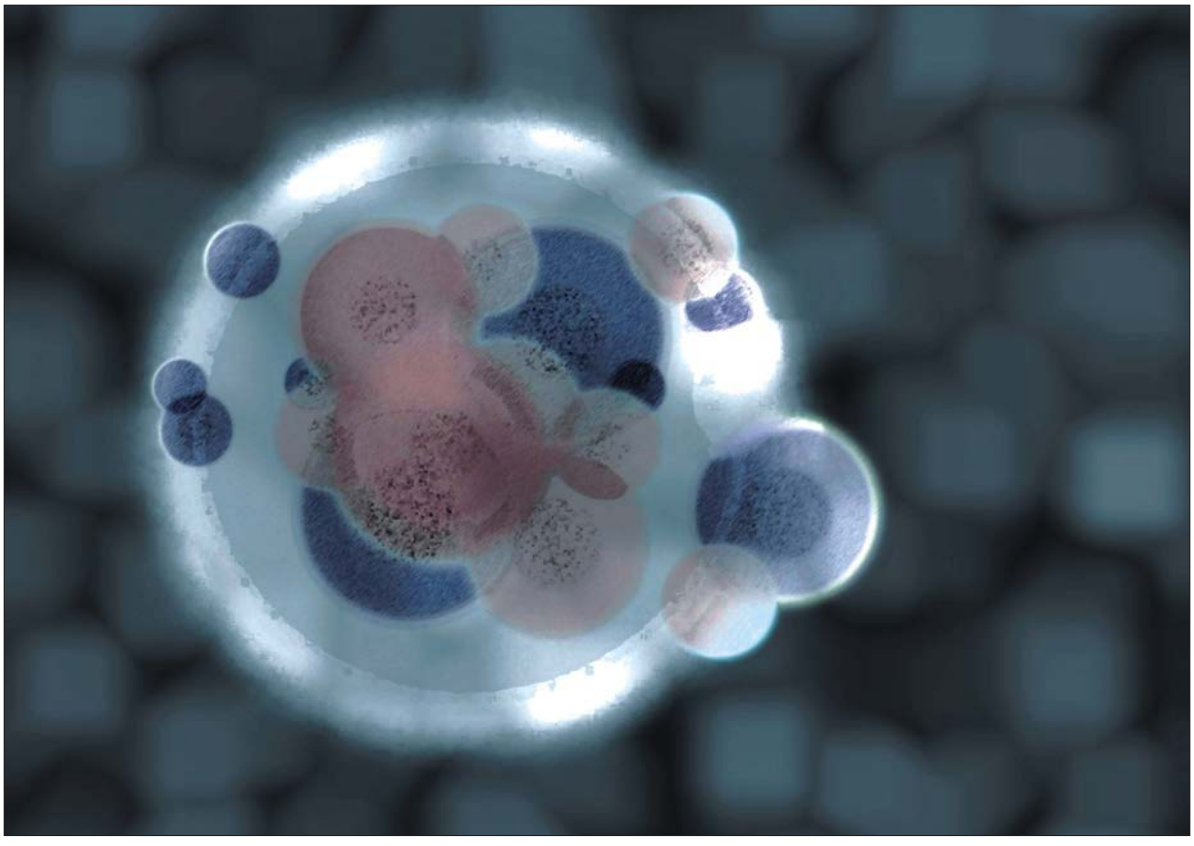

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Comparative Phytochemical Content of Leaves Extracts of Two *Annona senegalensis* Pers: The One from Togo and the Other Originates from Burkina Faso

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Abstract: In view to valorize the traditional medicine of Togo, a comparative phytochemical screening was undertaken on the leaves powder extracts of *Annona senegalensis* of Togo and the one originates from Burkina Faso. The different extracts were obtained by splitting (percolation) and by steeping the vegetable material with appropriate solvents. The main chemical groups of the extracts were characterized by the aid of specific chemical reactions. The 70% aqueous methanol and aqueous extracts were analysed using High-Performance Liquid Chromatography (HPLC). The rate of the relative humidity in Togolese leaves powder was higher (9.7±0.9%) compared to the one from Burkina Faso (7.0±1.1%) ($p<0.05$). The chemical tests in the limit of their sensitivity and their specificity allowed us to detect some various components hereafter: sterols/triterpenes, carotenoids, flavonoids, anthocyanosides, saponosides and tannins. The HPLC assay has confirmed the presence of some flavonoids, namely rutin and isoquercetrin (specimen from Togo) and the preceding compounds plus epicatechin and catechin derivatives (specimen from Burkina). These differences in the qualitative composition of bioactive compounds of extracts corroborate with the role of the ecosystem on the phytochemical constituents of the two species.

