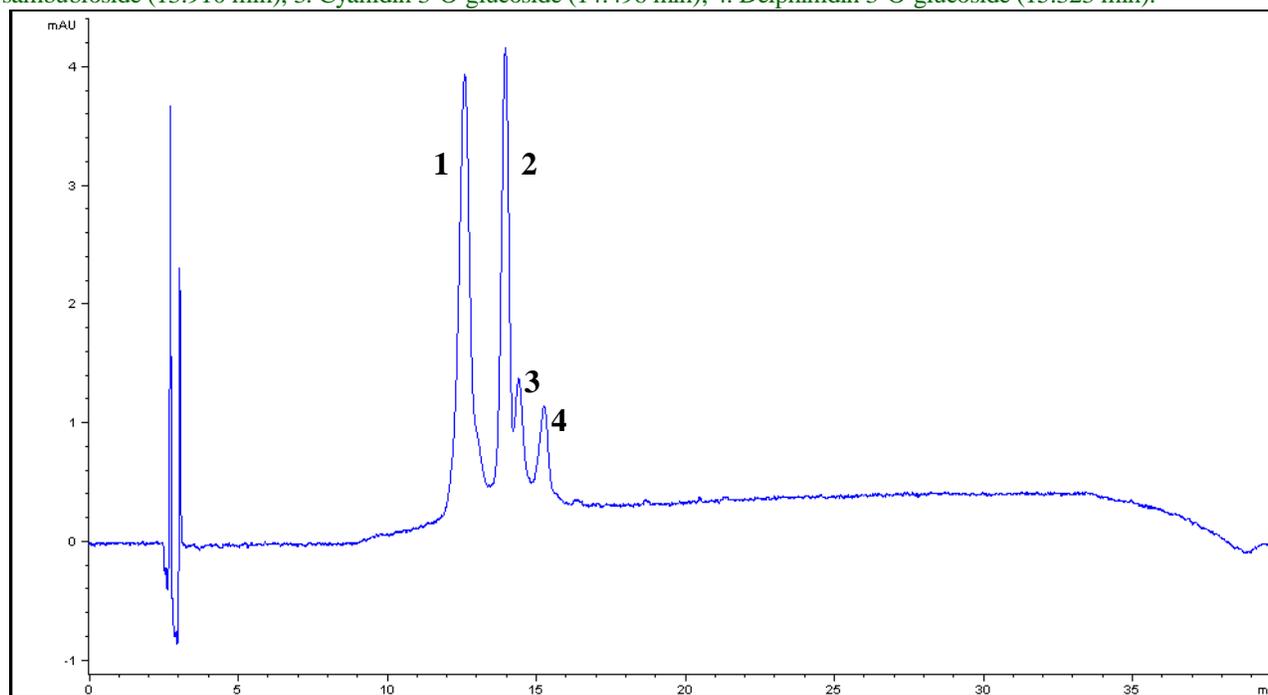
Fifty milligrams (50 mg) of freeze-dried extract were dissolved overnight with 5 mL of 0.1 % trifluoroacetic acid in methanol at 4 °C in a blender. Sample was centrifuged at 3000 rpm for 10 min. Supernatant was collected and filtered through a Millipore membrane (0.45 µm). The filtrate was twice diluted with purified distilled water. One hundred microliters (100 µL) of filtrate were injected by an Agilent 1100 series autosampler and chromatograms were monitored at 521 nm. The elution program was 5-15 % B (0-5 min), 15-25 % B (5-15 min), 25-100 % B (15-30 min) and 100 % B (30-40 min) with a flow rate of 0.8 mL/min. The

anthocyanins identification and peak assignments are based on their retention times, UV-VIS spectra comparing with standards and published data.

Results and discussion

The screening of plants for medicinal value has been carried out by numerous researchers with the help of phytochemical analysis (N'guessan et al., 2009; Kone et al., 2012; Obouayeba et al., 2014c and 2015d). Phytochemical screening is of paramount importance in identifying new source of therapeutically and industrially valuable compound having medicinal significance, to make the best and judicious use of available natural wealth. A number of medicinal plants have been chemically investigated by several researchers (Kone et al., 2004; Zirihi et al., 2007; Obouayeba et al., 2014a and 2015c). The selection of plant parts such as petals which yields maximum secondary metabolites is the prime or prerequisite step in this investigation.

