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Characterization of Leaf Phenolic Compounds of *Sabicea johnstonii* by HPLC-MS^n

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ABSTRACT

Sabicea johnstonii is reported among wild plant species used by Congolese people for certain purposes. The leaves of this plant are used for beverages. They are also used to lower blood pressure and to treat burns. In a recent study in vitro, it was shown that the scavenging capacity and the reducing power of the leaves decoction of *S. johnstonii* were mainly due to their polyphenols. They can be used to treat some health problems. The objective of this research work was to characterize the major phenolic compounds of these leaves by HPLC-MSn. Fresh leaves of *S. johnstonii*, collected at Masako, were drying and their phenols were extracted with methanol for their characterization by HPLC-MSn. These analyses showed the presence of ten major phenolic compounds: dicaffeoylquinic acid, three procyanidins, two quercetin O-glycosides, two kaempferol O-glycosides and two unidentified phenols. The presence of these compounds and the observed antioxidant activities in another study justify the use of these leaves in Congolese traditional medicine and as beverage.

1. Introduction

Plants containing polyphenolic compounds are sometimes used to treat many health problems. Epidemiological and experimental studies showed that these phenolic compounds play a role in preventing various diseases including cancer, diabetes and cardiovascular or neurodegenerative diseases [1, 2]. They can destroy free radicals and reduce the risk of these diseases. They can be used to treat other health problems such as to lower blood pressure and to treat burns.

Sabicea johnstonii (Rubiaceae) [3] is a spontaneous liana. Leaves and corolla of this plant are red-carmine and blackish (dry state) respectively. These leaves are consumed as tea by Kumu people of Masako (Forest reserve, DR Congo) [4]. They are sometimes used to lower blood pressure. The Lega people (DR Congo) used the powdered dry leaves of this plant to treat burns [5]. In the Ituri forest, Mbuti and Efe hunter-gatherers use it as a magic plant [6].

A recent study [7] on some wild plant teas collected at Masako showed that the leaf decoction of *S. johnstonii* contains saponins and polyphenols including flavonoids. It does not contain alkaloids, cyanides and oxalates. This study showed also that these polyphenols are responsible for the scavenging capacity and the reducing power observed.

It is known that HPLC-MS^n is a powerful tool for the analysis of natural substances. The purpose of the present research was to characterize by HPLC-MS^n the major phenolic compounds of the methanolic leaf extract of *S. johnstonii*.

2. Experimental Methods

2.1 Plant Material

Fresh leaves of *S. johnstonii* were collected in the forest reserve of Masako and were identified at the Faculty of Sciences (University of Kisangani, DR Congo). After drying in darkness at room temperature for five days in the Chemistry laboratory of this faculty, the leaf powder was packed for analysis in the "Polyphenols BIOTECH" laboratory (France).

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2.2 HPLC-MS^n Analysis

HPLC is a separation method of choice for natural substances analysis. Mass spectrometry provides information about the molecular mass and the fragmentation patterns of a compound.

The HPLC-MS^n system used for analysis consisted of a HPLC system Agilent technologies (Automatic injector and Diode-array detector 1200 series, Prontosil column type C18: 4 mm x 250 mm, 5 µm) and a Mass spectrometer Esquire 6000 (Bruker Daltonics Bremen) equipped with an electrospray ionization source.

Phenolic compounds of the leaf powder were extracted with methanol. Their separation was performed at 25 °C with solvents A (Water/Formic acid 0.1%) and B (Acetonitrile/ Formic acid 0.1%). The injection volume and the elution flow rate were 20 μL and 21.6 mL/min respectively. The elution program was used as shown in Table 1.

Table 1 Elution pattern of the extract

Time (minutes)	0	2	15	40	44	45	50	51	60
% Solvent B	1	1	10	35	50	100	100	1	1

The detection of phenolic compounds was carried out at 280 nm. Mass spectra were acquired using electrospray ionization in positive or negative mode. They were obtained in full scan MS mode from 150 to 1200. ESI-MS parameters were as follows: potential of the ESI source, 4 kV; capillary temperature, 350 °C; nebulizer, 35 psi; dry gas, 10l/min. Data were collected and treated with Hystar logical version 3.0. The MS^n fragmentations of these compounds and the literature data were used for their identification.

