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Total Phenolics and Total Flavonoid Contents, Antioxidant Activity and Flavonoids Identification by High-Performance Liquid Chromatography – Tandem Mass Spectrometry of *Odontonema strictum* (Acanthaceae) Leaves

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ABSTRACT

Different extracts of Odontonema strictum leaves are investigated for their total phenolic content, total flavonoid content and antioxidant properties using the DPPH (2,2-diphenyl-1-picryl-hydrazyl) radical scavenging capacity assay. The flavonoid diversities in the ethyl acetate and butanol extracts are also investigated by high-performance liquid chromatography–tandem mass spectrometry (LC-MS/MS). Total phenolic contents varies from 27.7 ± 1.5 mg GAE/g to 102.6 ± 0.9 mg GAE/g (GAE=Gallic Acid Equivalent) of dried extract and total flavonoid contents from 5.2 ± 0.2 mg QE/g to 170.7 ± 7.3 mg QE/g (QE=Quercetin Equivalent) of dried extract. The IC_{50} of DPPH inhibition is measured from 1.1 ± 0.1 to 3.9 ± 1.7 µg/mL. The antioxidant capacity correlates quite well with the total phenolic and the total flavonoid contents, indicating that phenolic compounds are major contributors to the antioxidant properties of the extracts. Lastly, based on LC-MS/MS analysis, we demonstrate that the flavonoid glycosides are constructed on the luteolin structure as aglycone.

Keywords: *Odontonema strictum*, Total phenolic content, Total flavonoid content, Antioxidant activity, Flavonoids glycosides, LC-MS/MS

INTRODUCTION

The prevalence of cardiovascular diseases in general and hypertension in particular remains high in sub-Saharan Africa [1-3]. These data reveal that hypertension is a public health issue and must be treated accordingly by developing strategies to prevent, detect, treat and effectively control hypertension. Conventional drugs against hypertension are too expensive for populations in developing countries, where nearly 80% of them resort to traditional medicine for their medical care [4]. The valorization of bioactive plants in efficient and cheap herbal medicine therefore constitutes a promising alternative, for both medical and economic points of views.

Odontonema strictum (Acanthaceae) is a plant used in Burkina Faso by the traditional medicine practitioners against hypertension. The aqueous extracts from the leaves of this plant were showed efficient to decrease hypertension. In a bio guided study, Kini et al. [5] and Ouédraogo et al. [6] demonstrated that the active molecules against hypertension are abundant in the ethyl acetate extracts that are mainly constituted by flavonoids. In addition to flavonoids, *Odontonema strictum* leaves contain tannins, saponins, sterols, triterpenoids and glycosides [7]. A tiliroside has also been identified in the *Odontonema strictum* leaves [8]. Flavonoids represent a large family of phenolic compounds present in fruits and vegetables in all parts of plants. They are known to have antioxidant properties [9,10]. This phytochemical family is also known to possess several biological properties, including anti-hypertensive [11], anti-HIV and anti-plasmodial

[12], anticancer [13] and antifungal [14] ones. Extracts rich in flavonoids also showed significant antifungal activities against phytopatogenic fungi [15].

Since our current interest includes assessing health-promoting phytochemicals [16], therefore we here investigate the total phenolic and total flavonoid contents of various extracts (water, ethanol, ethyl acetate, butanol) of *Odontonema strictum* leaves. The antioxidant activity of these extracts was also determined using the DPPH radical scavenging assay. Lastly, we intend to identify flavonoids in extracts using LC-MS/MS.

MATERIALS AND METHODS

Plant material and chemicals

Odontonema strictum leaves were collected in the garden of Department of Traditional Medicine, Institute for Health Sciences Research in Ouagadougou (Burkina Faso). The collected plant material were compared to a previous voucher specimen (N°8702) deposited in the Herbarium of the National Centre for Scientific and Technological Research (Ouagadougou, Burkina Faso). The leaves were shade dried, powdered and stored in an air tight container till further use.