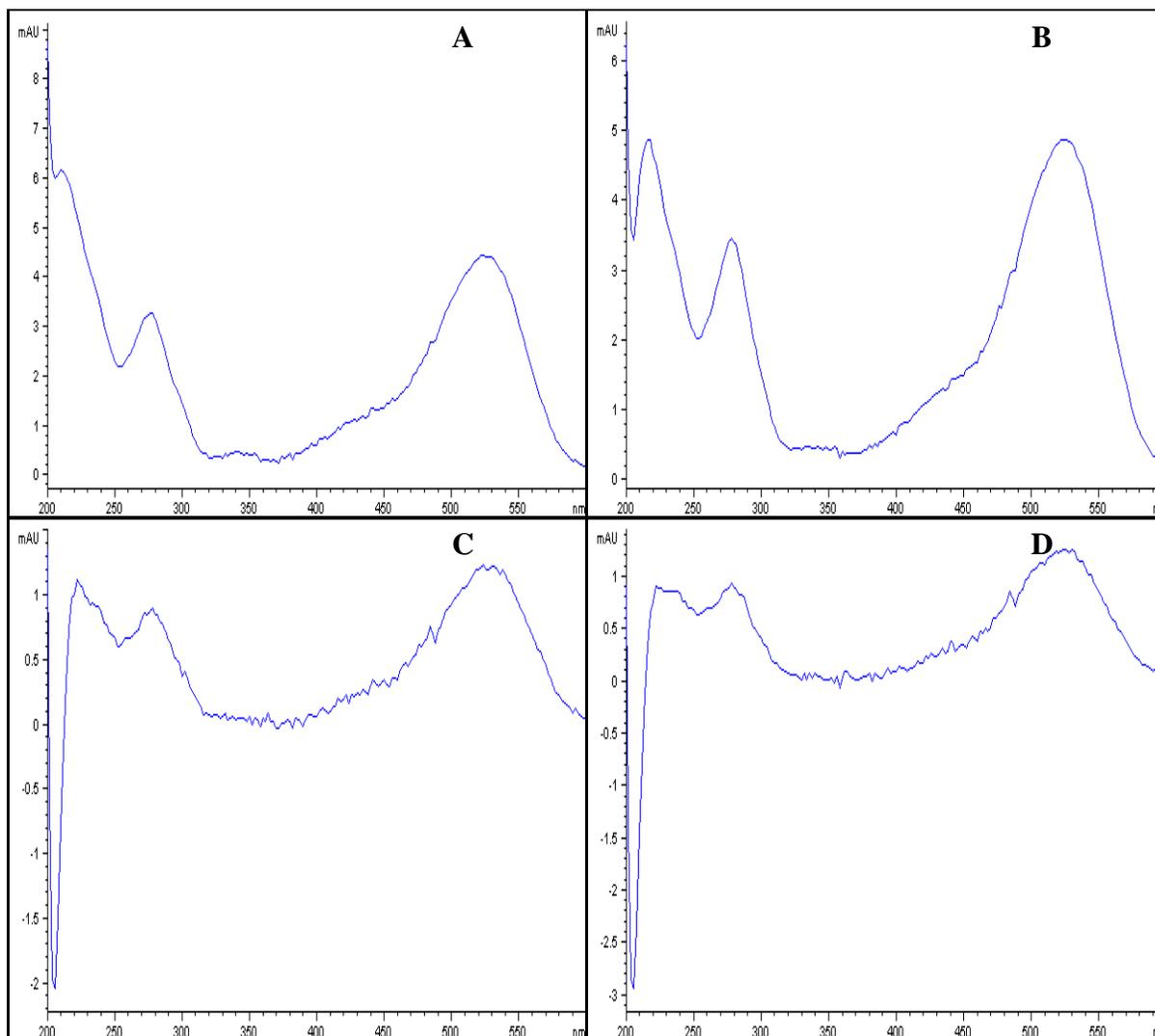
Fig. 2: HPLC chromatogram of some *Hibiscus* anthocyanins detected at 521 nm. Peaks were identified by comparison with reference standards when available or by ¹H NMR data (retention time). 1. Delphinidin 3-O-sambubioside (12.523 min); 2. Cyanidin 3-O-sambubioside (13.910 min); 3. Cyanidin 3-O-glucoside (14.496 min); 4. Delphinidin 3-O-glucoside (15.323 min).



