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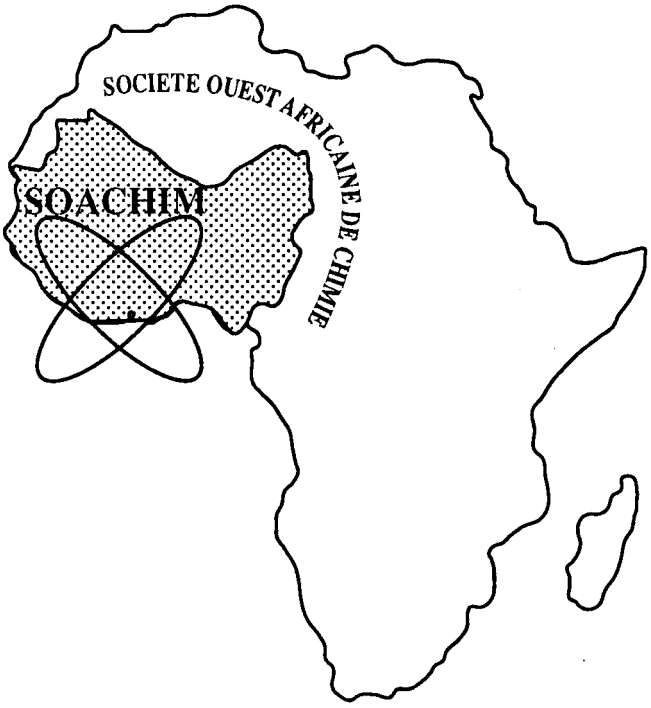

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Characterization of carotenoids by using HPTLC – MS^2 and evaluation of total antioxidant contents in *Cymbopogon giganteus* extracts from Burkina Faso

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Abstract. The natural carotenoids are more and more sought after for their coloring and bioactive properties. In this work, the carotenoid content was evaluated in the hexane extracts of different organs and six (06) carotenoids were characterized in the extracts of the leaves of Cymbopogon giganteus acclimatized in Burkina Faso. The contents of total carotenoids were estimated at 13.3 µg, 4.2 µg and 1.5 µg of β-carotene equivalent (EBC) / mg of dry extract, respectively in the hexanic extracts of leaves, stems and roots of *C. giganteus*. The major carotenoids that were identified by thin-layer chromatography-mass spectrometry (HPTLC-MS) for the first time in the extracts of this plant are lutein, 5,6-epoxy-lutein, violaxanthin, β-carotene, β-cryptoxanthine and Phytoène. Moreover, the total antioxidant contents evaluated by FRAP and DPPH methods vary between 28.6 and 34.1 µg TE/mg extract; 19.4 and 23.6 µg TE/mg extract; and 16.5 and 18.1 µg TE/mg dry extract respectively for leaves, stems and roots. Hexane extracts of the different parts of C. giganteus may be a potential source of natural antioxidants and carotenoids.

Keywords : Carotenoids, *Cymbopogon giganteus*; HPTLC/MS; antioxidants

Caractérisation des caroténoïdes par HPTLC – MS^2 et évaluation des teneurs en antioxydants totaux dans les extraits de *Cymbopogon giganteus* du Burkina Faso.

Résumé Les caroténoïdes d'origine naturelle sont de plus en plus recherchés pour leurs propriétés colorantes et bioactives. Dans ce travail des caroténoïdes, ont été caractérisés et quantifiés dans les différentes parties de *Cymbopogon giganteus* acclimatée au Burkina Faso. Les teneurs en caroténoïdes totaux ont été estimées à 13,3 ; 4,2 ; 1,5 µg d'équivalent de bêta carotène (EBC) / mg d'extrait sec, respectivement dans les extraits hexaniques des feuilles, des tiges et des racines de *C. giganteus*. Les caroténoïdes majeurs qui ont été identifiés par chromatographie sur couche mince couplée à la spectrométrie de masse HPTLC-MS pour la première fois dans les extraits de cette plante sont la lutéine, la 5,6-epoxy-lutéine, la violaxanthine, le β-carotène. Par ailleurs, les teneurs en antioxydants totaux évaluées par les méthodes FRAP et DPPH varient entre 28,6 et 34,1 µg d'ET/mg d'extrait ; 19,4 et 23,6 µg d'ET/mg d'extrait ; et 16,5 et 18,1 µg d'ET/mg d'extrait sec respectivement pour les feuilles, les tiges et les racines. Les extraits hexaniques des différentes parties de *C. giganteus* peuvent constituer une source potentielle d'antioxydants et de caroténoïdes naturels.