The chemicals used were analytical grades including: 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), Dimethyl sulfoxide (DMSO) (Sigma-Aldrich), gallic acid, quercetin dihydrate, Folin-Ciocalteau's phenol reagent (Sigma), Aluminium chloride hexahydrate (Sigma-Aldrich).

Extraction procedure

Decoction

Fifty (50) g of leaves powder were introduced into a 500 mL flask to which 500 mL of distilled water was added. The mixture was heated to reflux for 30 min. The solution was cooled at room temperature, filtered and centrifuged (Centriguge OSI, Italy) for 10 min at 2000 g. The extract obtained (OSA) was frozen and then lyophilized for total phenolic and total flavonoid contents determination and anti-oxidant activities assessment.

Maceration with ethanol

Fifty (50) g of leaves powder were introduced into a 1 L Erlenmeyer flask to which 500 mL of ethanol (70%) was added. The mixture was kept at room temperature for 24 h. The solution was then filtered and centrifuged (Centriguge OSI, Italy) for 10 min at 2000 g to obtain the hydro ethanol extract (OSE).

Flavonoids enriched extracts

Flavonoids were extracted according to the literature with slide modifications [17-19].

The leaves powder (300 g) of Odontonema strictum was defatted first with 1500 mL of cyclohexan at room temperature, by stirring with a magnetic stirrer. After filtration, the marc was extracted with 450 mL of ethanol 70% (v/v) by stirring on a magnetic stirrer for 24 h. The filtrated extracts were evaporated to dryness with a Rotavapor. The ethanol extract was dissolved in 500 mL of distilled water and extracted successively with ethyl acetate (3 × 300 mL) and *n*-butanol (3 × 300 mL). The solvents were then removed using a Rota vapor to give ethyl acetate (OSEA) and n-butanol (OSB) extracts. Water was also removed from the residual water fraction to give an aqueous extract (OSAR). The three extracts were subjected to total phenolic and flavonoid contents determination and antioxidant activities assessment.

Determination of total phenolic

Total phenolic content was determined using the Folin-Ciocalteu method [20-22]. A 1 mL sample aliquot of extract or gallic acid standard (0.5-50 µg/mL) was mixed with 1 mL (0.2 N) Folin–Ciocalteau's reagent. After vortexing and incubating at room temperature for 8 min, 2 mL aqueous sodium carbonate solution (7.5%) was added. The mixture was vortexed and kept at room temperature for 30 min. Absorbance of the blue-colour solution was recorded at 760 nm on a UV-Vis spectrophotometer Agilent 8453. The experiment was performed in triplicate. The results, determined using the equation of the calibration curve ($y=29.078x+0.0202$; $R^2=0.999$) and the total phenolic content was expressed as gallic acid equivalent (GAE).

Determination of total flavonoid content

The flavonoid content of the extracts was determined using the method described the literature [23,24]. In short, the extracts were individually dissolved in methanol at the concentration of 1 mg/mL. Then, the sample solution 0.5 mL was mixed with 0.5 mL of methanolic solution of aluminium chloride $AlCl_3$ (2%). After 1 h incubation at ambient

temperature, the absorbance of the supernatant was measured at 415 nm using UV-Vis spectrophotometer Agilent 8453. The experiment was performed in triplicate. The total flavonoid content was expressed as quercetin dihydrate equivalent from the equation of the calibration curve (y=35.6x–0.0379; R^2=0.9982).

Antioxidant activity using DPPH radical scavenging assay

Free radical scavenging activity of the extracts was determined by the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay described in literature [22,25,26] with slight modifications. Briefly, 10 µL of extract or fractions or standard was added to 200 µL of DPPH methanolic solution (0.04 $mg.mL^{-1}$) in a 96-well microliter plate and vortexed. After 30 min incubation in the dark at room temperature, the absorbance was measured at 490 nm using spectrophotometer BioRAd (model 680, Japan). Antiradical activity was defined as the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% and expressed as antiradical power ($ARP=1/IC_{50}$). All measurements were performed in triplicate.