Key words: *Annona senegalensis* from Togo, *Annona senegalensis* from Burkina Faso, leaf extracts, phytochemical contents

INTRODUCTION

The biodiversity of the nature, particularly the one of Togo remains largely unexplored. Since only 5-15% of the higher plants have been systematically investigated for the presence of bioactive compounds (Pieters and Vlietinck, 2005). One of the first steps leading to drug discovery and development process is the phytochemical screening of the plants used in traditional medicine. In the setting of the revalorization of the traditional medicine of Togo, we identified among the plants entering in the composition of the recipes used by the healer Sakiè E. a medicinal plant named *Annona senegalensis*. The healer uses the leaves macerate or decoction as medication in the localized or generalized oedema, the fever, the snakebite, the fractures and sprains (poultice for applying).

Annona senegalensis Persoon (Annonaceae) is a shrub or small tree with large ovate or oblong alternate leaves, widespread in the Saharan savannahs to Guinean (Neuwinger, 1996). Traditionally (in Togo and in many other countries of Africa), the leaves are used as medication in various diseases (diarrhoea, diseases of the joints, respiratory diseases, conjunctivitis, wounds, snakebite, jaundice, haemorrhoids, dracunculosis, feminine barrenness, convulsions, fever, asthenia) (Neuwinger, 1996).

In order to value the pharmacological activities of this plant, we were interested in this preliminary study in the extraction and the characterization of the active principles (phytochemical screening) of the leaves powder of *Annona senegalensis* originates from Togo. This screening has been undertaken simultaneously on the species collected at Burkina Faso where the study has been carried out as comparison.

MATERIALS AND METHODS

This study has been achieved during the year 2006 at the Institute of Research in Science of Health (IRSS/CNRST), Department of Medicine and Traditional Pharmacopeia, Ouagadougou (Burkina Faso).

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Collection of the plant material: The leafed twigs of the two plants were harvested in localities situated to 633 km in the south of Ouagadougou (Burkina Faso) on the Togolese territory at Pya-Kadjika (Kozah Prefecture) either to a few 437 km in the North of Lomé (Togo) for *Annona senegalensis* originates from Togo. The species from Burkina Faso has been obtained to about 40 km in the west of Ouagadougou in Kokologho on the road of Bobo-Dioulasso. The period of the collect was between the months of August and October 2005. The plant was then identified by the botanists of the Faculties of the Sciences of the Universities of Lomé and Ouagadougou.

The leafed twigs were dried safe from the sun, to the plain temperature (about 27 to 29°C) for 2 weeks. After removal of the twigs, the green dried leaves were ground into fine powder using a dry grinder provided with a sifter of 1.5 mm stitches. The ground samples were kept in a tight plastic bag (1 kg) for further extraction.

Determination of the relative humidity rate (RHR) of the leaves powder: Before submitting the plant powder to different operations of active principles extraction, one of the first parameters to determine was the relative content in water of this powder. To make it, an amount of 1 g (P) has been placed in the oven (105 to 110°C) during 1 h in order to get a dry weight (P') constant. Four tests allowed us to calculate the average RHR according to the formula:

$$\frac{P - P'}{P} \times 100'$$

The RHR of both leaves powder were compared using statistical t-test of Student.

Extraction of the active principles

Solvent partitioning extraction of vegetable material (percolation): The extraction was a partitioning method which separates the various chemical groups according to their physico-chemical properties using organic solvents of different polarities. One hundred grams of plant powder have been subjected to the successive extraction, in an adapted percolator, with the following solvents (5 to 8 times the volume of vegetable material amount): ether of petroleum, dichloromethane, ethyl acetate, distilled water. After each percolation, the residue was dried in the oven at 40°C in order to remove the residual solvent and then macerated for 5 h in the following solvent. After percolation, ether of petroleum (Eep), ethyl acetate (Eea) and water (Ea) extracts were collected and then freeze-dried and kept in desiccator for qualitative chemical analysis.

In this study, the dichloromethane solvent is used mainly to remove chlorophyll pigment from leaves powder.