3. Results and Discussion

HPLC-DAD-MS was used for characterizing phenolic compounds of the methanolic leaf extract of *S. johnstonii*. The Chromatogram of the HPLC separation is shown in Fig. 1. The analysis of this figure showed that leaves of *S. johnstonii* contain ten major phenolic compounds listed in Table 2.

Some of these compounds have a high molecular weight, suggesting they belong to the class of complex phenolic compounds.

MS^n fragmentations (positive or negative mode) of these compounds were used for obtaining more information on their structures. The results of these fragmentations are presented in Table 3.

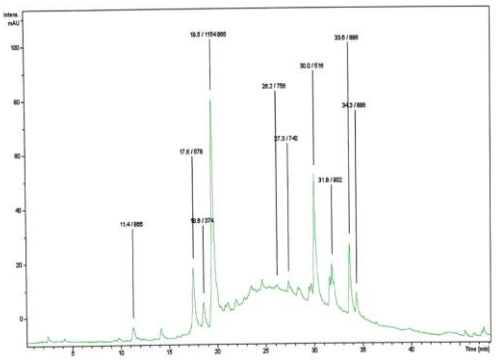

Fig. 1 HPLC Chromatogram of methanolic leaf extract of *S. johnstonii* monitored at 280 nm

Table 2 Major phenolic compounds of methanolic leaf extract of *S. johnstonii*

Compounds	1	2	3	4	5	6	7	8	9	10
Retention time (min)	11.4	17.6	18.6	19.6	26.2	27.6	30	31.8	33.6	34.3
MW (Da)	866	578	748	1154	756	740	516	908	886	886

Table 3 Ion fragments of major phenolic compounds of *S. johnstonii* obtained by MS^n

Compound	MS modes	MS^1 ions	MS^2 ions
1	$[M+H]^+$	867 $[M+H]^+$ 402	813 778 697 579 535 427 295
	$[M-H]^-$	865 $[M-H]^-$	847 739 695 613 577 403 363 281
2	$[M+H]^+$	579 $[M+H]^+$	561 427 352 291 247 225
	$[M-H]^-$	577 $[M-H]^-$	451 425 331 287 245 203
3	$[M+H]^+$	749 $[M+H]^+$ 731 713 647 624 609 577 569 537	
	$[M-H]^-$	747 $[M-H]^-$ 663 577 373 289 193	407 373 317 202
4	$[M+H]^+$	1155$[M+H]^+$ 953 867 763 731 663 579 482 355 279 163	1126 813 715 621 577 409 291 245
	$[M-H]^-$	1153 $[M-H]^-$ 679 707 576 353 191 129	1065 847 739 695 577 543 425 363 287
5	$[M+H]^+$	757 $[M+H]^+$ 679 611 577 465 303	725 611 501 465 401 303 167
	$[M-H]^-$	755 $[M-H]^-$ 720	755 669 591 489 410 367 325 300
6	$[M+H]^+$	741 $[M+H]^+$ 707 595 287	741 723 595 541 494 449 366 287 241 139
	$[M-H]^-$	739 $[M-H]^-$ 387	739 619 575 531 445 393 327 284 255
7	$[M+H]^+$	1033 $[2M+H]^+$ 499 163	319 163
	$[M-H]^-$	1031 $[2M-H]^-$ 515 $[M-H]^-$ 353	353 191
8	$[M+H]^+$	903 $[M+H]^+$ 757 601	885 757 611 455 412 303
	$[M-H]^-$	901 $[M-H]^-$ 515	755 301
9	$[M+H]^+$	887 $[M+H]^+$ 704 595 287	869 801 741 674 595 455 397 329 287
	$[M-H]^-$	885 $[M-H]^-$ 741 521	844 739 627 284
10	$[M+H]^+$	887 $[M+H]^+$ 741 631 544	741 475
	$[M-H]^-$	885 $[M-H]^-$ 745 521	739

3.1 Compound 1 (MW 886 Da)

MS^2 $[M+H]^+$ fragmentation of compound 1(m/Z 867) gave $[M+H-170]^+$ ion at m/Z 697 (loss of gallic acid) and $[M+H-288]^+$ ion at m/Z 579 (interflavonic bond cleavage) which produced an ion at m/Z 427 after losing 152 Da (loss of galloyl unit due to retro-Diels-Alder "RDA").