Identification of flavonoids by LC-MS/MS

Preparation of standards and sub fractions solutions

One (1) mg of each standard was firstly dissolved in 1000 µL of methanol HPLC grade to prepare stock solutions of 1 $mg.mL^{-1}$. The stock solutions were then diluted to obtain concentrations ranging between 100 to 10 $\mu g.mL^{-1}$. The standards available were: Apigenin, Genistein, Luteolin, Kaempferol, Quercetin, Isorhamnetin, Myricetin, Luteolin O-7-Glucoside, Linarin, Fortunellin, Kaempferol 7-O-neohesperidoside and Diosmin.

The sub fractions solutions are prepared in the same conditions as the flavonoid standards.

LC-MS/MS analysis

For flavonoid identification, both ethyl acetate (OSEA) and *n*-butanol (OSB) extracts were gathered in order to have most of the flavonoids of the plant in one extract. The extract obtained was called OSEB. This extract was first subject to Diaion HP-20 column chromatography, eluted with gradient of methanol and water (0–100% MeOH). The fractions collected were combined according to TLC profile, and those containing flavonoids compounds were subject to silica gel column using chloroform and methanol gradient (0-100%MeOH). The fractions from the silica gel column were analyzed by LC-MS/MS for flavonoids identification.

For the on-line LC-MS/MS analyses, a Waters Alliance 2695 liquid chromatography device was used. The HPLC device was coupled with the Waters Quattro Ultima mass spectrometer and consisted of a vacuum degasser, a quaternary pump and an auto-sampler. Sample volumes of 1 µL are injected. Chromatographic separation is performed on a non-polar column (Eclipse plus C18; 4.6 × 100 mm; 3.5 µm; Agilent) at 40°C. The mobile phase is programmed with a constant flow (1 mL/min) of 60% of eluent A (water, 0.1% formic acid) and 40% of eluent B (acetonitrile) during 15 min. The ESI conditions are follow: positive ion mode (*m/z*=M+1); capillary voltage 3.1 kV; cone voltage 40 V; source temperature 100 °C; desolvatation temperature 300 °C. Dry nitrogen is used as ESI gas with a flow rate of 50 L/h for the gas cone and 500 L/h for the desolvation gas. The single-stage LC-MS spectra are recorded by scanning the first quadrupole analyzer between *m/z* 250 and 1500. For the LC-MS/MS experiments, the ions of interest were mass-selected by the first quadrupole. The selected ions are then submitted to collision against argon in the trap cell of the tri-wave device and the laboratory frame kinetic energy (E_{lab}), typically at 80 eV, is selected to produce ion signals intense enough. Ions therefore produced are lastly mass measured with the ToF analyzer.

Data were processed using MassLynx version 4.1 software (Micromass, Manchester, UK).

Statistical analysis

All experiments were performed in triplicates and the data are represented as mean ± standard error of mean (SEM). Analysis of variance (ANOVA) enabled appreciating the differences between extracts for their antioxidant, polyphenolic and flavonoid contents using the statistical software Genstat, 14th Edition. The $p<0.05$ values were considered statistically significant.

RESULTS AND DISCUSSION

Five (5) different extracts were obtained from *Odontonema strictum* leaves, with different solvents, in order to have all secondary metabolites of the leaves. Table 1 details the extraction values of the extracts obtained. Water extract by decoction (OSA) gave the highest extractive value, followed by the residual water extract (OSAR) EtOAc and BuOH.

Total phenolic and flavonoid contents

The data presented in Table 1 indicate the highest total phenolic content at 102.6 ± 0.9 mg GAE/g for the ethyl acetate extract (OSEA), while the lowest total phenolic content at 27.7 ± 1.5 mg GAE/g of dried extract is measured in the residual aqueous fraction (OSAR). The n-butanol fraction (OSB) and the hydro ethanol extract (OSE) extract also present high phenolic contents with 97.8 ± 2.1 and 73.7 ± 4.9 mg GAE/g of dry extract, respectively.