The results of HPLC chromatogram detected at 521 nm showed two major peaks (1 and 2) and two minor peaks (3 and 4) corresponding to the anthocyanins were isolated from the petals extract of *Hibiscus sabdariffa* (Fig. 2). Peak assignments are based on matching UV-vis and identical HPLC retention time with known anthocyanins from a

reference library of compounds previously purified and identified by anthocyanins identified in petals of *Hibiscus sabdariffa*. Thus, four anthocyanins were isolated and identified from the petals of *Hibiscus sabdariffa* with two major anthocyanins such as the cyanidin 3-O-sambubioside and the delphinidin 3-O-sambubioside.

Fig. 3: UV-visible spectra of the four anthocyanin pigments isolated from petals of *Hibiscus sabdariffa*. A) Cyanidin 3-O-sambubioside ($\lambda_{\max} = 522$ nm); B) Delphinidin 3-O-sambubioside ($\lambda_{\max} = 525$ nm); C) Cyanidin 3-O-glucoside ($\lambda_{\max} = 530$ nm); D) Delphinidin-3-O-glucoside ($\lambda_{\max} = 527$ nm). Maximum wavelength (λ_{\max}).



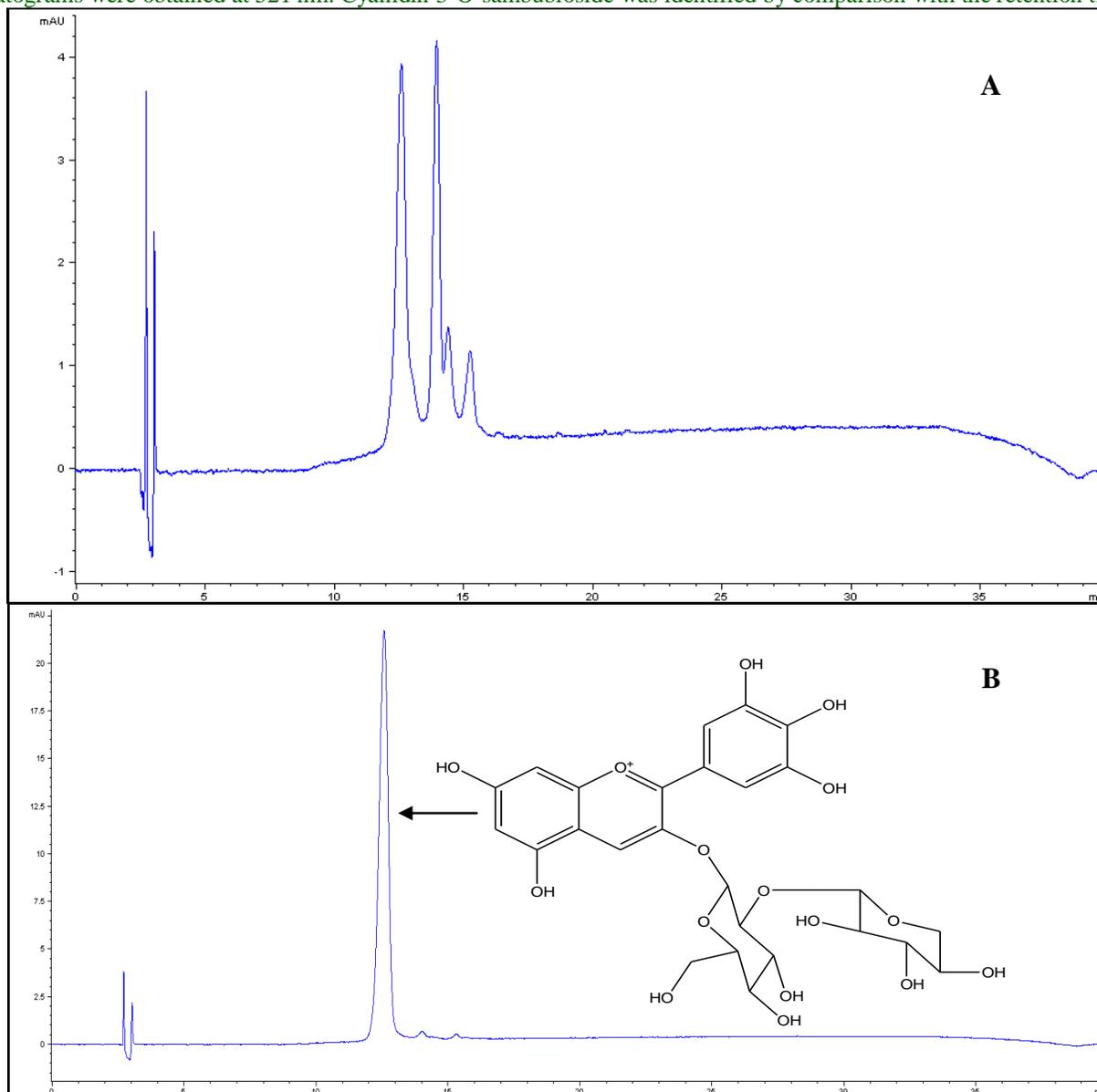
The two minor anthocyanins isolated and identified from the petals of this plant are the cyanidin 3-O-glucoside and the delphinidin 3-O-glucoside. However, on the four anthocyanins isolated and identified only two have been purified, it is the diglucosides namely cyanidin 3-O-sambubioside and delphinidin 3-O-sambubioside) as the show the Fig. 4 and 5. Our results are in accordance with those obtained by several authors (Kouakou et al., 2014; Obouayeba et al., 2014a; Borrás-Linares et al., 2015). These phytochemical compounds have pharmacological properties. Indeed, Delphinidin and its glycoside derivatives have significant antioxidant activity (Azevedo et al., 2009; Christian and Jackson, 2009). Several epidemiological studies have shown a protective