MS^2 $[M-H]^-$ fragmentation of this compound (m/Z 865) produced $[M-H-H_2O]^-$ ion at m/Z 847, $[M-H-126]^-$ ion at m/Z 739 (due to the loss of a heterocyclic ring fission "HRF" fragment), [M-H-Gallic acid]$^-$ ion at m/Z 695, $[M-H-252]^-$ ion at m/Z 613 (due to the loss of two HRF fragments) and $[M-H-288]^-$ ion at m/Z 577.

The resembled losses were observed by [8], [9], [10] and other researchers in the fragmentation of procyanidins.

The MS^2 $[M-H]^-$ fragmentation resembled to the fragmentation of a procyanidin trimer analyzed by [11] (m/Z: 865 $[M-H]^-$, 739, 695, 577, 451, 425, 287), [12] (m/Z: 865 $[M-H]^-$, MS^2: 739, 695, 577, 425, 287) or [13] (m/Z: 865 $[M-H]^-$, 847, 739, 695, 577).

The losses and fragments observed were characteristic of procyanidins, which allowed us to characterize this compound as a trimeric procyanidin ($C_{45}H_{38}O_{18}$, [13]).

3.2 Compound 2 (MW 578 Da)

MS^2 $[M+H]^+$ fragmentation of compound 2 (m/Z 579) gave $[M+H-H_2O]^+$ ion at m/Z 561, $[M+H-152]^+$ ion at m/Z 427 (loss of a galloyl moiety due to RDA) and $[M+H-288]^+$ at m/Z 291, corresponding to (epi) catechin.

MS^2 $[M-H]^-$ fragmentation of this compound (m/Z 577) produced $[M-H-126]^-$ ion at m/Z 451 (due to the loss of an HRF fragment), $[M-H-152]^-$ ion at m/Z 425 (loss of a galloyl moiety due to RDA) and [M-H-(epi) catechin]$^-$ ion at m/Z 287.

The similar losses were observed in the fragmentation of procyanidins by some other researchers [9-11].

Its MS^2 $[M-H]^-$ fragmentation resembled to the fragmentation of a procyanidin dimer analyzed by [13] (m/Z : 577 $[M-H]^-$, 451, 425, 407, 289, 245, 125) and [11] (m/Z : 577 $[M-H]^-$, 559, 451, 425, 407, 289 and m/Z : 579 $[M+H]^+$, 562, 427, 409).

These MS^2 spectra gave several product ions characteristic of dimeric procyanidins. Based on the above data, compound 2 was recognized as a procyanidin dimer ($C_{30}H_{26}O_{12}$, [13]).

3.3 Compound 3 (MW 748 Da)

MS^1 and MS^2 fragmentations of compound 3 on positive or negative mode did not provide sufficient structural information of this compound.

3.4 Compound 4 (MW 1154 Da)

MS^1 $[M+H]^+$ fragmentation of compound 4(m/Z 1155) gave $[M+H-288]^+$ ion at m/Z 867 (interflavonic bond cleavage) and $[M+H-576]^+$ ion at m/Z 579 (two interflavonic bond cleavages).

MS^2 $[M+H]^+$ fragmentation of this compound gave $[M+H-578]^+$ ion at m/Z 577 (loss of a dimer) and an ion at m/Z 291 corresponding to (epi)catechin.

MS^1 $[M-H]^-$ fragmentation of compound 4 (m/Z 1153) gave $[M-H-288]^-$ ion at m/Z 865 (interflavonic bond cleavage) and $[M-H-577]^-$ ion at m/Z 576.