Steeped extracts: Twenty five grams of leaves powder have been macerated during 5 h either with distilled water (10-12 volumes) for aqueous macerate (Maq) either with 70% (v/v) aqueous methanol (5-8 volumes) for aqueous methanol extract (Ehm). The extracts were separated from residue by filtration and centrifugation (2000 rpm for 15 min). The extract obtained was freeze-dried and kept in desiccator.

Hydrolysed extracts: This method aims to hydrolyse and characterize the heterosidic contents of the different extracts (Eae, Ehm, Maq). The 10% hydrochloric acid solution is added to the extract (5:3, v/v) in refluxing system and heated up for 30 min (Constantinescu *et al*., 1964). The extraction of genins has been performed using apolar solvent such as dichloromethane (organic phase), the anthocyanins (pigments) remained in the acidic aqueous phase.

Characterization of the chemical groups of the extracts: The qualitative chemical determination of the extracted constituents has been performed with the help of colourful specific reactions from Constantinescu *et al*. (1964) using suitable chemical reagents.

Ether of petroleum extract: The ether of petroleum is a suitable apolar solvent for the fat-soluble chemical principles extraction from vegetable material such as volatile and fatty compounds, sterols and triterpenes, emodols, carotenoids, basic alkaloids, coumarins, chlorophyll and aglycones issued from hydrolysed glycosides (Constantinescu *et al*., 1964).

Thus, the following specific reactions were tested in order to identify the phytochemical active principles of interest for present study:

Reaction of Liebermann-Burchard's: The dried ether of petroleum extract was dissolved in acetic anhydride and then in chloroform. By means of a pipette, concentrated sulphuric acid was added at the bottom of the tube containing the extract solution. At the contact zone of the two liquids, a brownish-red or violet ring denotes the presence of sterols and triterpenes.

Reaction of Bornträger's: A red coloration appears when 25% ammonia solution or 10% sodium hydroxide is added to the extract (3:1, v/v) in the presence of emodols (anthracenoside aglycones).

Reaction of Carr Price's: The carotenoids are identified by a blue coloration that turns to the red in presence of a saturated solution of antimony chloride in chloroform.

Reaction of Shibata or cyanidine test: This test is used to identify flavonic aglycones in 50% methanolic middle in presence of metallic magnesium and some drops of concentrated hydrochloric acid. A red or an orange coloration is a sign of the presence of flavonols or flavanones, respectively.

Ethyl acetate extract: The ethyl acetate essentially extracts some glycosides compounds such as the flavonosides and anthocyanosides. The lipophilic aglycones compounds such as flavonic genins were identified in the organic phase of hydrolysed ethyl acetate extract by means of Shibata's reaction as previously described. If the aqueous acidic phase of hydrolysed extract is red and it turns either to violet at a neutral pH, either to green or blue in alkaline medium (NaOH or KOH), the anthocyanin pigments are present.

During the concentration of the ethyl acetate extract under reduced pressure in a Rotavapor - Büchi 461 Water Bath, a precipitate appears at the end of the operation. This precipitate which becomes insoluble in the solvent of extraction but soluble in the ethanol 95° has been washed 3 to 4-fold with the ethyl acetate and recovered as powder. The Shibata's reaction tested on this precipitated fraction has induced a strong red coloration. The carotenoids were identified with the help of the Carr Price's reaction previously described under ether of petroleum extract.

Aqueous methanol extract: Aqueous methanol (70%, v/v) extract may contain many active principles of natural constituents as for example: polyphenols (tannins), reducing compounds, alkaloid salts, polyphenolic glycosides (anthracenosides, flavonosides), sterol glycosides (cardiotoniques, saponosides), triterpene glycosides, anthocyanosides (Constantinescu *et al*., 1964).

The chemically active constituents extracted were identified by means of some specific colourful reactions within 70% aqueous methanol or its hydrolysed extract.

In the aqueous methanol extract, we were particularly interested in the identification of following groups.

The tannins were characterised using a 2% solution of ferric chloride. The occurrence of a blackish blue colour shows the presence of gallic tannins and a green blackish colour indicates catechol tannins.

The saponins (saponosides) were identified by shaking 2 mL of diluted solution (1:1) in a test-tube of 1.6 cm diameter for 15 min. The occurrence of a foam column of at least 1 cm in height, persisting minimum 15 min, indicates the presence of saponins.

The flavonosides and anthocyanosides were detected as previously described under ether of petroleum and ethyl acetate extracts, respectively.