Mots clés : Caroténoïdes, *Cymbopogon giganteus*; HPTLC/MS ; antioxydants

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1. Introduction

Synthetic antioxidants are nowadays questioned for toxicological risks [1]. Thus, natural substances such as carotenoids, known for their antioxidant properties and their involvement in the prevention of diseases related to oxidative stress, are increasingly sought after by the food industry. They are widespread compounds in plants. [2] They have a very remarkable coloring power and as a result, are used as natural colorants by food industries [3]. In addition, carotenoids have a functional interest based mainly on their antioxidant properties. The antioxidant activity of these compounds is mainly due to their high reactivity towards free radicals in the body. Carotenoids are also known for their important role in the prevention and protection of the body against certain pathologies such as cancer, cardiovascular diseases [3-5].

However, the chemical synthesis of these compounds is very expensive and is increasingly questioned nowadays due to toxicity risks. The search for new natural sources of these compounds could constitute an alternative to the use of synthetic molecules [6, 7].

Our recent previous work on solvent extracts of the species showed a high presence of carotenoids in hexane extracts of leaves, flowers and roots. [9]. Thus, we focused our investigations on Cymbopogon giganteus, an aromatic plant growing spontaneously in the savannahs of Asian and African tropical regions. To our knowledge, no study has yet focused on the non-volatile extracts of this plant.

The objective of the present work is to characterize by thin layer chromatography coupled with mass spectrometry, the main carotenoids of the extracts of *Cymbopogon giganteus* (Medicinal plant) and, consequently, to evaluate the antioxidant potential of the hexanic extracts of the different parts of this plant in view of its valorization as a natural source of antioxidant and carotenoids.

2. Material and methods

2.1 Plant material

The plant material is composed of the different parts of *Cymbopogon giganteus* (roots, stems and leaves). It is collected in the experimental field (12°25'28.2"N; 1°29'15.06" W) on the site of l'Institut de Recherche en Sciences Appliquées et Technologie (IRSAT), in October 2017. After identification by Dr. Issouf ZERBO of the Joseph Ki-ZERBO University, a specimen was deposited in the herbarium of Unité de Formation et de Recherche en Sciences de la Vie et de la Terre (UFR/SVT) under the code 6895. The plant material was well washed and dried in the shade at room temperature for 15 days and then ground.

2.2 Chemical reagents.

The solvents and reagents used are all analytical or HPLC grade. β-carotene, Trolox, 2, 4,6-tripyridil-s-triazine (TPTZ), iron (III) chloride hexa-hydrate, sodium acetate tri-hydrate, gallic acid, 2,2-diphenyl-1-picryhydrazyl radical (DPPH), and potassium hydroxide are the reagents used. hexane, methanol, dimethyl sulfoxide (DMSO) and ethyl acetate are the solvents used. They come from SIGMA.

2.3 Extraction

Extracts of the different organs of *Cymbopogon giganteus* (roots, stems and leaves) are obtained by maceration with hexane. The extraction is repeated several times. After filtration, the different filtrates are collected and concentrated dry at low temperature (≤40 °C) and protected from light.

2.4 Determination of total carotenoids

The total carotenoid contents of the extracts are evaluated following the method described by Koala and al. [10], slightly modified. After a suitable dilution, the absorbances of the extracts are read at 450 nm with a 96-well quartz microplate (MP96 spectrophotometer, SAFAS). Values of total carotenoid contents are obtained by relating the absorbances of the extracts to a standard curve ($y = 25.56x + 0.016$; $R^2 = 0.999$; $y = Absorbance\ and\ x = content$) established using β-carotene as a standard. Values are expressed as β-carotene equivalents per gram of extract (EBC/g). All measurements are performed three times.

2.5. Determination of the total antioxidant content of the extracts

2.5.1. Method using 2, 2-diphenyl-1-picrylhydrazyl (DPPH)

The antiradical capacity of each extract to reduce the DPPH▪radical is determined following the method of Brand-Williams et al. (1995). Approximately 50 µL of each extract is added to 200 µL of methanolic DPPH solution (Concentration 0.04 mg/mL). After 10 min of incubation at 37 °C, the absorbances are read at 515 nm. The antiradical capacity of the extracts is determined by relating the read absorbances to the calibration curve established using trolox as standard ($y = -18.109x + 0.5889$; $R^2 = 0.9899$; $y = Absorbance\ and\ x = content$). Results are expressed as microgram trolox equivalent per milligram of extract (µg TE/mg). All measurements are repeated three times.