MS^2 $[M-H]^-$ fragmentation of this compound gave $[M-H-2CO_2]^-$ ion at m/Z 1065, $[M-H-306]^-$ ion at 847 (loss of a RDA fragment with 168 Da plus a benzofuran-forming "BFF" fragment with 138 Da), $[M-H-414]^-$ ion at m/Z 739 (interflavonic bond cleavage plus the loss of an HRF fragment with 126 Da) and $[M-H-576]^-$ at m/Z 577 (two interflavonic bond cleavages).

Procyanidin consist of monomers ranging from dimers to polymers. The presence of (epi) catechin (290 Da) as monomer was also observed on positive mode (MS^2) at m/Z 663 [953-(epi) catechin]$^+$ and at m/Z 287 on negative mode [577-(epi) catechin]$^-$.

On positive mode, the loss of 288 Da was also observed after elimination of this moiety from the ion of m/Z 867. On this mode, the ions of m/Z 731, 715 and 663 lost 152 Da, 306 Da (or 138 Da) and 308 Da (loss of gallic acid 170 Da plus a BFF fragment 138 Da) giving rise to product ions of m/Z 579, 409 (or 577) and 355 respectively.

The anions of m/Z 847, 695 and 577 lost 152 Da (elimination of a galloyl unit) giving rise to product ions of m/Z 695, 543 and 425 respectively.

The MS^2 $[M-H]^-$ fragmentation resembled to the fragmentation of a procyanidin tetramer analyzed by [12] (m/Z : 1153 [M-H],MS^2 983, 865, 695, 577).

These fragmentations produced losses and fragment ions characteristic of trimeric procyanidins. Based on the above data, compound 4 was characterized as a procyanidin tetramer ($C_{60}H_{50}O_{24}$, [13]).

3.5 Compound 5 (MW 756Da)

MS^1 and MS^2 fragmentations of compound 5 on positive mode gave a [M+H-Deoxyhexosyl]$^+$ ion at m/Z 611, [M+H-2Deoxyhexosyl]$^+$ ion at m/Z 465 and [M+H-2Deoxyhexosyl-Hexosyl]$^+$ ion at m/Z 303, corresponding to quercetin (aglycone).

The presence of quercetin (302 Da) as aglycone was also observed on negative mode (MS^2) at m/Z 367 [669-Qercetin]$^-$ and at m/Z 300 (from homolytic cleavage, [14]).

MS^1 or MS^2 fragmentation of this compound on positive mode gave an ion at m/Z 611, after losing 146 Da (deoxyhexosyl unit), suggesting it attachment on quercetin skeleton. The cleavage of 308 Da from the m/Z 611 ion gave a protonated quercetin, what suggested also the attachment of a deoxyhexosylhexose or coumaroylhexose on quercetin skeleton.

These fragmentations were typical of flavonoid O-glycosides. Based on these data, compound 5 was a quercetin O-glycoside, its structure might

be either quercetin O-deoxyhexoside-O-deoxyhexosylhexoside or quercetin O-deoxyhexoside-O-coumaroylhexoside. This compound is an isomer of quercetin 3-O-rutinoside -7-O-rhamnoside and quercetin 3-O-deoxyhexosylhexoside -7-O- deoxyhexoside analyzed previously by few workers [10, 15].

3.6 Compound 6 (MW 740 Da)

MS^1 fragmentation of compound 6 on positive mode produced $[M+H\text{-}Deoxyhexosyl]^+$ ion at m/Z 595 and $[M+H\text{-}2Deoxyhexosyl\text{-}Hexosyl]^+$ ion at m/Z 287, corresponding to kaempferol (aglycone).

On the same mode, MS^2 fragmentation of this compound gave $[M+H\text{-}H_2Ol]^+$ at m/Z 723, $[M+H\text{-}Deoxyhexosyl]^+$ ion at m/Z 595, $[M+H\text{-}2Deoxyhexosyl]^+$ ion at m/Z 449 and $[M+H\text{-}2Deoxyhexosyl\text{-}Hexosyl]^+$ ion at m/Z 287 (aglycone).