Aqueous steeped extract: Water can extract from vegetable products hydrosoluble (polar) constituents such as saponosides, polyphenols (tannins, flavonosides, anthocyanosides), glucides, alkaloid salts (Constantinescu *et al*., 1964). The previously chemical active principles of interest (flavonosides, tannins, saponosides) were identified using the tests described under aqueous methanol extract. We tested also the organic phase of the hydrolysed aqueous extract for the detection of lipophilic constituents sterols/triterpenes, flavonic aglycones as previously described. The anthocyanins (pigments) were detected in the acidic aqueous phase of hydrolysed aqueous extract (see under ethyl acetate extract).

High performance liquid chromatography (HPLC) analysis of the extracts: The HPLC profiling of 70% aqueous methanol and aqueous steeped extract was carried out in the Laboratory of Toxico pharmacology of CHU of Clermont Ferrand (France) using a HPLC/UV System (Waters 2690 Separations Module) equipped with an autosampler, a Waters® 2696 pomp, a Symmetry C8 Waters® column and a Diode Array Detector. Data were processed and analyzed using Millenium^{32} Software (Waters Product). The samples were separated at 30°C on a 5 μm C18 column (4.6 mm × 25 cm). The solvents of elution were (A) 0.6% monosodium orthophosphate buffer and (B) acetonitrile for HPLC. The internal standard was Proadifen (SKF525A). Quercetin (Extrasynthese S. A., France) and rutin (Sarsynthese, France) were used as standard flavonoids. The HLPC was monitored at 200-350 nm and the two main criteria used for identification of molecules were the time of retention and the UV spectrum which were compared to those of Waters Toxicol data base.

RESULTS AND DISCUSSION

Determination of the relative humidity rate (RHR) of the leaves powder: The content in relative humidity of the leaves powder of *Annona senegalensis* of Togo and the one harvested in Burkina Faso was respectively 9.7±0.9% and 7.0±1.1%.

Characterization of the chemical groups of the extracts: In Table 1, sterols/triterpenes, carotenoids, flavonoids, anthocyanosides, saponosides and catechol tannins were

Table 1: Comparative phytochemical screening of the leaves powder partitioning (percolation) extracts of *Annona senegalensis* of Togo and the one originates from Burkina Faso

Extracts	Phytochemical content tested	Tg*	Bf*
Ether of petroleum extract (Eep)	Sterols/triterpenes	+++	+++
	Aglycones anthracenosides (emodols or emodins)	nd	nd
	Flavonic aglycones	+	nd
	Carotenoids	+++	+++
Ethyl acetate extract (Eea)	Flavonosides	++	+++
	Caroténoides	±	++
	Anthocyanosides	++	++
Aqueous extract (Ea)	Anthocyanosides	+++	+++
	Saponosides	++	++
	Polyphenols (catechol tannins)	+++	+++

nd: No detected; ±: Doubtful reaction; +: Weakly positive reaction; ++: Moderately positive reaction; +++: Strongly positive reaction, Tg*: Vegetable material from Togo; Bf*: Vegetable material from Burkina Faso

Table 2: Comparative phytochemical screening of the steeped leaves powder and hydrolysed aqueous extracts (Maq) of *Annona senegalensis* of Togo and the one originates from Burkina Faso

Extracts	Phytochemical content tested	Tg*	Bf*
70% aqueous methanol extract (Ehm)	Flavonosides	+	++
	Anthocyanosides	+++	+++
	Saponosides	+++	+++
	Polyphenols (catechol tannins)	+++	+++
Steeped aqueous extract (Maq)	Flavonosides	++	+++
	Anthocyanosides	+++	+++
	Saponosides	++	++
	Polyphenols (catechol tannins)	+++	+++
Hydrolysed Maq (organic phase)	Flavonic aglycones	nd	++
	Sterols/triterpenes	++	++

nd: No detected; +: Weakly positive reaction; ++: Moderately positive reaction; +++: Strongly positive reaction, Tg*: Vegetable material from Togo; Bf*: Vegetable material from Burkina Faso

detected in different extracts obtained by partitioning extraction. Concerning carotenoids and flavonoids in Eea, the reaction of identification was doubtful or moderately positive respectively for Togolese sample.

The flavonic aglycones (Eep) were identified in Togolese sample but not in Burkinabe corresponding sample. Emodols were not detected in both samples.