2.5.2 Method using FRAP (Ferric Reducing Antioxidant Power)

In this method, the FRAP reagent used is a solution of a ferric salt complex, Fe(III) $(TPTZ)_2 Cl_3$ (TPTZ = 2,4,6-tripyridyl-s-triazine) [12, 13]. The FRAP reagent is prepared by mixing 1 mL of TPTZ (1 mL, 10 mM in 40 mM HCl), 10 mL of sodium acetate buffer (pH = 3.6), and 1 mL of aqueous solution of de $FeCl_3, 6H_2O$ (1 %).

At 30 µL of distilled water and 20 µL of extract, 200 µL of FRAP solution is added. Then, 10 min later, the absorbance of the intense blue recoloration is read at 593 nm with the microplate reader (spectrophotometer MP96, SAFAS). The absorbance values are reported on a calibration curve established using Trolox as the reference antioxidant. The antioxidant contents, determined from the equation of the calibration curve ($y = 40.648x + 0.3419$; $R^2 = 0.9904$; $y = Absorbance\ et\ x = content$), are expressed as µg Trolox Equivalent (TE) per milligram of extract. All measurements are also repeated three times.

2.6 Structural identification of carotenoids

The hexanolic crude extract was submitted to saponification by addition of 30% methanolic KOH during three (03) hours in the dark and at room temperature to remove chlorophyll and fatty acids [10]. After liquid-liquid partitioning, the organic phase is extracted several times and then concentrated to dryness under vacuum. The residue is dissolved in minimal hexane (≈1 mL) and then kept in a refrigerator at 4 °C for 72 h. The separated major carotenoids were identified by HPTLC-MS coupling.

After developing the HPTLC plate in the hexane/ethyl acetate elution system (96/4 v/v), the individual spots were analyzed by the CAMAG TLC-MS interface system. The detector used is a Bruker microTOF-Q mass spectrometer, operating in positive ESI mode with a scan range from m/z 530 to 600.

2.7. Statistical study

The experiments were repeated at least three times and the results presented are expressed as the mean ± standard deviation calculated at the probability threshold of less than or equal to 95%. An analysis of variance (ANOVA) was used to assess the differences between the different organs of *Cymbopogon giganteus* in terms of total carotenoids and antioxidants, using the SPSS statistical software. Different letters in a column indicate a significant difference (P<0.001) between the contents according to the organs of *Cymbopogon giganteus*.

3. Résultats et discussion

3.1 Total carotenoids and total antioxidants contents.

The total carotenoid and antioxidant contents of the extracts of the different organs (roots, stems and leaves) of *Cymbopogon giganteus* are presented in table I. The results show that the total carotenoid content of *Cymbopogon giganteus* extracts varies from 1.5 µg EBC/mg dry extract for the roots to 13.3 µg EBC/mg dry extract for the leaves. The stems contain 4.2 µg EBC/mg. The analysis of the data in the table shows that the carotenoid contents are higher in the aerial parts, particularly the leaves of the plant. Indeed, in chlorophyllous plants, carotenoids are accessory pigments of photosynthesis. They play a role of light collector by transferring to the chlorophyll the light energy that they absorb in the ranges of the spectrum located between the violet and the red [4, 5].

The results also show the antioxidant contents, evaluated by two methods (DPPH reagent method and FRAP reagent method) and expressed in microgram (µg) of Trolox equivalent per milligram (mg) of dry extract (µg TE/mg dry extract). Statistical analysis (ANOVA: analysis of variance) of the antioxidant activities of the extracts of *Cymbopogon giganteus* showed that the differences in the antioxidant activities of the organs of this plant are significant (P<0.01).

The antioxidant activity of *Cymbopogon giganteus* extracts obtained by DPPH method varied from 18.1 to 34.1 µg of Trolox equivalent per milligram of dry extract, a twofold variation. Indeed, the leaf extract of *Cymbopogon giganteus* showed the highest antioxidant activity (34.1 µg TE/mg dry extract) followed by the stem extract (23.6 µg TE/mg dry extract). The root extract had the lowest antioxidant activity with a value of 18.1 µg TE/mg dry extract.

With the FRAP method, based on the reduction of ferric ions, the antioxidant activity ranges from 16.5 to 28.6 µg TE/mg dry extract. As observed in the case of DPPH, the extract from the leaves of *Cymbopogon giganteus* shows the highest antioxidant activity compared to the stems and roots respectively.