As far as the flavonoids are concerned, the highest flavonoid content is measured in the ethyl acetate extract (OSEA) with 170.7 ± 7.3 mg QE/g extract dry weight and the lowest content in OSAR at 5.2 ± 0.2 mg QE/g of dry extract. As shown in the Table 1, the total phenolic and flavonoid contents are ranked in the following order: OSEA>OSB>OSE>OSAR. The OSA has not been classified here because the dry extract is not fully dissolved during the experiment for total flavonoids quantification. Previously, Kini et al. [5] found that flavonoids C-heterosides and O-heterosides are present in *O. strictum* leaves powder at the concentration of 0.37% and 1.13%, respectively.

As the antioxidant activities of extracts are directly correlated with their total phenolic and flavonoid contents, it is likely that the OSEA will reveal the strongest antioxidant activity.

Antioxidant activity using DPPH radical scavenging assay

Antioxidant assay based on the radical DPPH$^{\bullet}$ is among the most popular spectrophotometric method for determining the antioxidant capacity of foods, beverages and vegetable extracts [27,28]. In the DPPH assay, the stable DPPH$^{\bullet}$ radical in methanol solution is reduced to diphenyl-picrylhydrazine (yellow-coloured solution) in presence of antioxidants due to the formation of the [DPPH+H] by hydrogen atom transfer to the free radical (DPPH$^{\bullet}$).

Radical scavenging activity of *O. strictum* leaves extracts are presented in Table 1. A lower IC_{50} value indicated higher antioxidant activity. The measured IC_{50} values range from 1.1 ± 0.2 to 3.9 ± 1.7 µg/mL. In line with the total phenolic and flavonoid contents, the ethyl acetate extract (OSEA) presents the highest antiradical power compared to the others with an IC_{50} at 1.1 ± 0.2 µg/mL. The *n*-butanol extract (OSB) also exhibits a high free radical scavenging activity with IC_{50} at 1.6 ± 0.5 µg/mL, followed by OSE at IC_{50}=1.8 ± 0.5 µg/mL.

To correlate the results obtained with the different methods, a regression analysis is performed (correlation coefficient (R)) and presented in Table 2. A good correlation appears between the total flavonoid contents and the DPPH assay (R=0.9733). Similarly, the DPPH data also correlate quite well with the total phenolic compound concentration determined by the Folin-Ciocalteu method (R=0.9016). The lowest correlation appears between the total phenolic content assay and the total flavonoid content (*R*=0.7510).

Table 1: Extractive values, total phenolic contents, flavonoid contents and DPPH radical activities of five extracts of *O. strictum* leaves

Extracts	Extractive value (%)	*TPC (mg GAE/g extract DW)	**TFC (mg QE/g extract DW)	***IC_{50} (mg/mL)	****ARP
		Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
OSA	26.2	38.0 ± 0.7 ^{b}	-	3.9 ± 1.7 ^{b}	0.25
OSE	8.6	73.7 ± 4.91^{c}	36.4 ± 1.9 ^{b}	1.8 ± 0.5^{ab}	0.56
OSEA	1.8	102.6 ± 0.9 ^{d}	170.7 ± 7.3 ^{d}	1.1 ± 0.2 ^{a}	0.93
OSB	3.3	97.8 ± 2.1 ^{d}	55.9 ± 2.3 ^{c}	1.6 ± 0.4 ^{a}	0.62
OSAR	13	27.7 ± 1.5 ^{a}	5.2 ± 0.2 ^{a}	2.9 ± 0.5 ^{ab}	0.34

Data are expressed as mean ± SEM of triplicate experiments. In each column, the values with the various letters are different (P<0.05)

GAE: Gallic Acid Equivalent; QE: Quercetin Equivalent; DW: Dry Weight; OSA: Decoction Extract; OSE: Ethanol Extract; OSEA: EtOAc Extract; OSB: BuOH Extract; OSAR: Residual Water Extract After EtOAc and BuOH

* Total Phenolic Content in mg of gallic acid equivalent in a g of extract; ** Total Flavonoid Content in mg of quercetin equivalent in a g of extract; *** Amount of antioxidant necessary to decrease the initial DPPH concentration to the half