The presence of kaempferol as aglycone was also observed on negative mode (MS^2) at m/Z 284 (from homolytic cleavage, [14]).

MS^1 or MS^2 fragmentation of this compound on positive mode gave an ion at m/Z 595, which showed the presence of a deoxyhexose moiety with 146 Da on quercetin skeleton. The cleavage of a deoxyhexosylhexose or coumaroylhexose moiety with 308 Da from the m/Z 595 ion gave a protonated kaempferol. This showed also the presence of this 308 Da moiety on kaempferol skeleton.

Fragmentation patterns were typical of flavonoid O-glycosides. The spectra $[M+Hl]^+$ of compound 6 presented two MS^1 fragments (m/Z : 597 and 287) and three MS^2 fragments (m/Z : 595, 449 and 287) which resembled the spectrum $[M+H]^+$ of kaempferol-3-O-2'', 6''-dirhamnosyl-glucoside (MW 740 Da) analyzed by Lin et al[16].

These data showed that compound 6 was a kaempferol O-glycoside, which might be either kaempferol O-deoxyhexoside-O-deoxyhexosylhexoside or kaempferol O-deoxyhexoside-O-coumaroyllhexoside ($C_{33}H_{40}O_{19}$). Compound 6 is the isomer of kaempferol-3-robinoside-7-0-rhamnoside (Robinin) [17].

3.7 Compound 7 (MW 516 Da)

MS^1 $[M+H]^+$ fragmentation of compound 6 generated $[M+H\text{-}H_2O]^+$ ion at m/Z 499 and $[M+H\text{-}Caffeoyl\text{-}Quinic\ acid]^+$ ion at m/Z 163, which corresponds to caffeoyl unit.

MS^2 $[M+H]^+$ fragmentation of this compound produced also a $[M+H\text{-}Caffeic\ acid\text{-}Quinic\ acid]^+$ ion at m/Z 163.

MS^1 $[M-H]^-$ fragmentation of compound 6 generated a single ion at m/Z 353 $[M\text{-}H\text{-}Caffeoyl]^-$, which corresponds to caffeoyl quinic acid.

MS^2 $[M-H]^-$ fragmentation of the molecular ion of this compound gave an ion at m/Z 353 $[M\text{-}H\text{-}Caffeoyl]^-$, which fragmented to produce an ion at m/Z 191 $[M\text{-}H\text{-}2Caffeoyl]^-$, corresponding to quinic acid.

This indicated that two caffeoyl units are attached to quinic acid and allowes us to suggest that compound 7 is dicaffeoyl quinic acid ($C_{25}H_{24}O_{12}$).

These fragmentation data are in agreement with the fragmentation of hydroxycinnamates reported earlier [18, 19]. They are also in agreement with the fragmentation of caffeoylquinic acid found by Parveen et al [20].

3.8 Compound 8 (MW 902 Da)

MS^1 $[M+H]^+$ fragmentation of compound 8 produced an ion at m/Z 757 $[M+H\text{-}Deoxyhexosyl]^+$ which generated an ion at at m/Z 601$[M+H\text{-}Quercetin]^+$.

MS^2 $[M+H]^+$ fragmentation of this compound produced $[M+H\text{-}H_2O]^+$ ion at m/Z 885, $[M+H\text{-}Deoxyhexosyl]^+$ ion at m/Z 757, $[M+H\text{-}2Deoxyhexosyl]^+$ ion at m/Z 611 and $[M+H\text{-}2Deoxyhexosyl\text{-}\ Deoxyhexosylhexosyl]^+$ ion at m/Z 303 (Quercetin).

MS^2 $[M-H]^-$ fragmentation of compound 8 produced two ions : $[M\text{-}H\text{-}Deoxyhexosyl]^-$ ion at m/Z 755 and $[M\text{-}H\text{-}2Deoxyhexosyl\text{-}Deoxyhexosylhexosyl]^-$ ion at 301 (Quercetin aglycone).