Thus, there is a strong correlation ($R^2 = 0{,}98$) (Table II) between the two methods used for the evaluation of the antioxidant potential of *Cymbopogon giganteus* extracts. This correlation can be explained by the fact that antioxidants are compounds capable of donating an electron or a hydrogen atom for the reduction of free radicals. Arnous et al (2002) also reported a strong correlation between the free radical scavenging capacity of

Table I: Carotenoid and total antioxidant contents of *Cymbopogon giganteus* extracts

Extracts	Total carotenoids contents (TCT) en µg EBC/mg	Total antioxidants contents (µg ET/mg)	
		DPPH	FRAP
Leaves	13.3±0.04 a	34.1 ±3.0 A	28.6±2.18 a'
Stems	4.2±0.05 b	23.6±0.2 A	19.4±0.03 a'
Roots	1.5±0.01 c	18.1±1.0 A	16.5±0.5a'

The results of total carotenoids and antioxidants contents are expressed as mean ± standard deviation. Multiple comparisons between organs are performed by the "multivariate analysis of SPSS". Means in each column followed by a different letter are significantly different (P < 0.001)

Table II : Correlation coefficient (R^2)

	DPPH	FRAP
TCT	0.98	0.99
FRAP	0.98	

DPPH and the ferric ion reduction capacity in wines.

The results of the antioxidant capacity measurements of *Cymbopogon giganteus* extracts are also correlated with their total carotenoid contents. It appears that the antioxidant activities of the extracts of this plant, obtained by the FRAP and DPPH radical methods, correlate strongly with their total carotenoid contents (Table II). These results would indicate a relationship between the contents of total carotenoids in the extracts of *Cymbopogon giganteus* and their antioxidant capacities. Therefore, the presence of carotenoids in *Cymbopogon giganteus* extracts would contribute significantly to their antioxidant properties. Indeed, carotenoids are part of the micronutrients that participate in the body's defenses against reactive oxygen species [14, 15]. They are essentially singlet oxygen scavengers, but they can also neutralize free radicals [14, 15]. The mechanisms by which carotenoids protect biological systems from singlet oxygen damage would consist of a set of physical and chemical reactions between the carotenoids and the "excited" oxygen molecule [16, 17, 18, 19]. The excitation energy from the singlet oxygen would be transferred to the pigment and then dissipated at the conjugated double bonds [16, 17, 18, 19]. Lycopene, carotenoids with more conjugated double bonds, is the most efficient scavenger of singlet oxygen followed by γ-carotene [16, 17, 18, 19].

At the cellular level, lutein is the most protective carotenoid against lipid peroxidation followed by lycopene and canthaxanthin, with α-carotene and β-carotene being the least active [16, 17, 18, 19, 20, 21].

Globally, the carotenoids brought by the diets would thus reinforce the antioxidant defenses of the body, in synergy with other compounds such as vitamin E, vitamin C, polyphenols.

3.2 Identification of the major carotenoids of *Cymbopogon giganteus* leaves extract

The carotenoids in leaves hexane extract of *Cymbopogon giganteus*, separated by thin-layer chromatography, are identified on the basis of information obtained from chromatographic elution and tandem mass spectrum. Analysis of HPTLC-MS/MS spectra of the most intense spot (R_f = 0.8) with the hexane/ethyl acetate 96/4 v/v) solvent system as eluent shows that this spot consists of several compounds.

On the positive mode electrospray mass spectrum (ESI+, Figure 4), we observe about ten quasi-molecular ions *m/z* 535.42 $[M+H]^+$; *m/z* 536.43 $[M]^{+\cdot}$; *m/z* 545.39 $[M+H]^+$; *m/z* 549.39 $[M+H]^+$; *m/z* 551.42 $[M+H]^+$; *m/z* 553.42$[M+H]^+$; *m/z* 565.40 $[M+H]^+$; *m/z* 567.41 $[M+H]^+$; *m/z* 569.42 $[M+H]^+$ et 583.41 $[M+H]^+$. Eight (08) of these peaks could be identified and correspond respectively to masses of 600 u; 584 u; 582 u; 568 u; 566 u; 552 u; 544 u and 536 u. Six of these masses are characteristic of the molecular weight of some known carotenoids. These molecules are grouped in Table III.

- ***Identification of compound A***

The mass spectrum shows an intense peak at m/z 567.42 $[M+H-H_2O]^+$ corresponding to the fragment ion of the lutein 5,6-epoxide molecule at m/z 585 $[M+H]^+$ which has lost a water molecule. The presence of an allylic hydroxyl group in this molecule allows for resonance stabilization of the fragment ion after the loss of water. Hence the intensity of the peak at m/z 567.42 $[M+H-H_2O]^+$ compared to that of the molecular ion at m/z 585 $[M+H]^+$ and the second fragment ion at m/z

549 $[M + H - 18 - 18]^+$ corresponding to the loss of two water molecules.