**** ARP (antiradical power)=1/IC_{50}

Values are expressed in mean ± SEM

Table 2: Correlation coefficient (R) between assays

	DPPH	TPC
TPC	0.9016	-
TFC	0.9733	0.751

TPC: Total Phenolic Content; TFC: Total Flavonoid Content; DPPH: 2,2-diphenyl-1-picryl-hydrazyl

The antioxidant activities determined using the DPPH method is in agreement with the results of the Total Phenolic and Total Flavonoid contents and the scavenging activity of the plant extracts might then be attributed to the presence of phenolic compounds [29]. Indeed, several studies reported strong correlation between antioxidant activities of plants extracts and their phenolic content [30-32]. It is also well-known that flavonoids have good antioxidant properties [9,10,33]. This might justify the good correlation between DPPH radical scavenging assay and TFC with a correlation coefficient R at 0.9733. Antioxidant properties of phenolic compounds are linked to their aromatic rings bearing one or several hydroxyl groups and are therefore potentially able to quench free radicals by forming resonance-stabilized phenoxyl radicals [22,34].

The evaluation of the total phenolic and total flavonoid contents as well as the antioxidant properties of *Odontonema strictum* leaves extracts is reported here for the first time. The literature shows a correlation between anti-inflammatory, analgesic and antioxidant activities of the methanol and dichloromethane extracts [35]. Therefore, the ethyl acetate and butanol extracts of *Odontonema strictum* leaves are also likely to present good anti-inflammatory and good analgesic activity, since they are rich in polyphenols, particularly flavonoids, but this is not cover by this study.

Identification of flavonoids by LC-MS/MS

We submit the extract OSEB rich in flavonoid to LC-MS analysis in order to identify flavonoids. Upon LC-MS, four significant signals, likely to correspond to flavonoids, are detected at 15.67, 16.43, 18.16 and 18.18 min (Table 3). The Linarin standard is eluted after 19.41 min and the similarity between the elution times of the detected molecules with the Linarin standard could be taken as indication to identify the flavonoid nature of the 4 compounds.

First of all, as exemplified in Table 3, the five observed retention times correspond to protonated molecules $[M+H]^+$, detected at respectively *m/z* 551, 565, 565, 593 and 593. Interestingly, we are then facing three different ion compositions, tentatively ascribed in Table 3.

All the standards available, i.e., Apigenin, Genistein, Luteolin, Kaempferol, Quercetin, Isorhamnetin, Myricetin, Luteolin O-7-Glucoside, Linarin, Fortunellin, Kaempferol 7-O-neohesperidoside and Diosmin, are not detected within the extract OSEB. A previous study reveals that most of the flavonoids in *Odontonema strictum* leaves possess the luteolin aglycon [5]. We then decided to perform LC-MS/MS analysis using the luteolin aglycon as the starting point. It must also be reminded that the neutral losses of 132, 162 and 146 mass units enable to identify pentosides (xylose or arabinose), hexosides (glucose or galactose) and deoxyhexoside (rhamnose), respectively [36].

Upon collisional activation (Figure 1), the *m/z* 551 ions undergo two consecutive losses of 132 u, revealing the presence of two pentose residues (xylose or arabinose) attached to the aglycone moiety. The two sugars can be attached to the flavonoid aglycone either at two different positions (di-*O*-glycosides, di-*C,O*-glycosides) forming monosaccharides or at the same position (*O*-diglycosides, *C,O*-diglycosides) forming a disaccharide. For the time being, two basic methods have been described to distinguish these regioisomers. In one approach, they can be distinguished by their product ion spectra, through the analysis of the Y1, Y0 and Z1 ions (Figure 2) [37,38]. The presence of the *m/z* 419 (Z1) fragment implies that one sugar unit is linked to another sugar and not directly to the aglycone, indicating an *O*-diglycosyl structure [39,40]. In theory, flavonoids could be glycosylated in any position, but *O*-glycosylation occurs mainly at position 7, as in flavones, isoflavones, flavanones and flavonols. So, the common *O*- glycosylation position of flavone is indicated in Figure 3 [41]. Taking all these results into account, together with the reported data [5], revealing that the flavonoids in *Odontonema strictum* leaves have the luteolin aglycone, we propose that the compound **1** may be a luteolin-7-diglycosyl with two pentoses (Figure 4).