effect against coronary heart disease and the consumption of anthocyanins (Mink et al., 2007; Ojeda et al., 2010). Regarding cyanidin and its glycoside derivatives, they have antioxidant properties (Salazar-Gonzalez et al., 2012; Azevedo et al., 2009) and by scavenging free radicals, it protects cells from oxidative damage and reduces the risk of cardiovascular damage (Mink et al., 2007; Ojeda et al., 2010) and certain cancers. This clearly shows the use of this plant as herbal medicine. Thus, several works were showed that the anthocyanins are the major constituents in *Hibiscus* extract (Du and Francis, 1973; Lin et al., 2007; Obouayeba et al., 2015d; Kouakou et al., 2015), which natural water-soluble pigments are belonging to

the class of flavonoids (Smith et al., 2000). The significant presence of anthocyanins in flowers of *H. sabdariffa* indicates that this plant can play an important role in industries (food, textile, pharmaceutical and cosmetic). Indeed, Okonkwo,

(2010) have shown that anthocyanins were potential natural dyes for these industries. In addition, these results were further supported by the results of the UV visible spectral properties (Fig. 3) that were corroborated with those of Du and Francis (1973).

Fig. 4: HPLC Chromatograms. A) *Hibiscus* anthocyanins from petal extract; B) Cyanidin 3-O-sambubioside. Chromatograms were obtained at 521 nm. Cyanidin 3-O-sambubioside was identified by comparison with the retention time.