- ***Identification of compound B***

The HPTLC/MS spectrum (ESI+) showed two fragment ions at m/z 583 $[M + H - H_2O]^+$ and at m/z 565 $[M + H - 2H_2O]^+$ which would correspond to the loss of one and two water molecules, respectively. These fragment ions would be derived from the cleavage of the molecular ion at m/z 601 $[M + H]^+$ which would correspond to violaxanthin of chemical formula $C_{40}H_{56}O_4$. The scan range from m/z 530 to 600 and the instability of the molecular ion that could be formed by violaxanthin would explain the absence of the peak at m/z 601 $[M + H]^+$ on the spectrum.

- ***Identification of compound C***

Lutein and zeaxanthin are isomers. Their molecular ion comes out at m/z 569.42 $[M + H]^+$ in agreement with the mass calculated using the formula ($C_{40}H_{56}O_2$). They differ only in the position of a double bond on the rings (Figure 1). These carotenoids with very similar structures can be differentiated by comparing the intensities of specific fragment ions. Only lutein contains an allylic hydroxyl group, thus easier to remove than the other hydroxyl group on the secondary carbon atom with adjacent saturated C-C bonds. The ion formed at m/z 551.4 $[M + H - H_2O]^+$ (Figure 1) is stabilized by mesomeric effect. Therefore, it has a more intense peak than that of the molecular ion of zeaxanthin at m/z 569.4 $[M + H]^+$. Unlike lutein, zeaxanthin, which lacks an allylic hydroxyl group, would show a mass spectrum with a base peak m/z 569.4 more intense than that of the fragment m/z 551.4.

Table III: Characteristic masses of the molecular weight of some known carotenoids

Compound	Molecular Ions	Fragmented Ions (m/z)	Probable Molecule
A	$585[M + H]^+$	$567\ [M + H - H_2O]^+, 549\ [M + H - 2H_2O]^+$	Taraxanthin (5,6-epoxy-lutein)
B	$601[M + H]^+$	$583[M + H - H_2O]^+, 565[M + H - 2H_2O]^+$	Violaxanthin
C	$569[M + H]^+$	$551\ [M + H - H_2O]^+$	Lutein
D	$553[M + H]^+$	$535\ [M + H - H_2O]^+$	β-cryptoxanthin
E	$545[M + H]^+$	-	Phytoene
F	$536[M]^+$	-	β-carotene or α-carotene