As revealed in Table 3, two isomers are detected at *m/z* 565 and correspond to molecules eluted after 16.43 and 18.18 min. The corresponding CID spectra are presented in Figures 3 and 5, respectively. Upon CID, the *m/z* 565 ions of the flavonoid isomer eluted after 16.43 min mostly undergo the consecutive losses of 146 and 132 u, leading to the fragment ions at *m/z* 419 and 287, respectively. As already reminded, a 146 u loss is characteristic of a rhamnose, whereas the 132 u correspond to a pentose loss. The rhamnose and the pentose might be attached on the same oxygen atom, probably *O*-7 [38,42] and the proposed structure for this compound **2** is shown in Figure 4.

Table 3: Retention time and MS^2 fragmentation of *Odontonema strictum* flavonoids. The linarin standard (compound **4**) has been introduced in the extract for comparison.

Compositions	Retention times (min)	Parent ions [M+H+]+	MS^2 fragment ions (%)
1 ($C_{25}H_{26}O_{14}$)	15.67	551	551 (100); 419 (15); 287 (20);
2 ($C_{26}H_{28}O_{14}$)	16.43	565	565 (100); 419 (15); 287 (30)
3 ($C_{26}H_{28}O_{14}$)	18.18	565	565 (68); 433 (20); 301 (100)
4 ($C_{28}H_{32}O_{14}$) **Linarin**	19.41	593	593 (100); 447 (50); 285 (15)
5 (NI)*	18.16	593	593 (100); 287 (80)

*NI: Not Identify

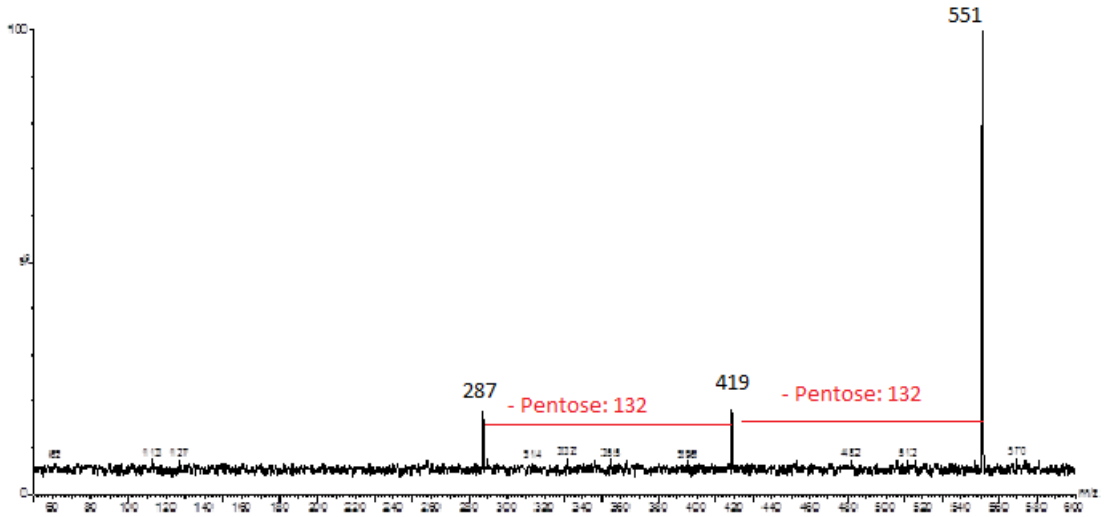


Figure 1: LC-MS/MS analysis of extract OSEB: collision-induced dissociation (CID) mass spectrum of the *m/z* 551 ions