In Table 2, the main phytochemical constituents detected in steeped extracts (Ehm, Maq) were: polyphenols (catechol tannins, flavonosides, anthocyanosides.), saponosides. Sterols/triterpenes were identified in organic phase of hydrolysed aqueous steeped extract (Maq) in both samples; whereas, flavonic aglycones were found only in the apolar phase of the sample originates from Burkina Faso.

The aqueous phase of hydrolysed aqueous extract (Maq) was constituted of mainly anthocyanins in both species.

HPLC analysis of the extracts: The HPLC chromatogram of Ehm (Fig. 1) and Maq (Fig. 2) confirmed the presence of flavonoids including rutin and isoquercetrin (flavonols) respectively in Togolese sample; whereas rutin, isoquercetrin, epicatechin and catechin derivatives (flavanols) were detected in extracts of the specimen from Burkina.

The determination of the relative humidity rate was justified by the fact that this parameter has been considered in weighing the plant powder for the different tests. Indeed, the weighted samples were carried out with 5% of residual humidity (European pharmacopoeia) in the plant powder (dry weight). The difference in the RHR detected in both plant powders may be explained by the difference of climatic conditions between the two sites of origin of the plant: Pya-Kadjika in Togo (savannah climate) and Kokologho in Burkina (Sub Saharan climate). The site of origin of Togolese species is the most watered compared to the one of Burkina Faso; this may explained the significantly highest RHR in the plant powder originates from Togo (9.7±0.9%)($p<0.05$).

In the limit of the sensitivity and specificity of the characterization reactions used, our results showed that the profile of chemical content of the fractioned extracts obtained from the two plants was almost similar, the main chemical groups detected being: sterols/triterpenes, carotenoids, flavonoids, anthocyanosides, saponosides and catechol tannins (Table 1). The precipitated fraction issued from ethyl acetate concentration has reacted positively to Shibata's test with an intense red coloration; that suggests the presence of flavonoids especially flavonols at least in this extract. The solvent like ethyl acetate is the favourite solvent for flavonoids extracting from leaves as reported by Ibewuike *et al.* (1997) and Aderogba *et al.* (2003, 2004, 2005).

The chemical composition of the extracts from the leaves powders, obtained by steeping showed a similar chemical profile for both plants (Table 2). On the other hand, the differences observed were about the flavonosides and their flavonic aglycones in different extracts; the coloration of the characterization tests was less intense in Eea, Ehm and Maq for the Togolese species. Concerning the flavonoids, these results were confirmed using HPLC assay of 70% aqueous methanol and aqueous extracts. The HPLC profiles (Fig. 1, 2) showed the presence of rutin and isoquercetrin (flavonols) in Togolese sample; whereas in addition to the preceding flavonols, epicatechin and catechin derivatives (flavanols) were detected in the extracts of specimen from Burkina. The concomitant presence of flavonols and flavanols in the same plant is normal since they belong to the same biosynthesis pathway of polyphenolic flavonoids (Rice-Evans *et al.*, 1996). The present findings corroborates with those reported by other authors who have notified the presence of flavonoids (quercetin and quercetrin) and sterols (Mackie and Misra, 1956), tannins and saponins (Ogbadoyi *et al.*, 2007) in the plant leaf.

These differences in the qualitative phytochemical content of the extracts could be due to some factors such as the climatic conditions (Reynolds, 2002; Pieters and