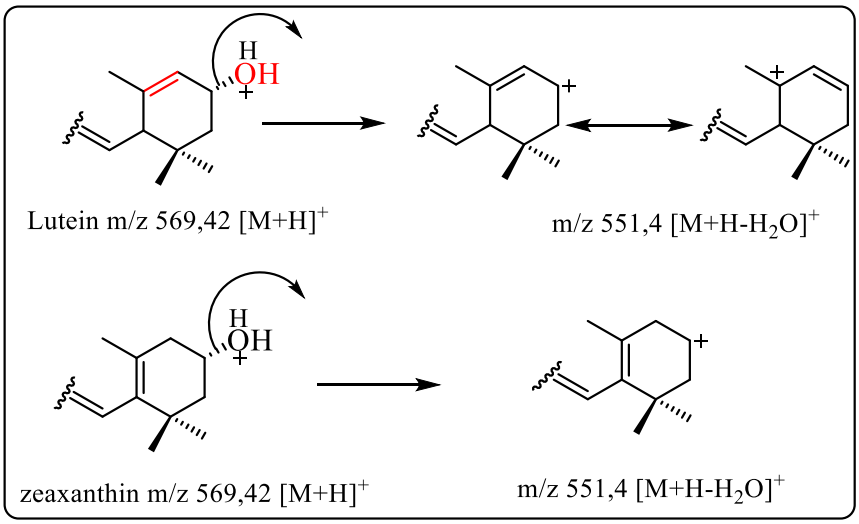


Figure 1: Schematic diagram of the fragmentation of lutein and zeaxanthin

- ***Identification of compound D***

β-cryptoxanthin and α-cryptoxanthin have the same molecular formula ($C_{40}H_{56}O$) and, therefore, the same pseudomolecular ion (m/z 553.42). They also have the same chromophore or carbon chain. The only structural difference, lies in the position of the hydroxyl group and the double bond of the second ring of the molecule as shown in figure 2. The hydroxyl group of α-cryptoxanthin is allylic and therefore easily removed compared to the hydroxyl group of β-cryptoxanthin, which is bonded to a secondary atom with neighboring saturated C-C bonds. The differentiation between these two molecules (α- and β-cryptoxanthin) is done by comparing the peak intensity of the m/z 553.42 $[M+H]^+$ molecular ion (Figures 4 and 5) with that of the m/z 535.42 $[M+H-H_2O]^+$ fragment. The mass spectrum of the Cymbopogon giganteus leaf extract (Figure 4 and 5) showed a more intense peak of the molecular ion m/z 553.42 (Figure 4 and 5) compared to the fragment ion peak m/z 535.42. This confirms that the peak at m/z 553.42 would correspond to β-cryptoxanthin.

On the other hand, zeinoxanthin is a carotenoid molecule that has the same gross formula and chromophore as α-cryptoxanthin but differs in the position of the OH group (Figure 2), could be identified by a more intense peak of the molecular ion m/z 553.42 compared with that of the fragment m/z 535.42. The high intensity of the molecular ion peak of the *Cymbopogon giganteus* leaves extract shows that the peak could be zeinoxanthin as well as β-cryptoxanthin.

- ***Identification of compound E***

The mass spectrum shows a peak at m/z 545.42 $[M+H]^+$ corresponding to the compound of molecular weight 544. This molecular ion could be identified as that of phytoene with chemical formula $C_{40}H_{64}$.

- ***Identification of compound F***

The mass spectrum also shows a molecular ion at m/z 537.44 $[M+H]^+$ which is in agreement with the gross formula ($C_{40}H_{56}$). This peak could be lycopene, beta-carotene or one of these isomers (alpha, gamma, epsilon-carotene). Among these carotenes, beta-carotene is the most commonly found in plants. β-carotene, also one of the most studied carotenes, has provitamin A activity as do β-cryptoxanthin and α-carotene making the plants that produce them sources of vitamin A [4, 5 ; 16-21].

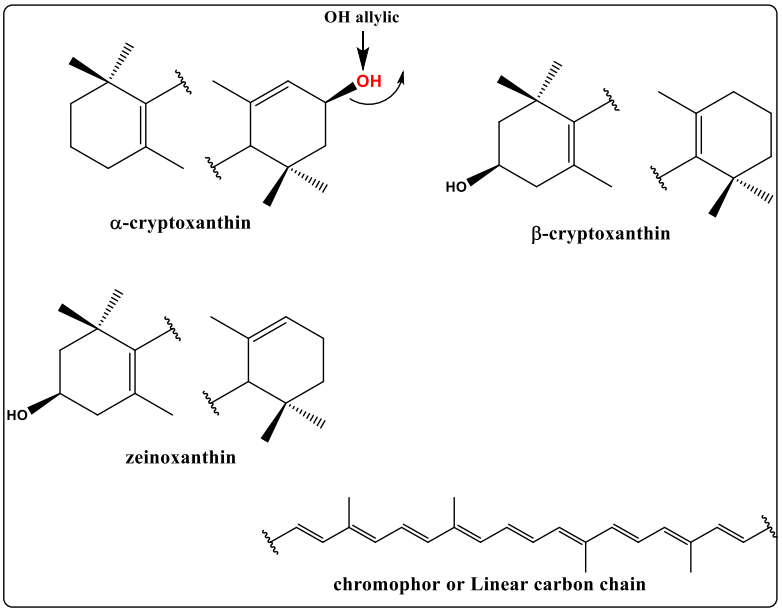
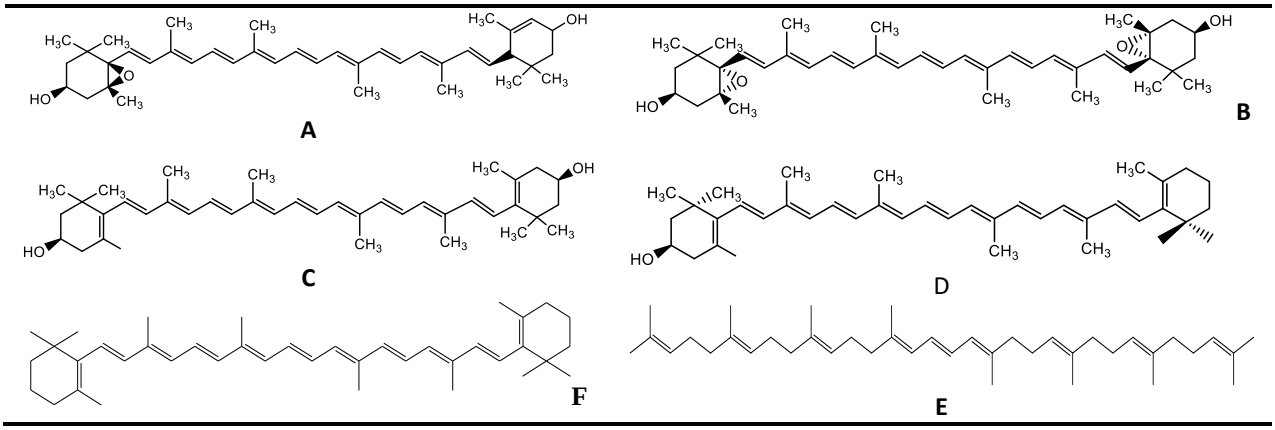