MS^2 fragmentation of this compound on positive mode gave an ion at m/Z 611 after losing 292 Da, which showed an O-glycosylation of quercetin with a deoxyhexosyldeoxyhexose. The cleavage of 308 Da from the m/Z 611 ion gave a protonated quercetin, what suggested another O-glycosylation of quercetin with a deoxyhexosylhexose or a coumaroylhexose.

Like compound 6, the fragmentation patterns of compound 8 were typical of flavonoid O-glycosides. These data allow us to suggest that compound 8 is quercetin O-deoxyhexosyldeoxylhexoside-O-deoxyhexosylhexoside or quercetin O-deoxyhexosyldeoxylhexoside-O-coumaroylhexoside ($C_{39}H_{50}O_{24}$).

This compound (MS^2 m/Z : 901 $[M-H]^-$, 755, 301) is the isomer of two unidentified conjugated quercetin (MS^n m/Z : 901 $[M-H]^-$, 755, 609, 301 and 901 $[M-H]^-$, 755, 609, 463, 301) present in tea and analyzed by Del Rio et al [21].

3.9 Compound 9 (MW 886 Da)

MS^1 $[M+H]^+$ fragmentation of compound 9 produced only three fragments as ions: $[M+H\text{-}Deoxyhexosyl]^+$ ion at m/Z 741, $[M+H\text{-}2Deoxyhexosyl]^+$ ion at m/Z 595 and $[M+H\text{-}2Deoxyhexosyl\text{-}Deoxyhexosylhexosyl]^+$ ion at m/Z 287 (kaempferol aglycone).

MS^2 $[M+H]^+$ fragmentation of this compound produced $[M+H\text{-}H_2O]^+$ ion at m/Z 869, $[M+H\text{-}Deoxyhexosyl]^+$ ion at m/Z 741, $[M+H\text{-}2Deoxyhexosyl]^+$ ion at m/Z 595 and $[M+H\text{-}2Deoxyhexosyl\text{-}\ Deoxyhexosylhexosyl]^+$ ion at m/Z 287.

The presence of kaempferol (286 Da) as aglycone was also observed on positive mode (MS^2) at m/Z 455 [741-Kaempferol]$^-$ and on negative mode (MS^2) at m/Z 284 (from homolytic cleavage, [14]).

The presence of ion fragments at m/Z 595 (loss of 292 Da) and m/Z 287 (loss of 308 Da) in the MS^1 or MS^2 fragmentation of compound 9 on positive mode showed the glycosylation of kaempferol with a deoxyhexosyldeoxyhexose and a deoxyhexosylhexose or coumaroylhexose.

These fragmentations were typical of flavonoid O-glycosides. These data allowed us to assign the structure of compound 9 as kaempferol O-deoxyhexosyl-deoxylhexoside-O-deoxyhexosylhexoside or kaempferol O-deoxyhexosyldeoxylhexoside-O-coumaroylhexoside ($C_{39}H_{50}O_{23}$). This compound is the isomer of two unidentified kaempferol conjugate (885$[M-H]^-$, 739, 593, 285) analyzed by Del Rio et al [21].

3.10 Compound 10 (MW 886 Da)

MS^1 or MS^2 $[M+H]^+$ fragmentation of compound 10 produced $[M+H\text{-}146]^+$ ion at m/Z 741 (loss of deoxyhexosyl unit). The same loss was observed at m/Z 739 on negative mode (MS^2). Although the compound 10 has the same molecular weight as the compound 9, his MS^1 and MS^2 fragmentations on positive or negative mode did not provide sufficient information about its structure.

4. Conclusion

Leaves of *S. johnstonii* contain ten major phenolic compounds: dicaffeoylquinic acid, three procyanidins, four flavonoid O-glycosides (two quercetin and two kaempferol derivates) and two unidentified phenols.

These leaves are another source of natural antioxidants which may have positive effects on certain diseases and burns. This justifies their use in treating some health problems.

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