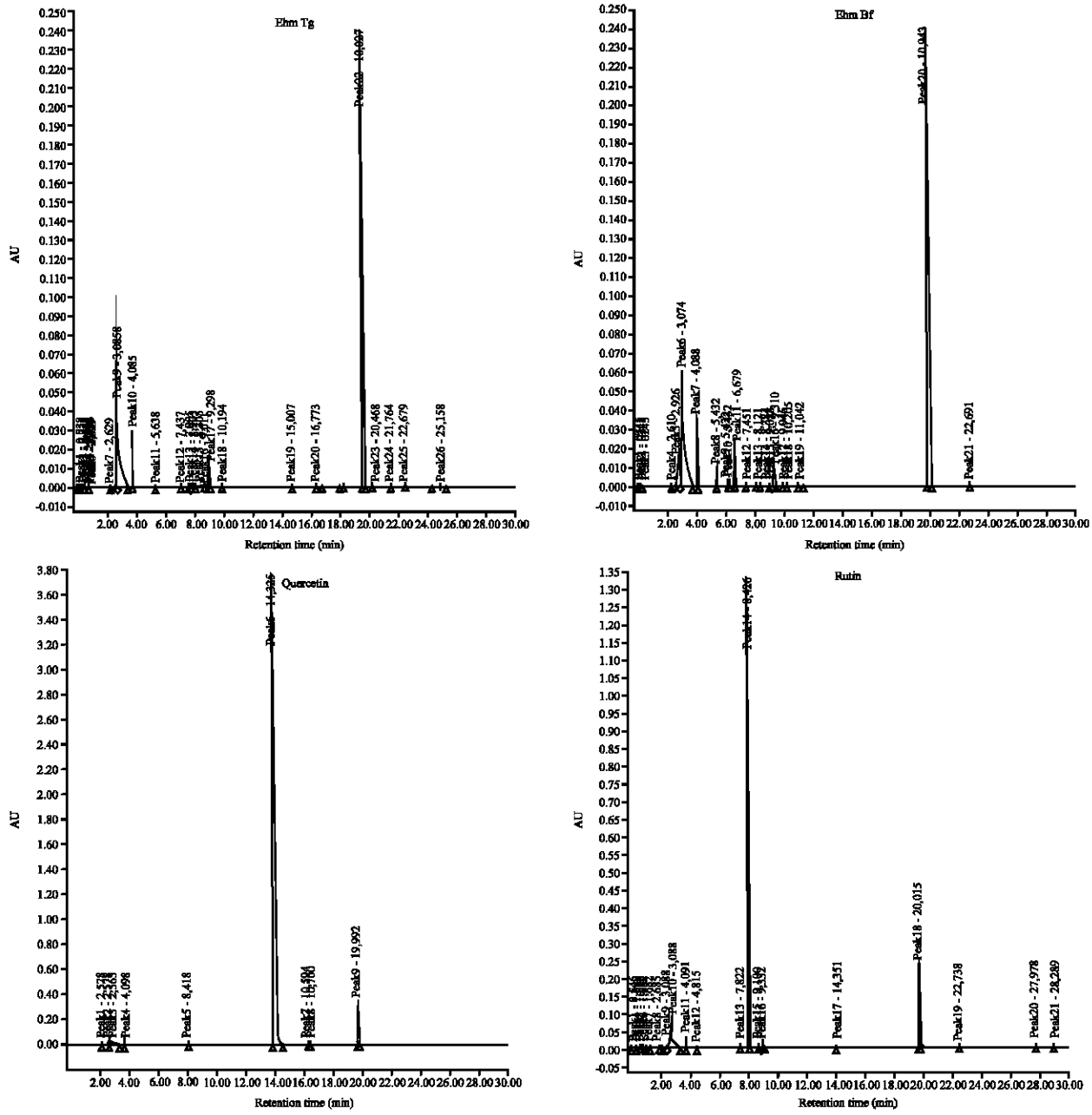

Fig. 1: The HPLC profiles of 70% aqueous methanol leave extract of *Annona senegalensis* from Togo and the one originates from Burkina Faso compared with the standard flavonoids Quercetin (14,325) and Rutin (8,423); Ehm Tg: Rutin (8,385); Ehm Bf: Rutin (8,384); Epicatectin (6,679); Catechin derivatives (5,432)

Vlietinck, 2005; Metting and Pyne, 2007), the geological environment (Gomes and Silva, 2007) of the sites of harvest, the period of the harvest (maturation), the enzymatic content responsible for the biosynthesis pathways (Pieters and Vlietinck, 2005), the regulation of gene expression by environmental factors (Boudet, 2007). Indeed, if one considers that the biosynthesized substances of the plant let it to adapt to its environmental conditions (role of signal, defense against predators and parasites, resistance against harmful bugs and diseases) (Pieters and Vlietinck, 2005), the one of Burkina being more arid, these differences could be explained.

The flavonic aglycones have not been detected in the organic phase of the Maq acidic hydrolysed (sample