Figure 2: Representative structures of α and β-cryptoxanthin and zeinoxanthin

Figure 3: Proposition of Chemical structures of compounds identifed from *Cymbopogon giganteus* leaves: 5,6-époxylutein (A), Violaxanthin (B), Lutein (C), β-cryptoxanthin (D), Phytoene (E), and β Carotene (F).



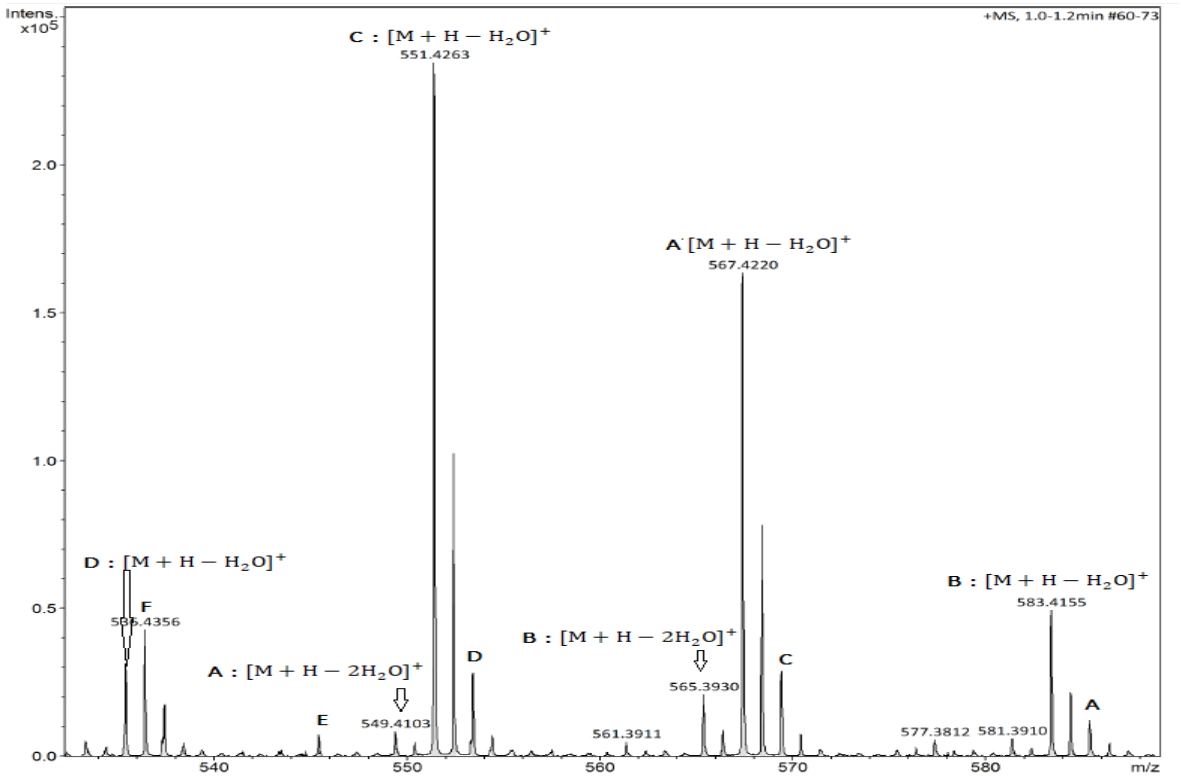


Figure 4: Mass Spectra of spot at Rf = 0,8

4. Conclusion

The present work is a contribution to the study of non-volatile compounds of *Cymbopogon giganteus*. The results of this study showed that the hexanolic extract of leaves is richer in carotenoids (13.3 µg EBC/mg) than those of stems (4.2 µg EBC/mg) and roots (1.5 µg EBC/mg), respectively. HPTLC-MS/MS analysis of the leaves extracts identified the major carotenoids as Taraxanthin (5,6-epoxy-lutein), Violaxanthin, Lutein, β-cryptoxanthin, Phytoene and β-carotene. The evaluation of antioxidant activity of *Cymbopogon giganteus* leaves, stems and roots extracts also showed a high antioxidant potential of the leaves extract (34.1 µg TE/mg) compared to the stems extract (23.6 µg TE/mg) and the roots extract (18.1 µg TE/mg) regardless of the method used (DPPH and FRAP).