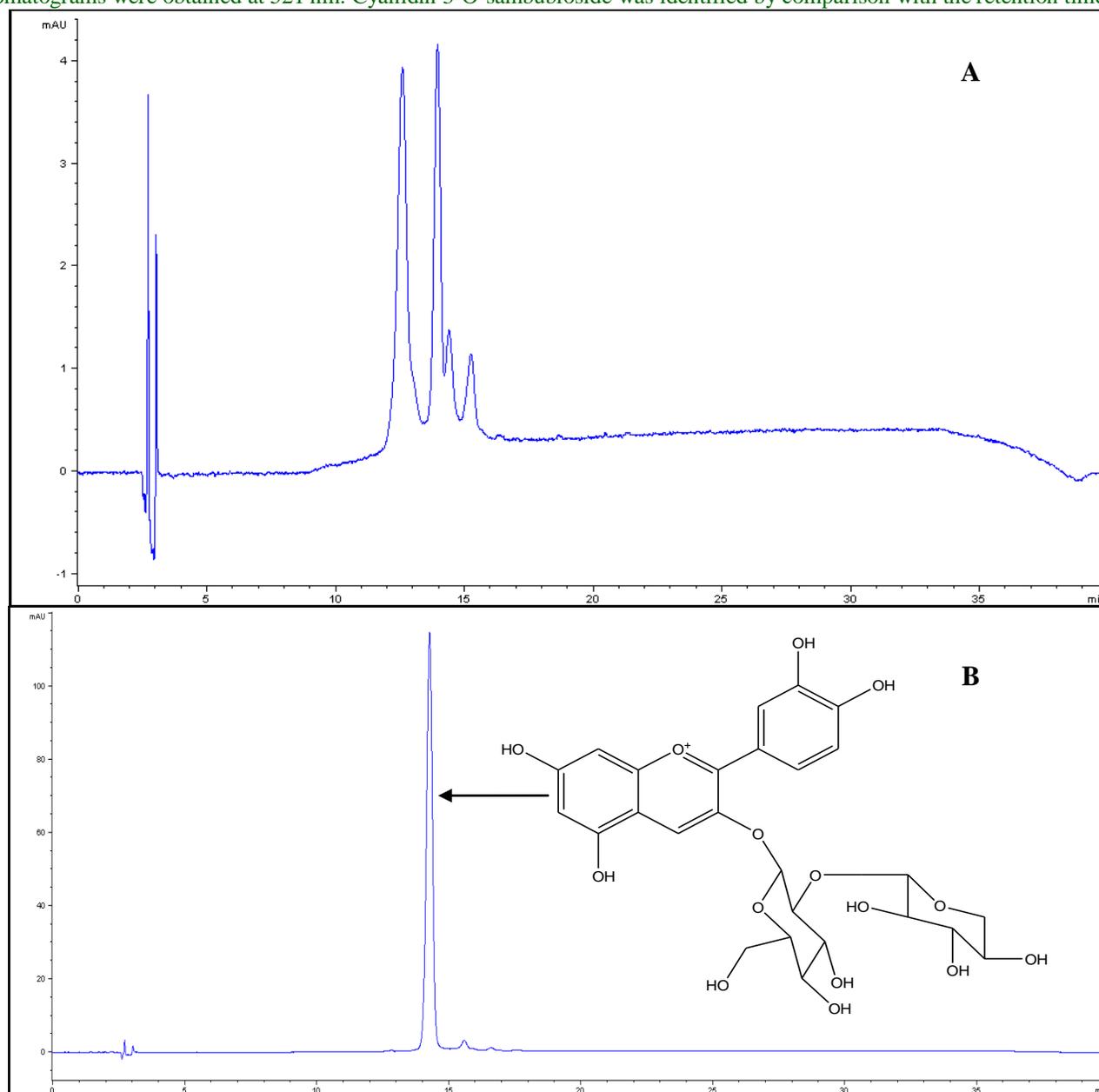


Conclusion

Hibiscus sabdariffa is a medicinal and food plant rich in phytochemical compounds such as polyphenols in particular in anthocyanins, of interest responsible for its pharmacological properties. The juice of flowers of *H. sabdariffa* L., commonly known as Bissap is used

in the preparation of local nonalcoholic cold beverage and as a hot drink. In Cote d'Ivoire, this production of a nonalcoholic drink called Bissap that is prepared from the red petals is popular. The use of *Hibiscus sabdariffa* petals as natural antioxidants, natural colorants, and an ingredient of functional foods seems to be promising.

Fig. 5: HPLC Chromatograms. A) Hibiscus anthocyanins from petals extract; B) Delphinidin 3-O-sambubioside. Chromatograms were obtained at 521 nm. Cyanidin 3-O-sambubioside was identified by comparison with the retention time.



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