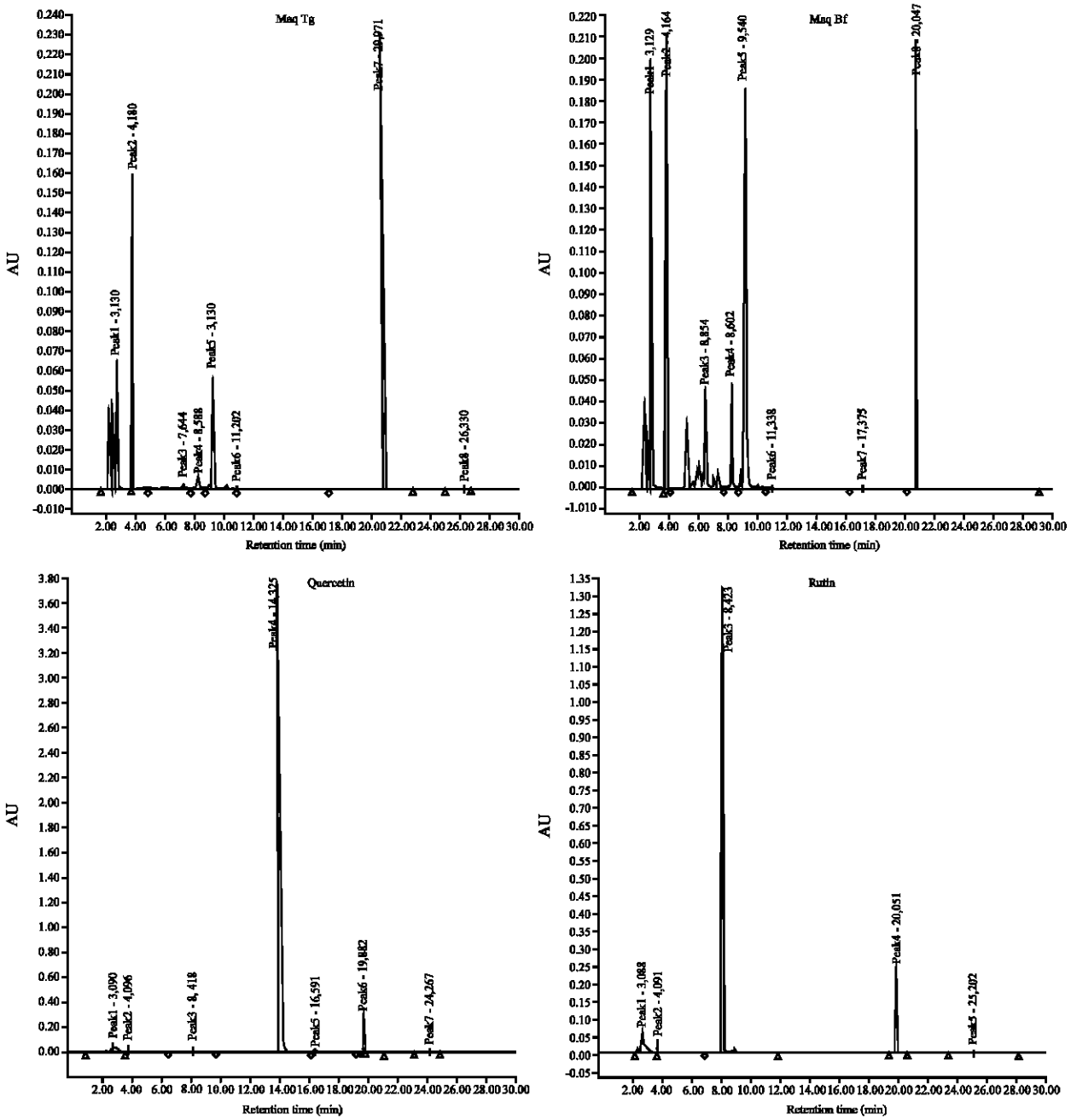

Fig. 2: The HPLC profiles of aqueous leave extract of *Annona senegalensis* from Togo and the one originates from Burkina Faso compared with the standard flavonoids Quercetin (14,325) and Rutin (8,423); Maq Tg: Isoquercetrin (9,303); Maq Bf: Rutin (8,377); Isoquercetrin (9,303); Epicatechin (6,678); Catechin derivatives (5,430)

originates from Togo). An apolar solvent extraction in the previously described conditions of the hydrolysis without heating gave a positive result to the Shibata's test. This finding suggests the heat sensitivity of these compounds.

The qualitative phytochemical composition observed in this study corroborates with the role of the environmental conditions of growth for each specimen, especially with the hostile environmental conditions.

CONCLUSION

This preliminary study, carried out in our conditions of work, shows that the phytochemical profile of the two

Annona involves some differences (RHR, phytochemical content, extract outputs) which could be due to the ecosystem and numerous other factors (maturation state, enzymatic content, regulation gene expression, conditions of work). These differences in qualitative bioactive constituents of extracts may have some outcomes on the pharmacological activities of each plant.

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