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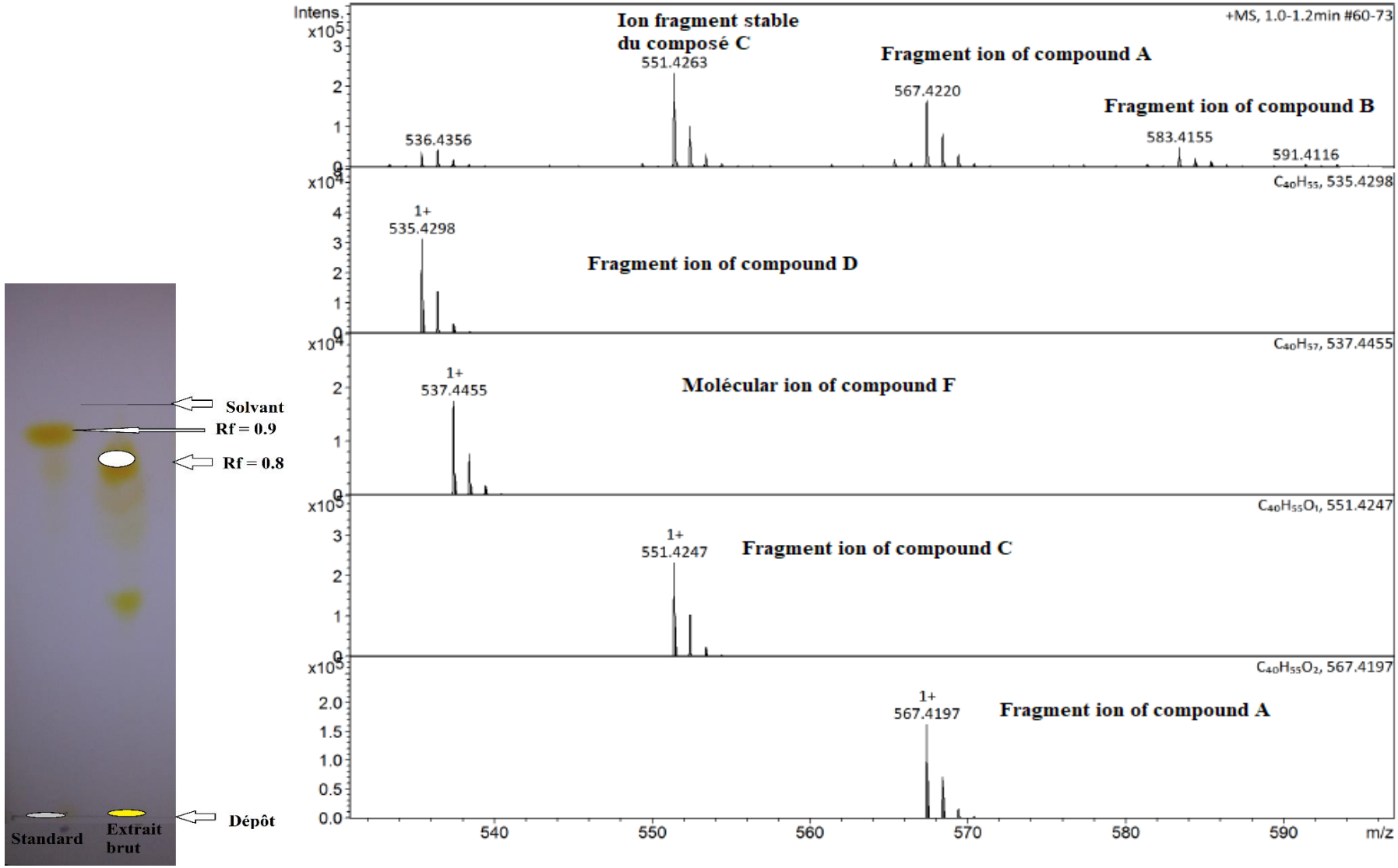

Figure 5: Chromatogram HP-TLC of leaves extract (a), Tandem mass spectra of the different compounds (b)

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