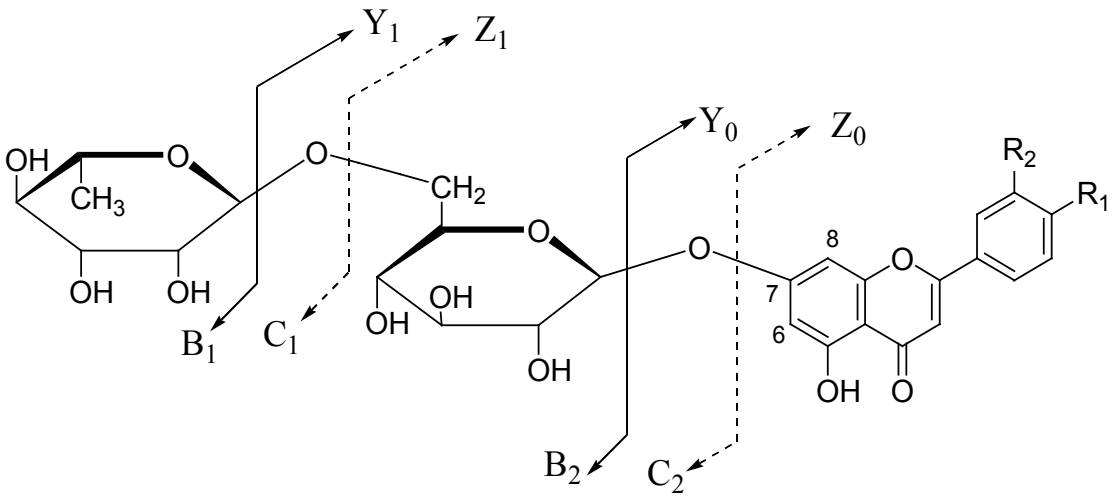


Figure 2: Collision-induced dissociation of protonated flavonoids: nomenclature for *O*-glycoside decomposition pathways and fragment ions [38]

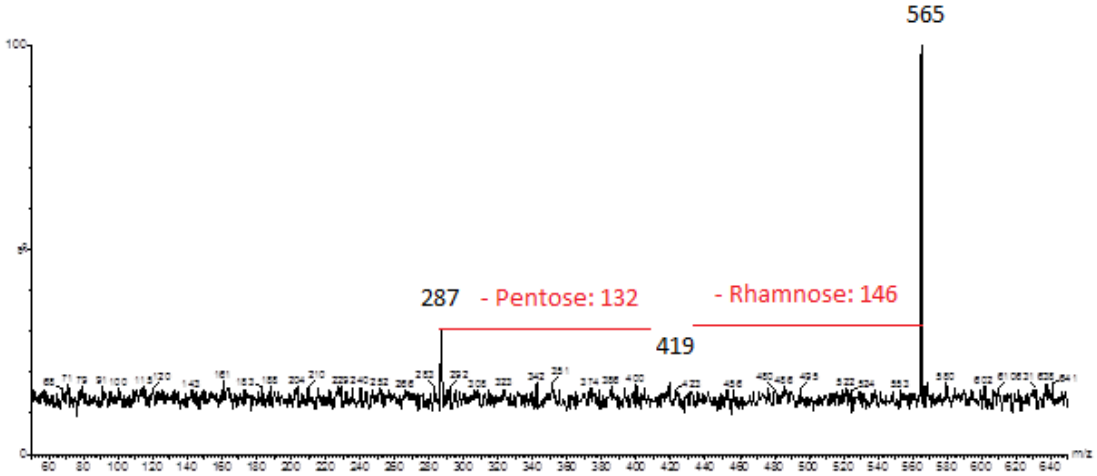
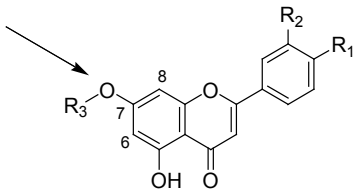


Figure 3: LC-MS/MS analysis of extract OSEB: collision-induced dissociation (CID) mass spectrum of the *m/z* 565 ions (t_R=16.43 min)



Flavone

N° Compounds	R1	R2	R3
1	OH	OH	Pent1 – Pent2
2	OH	OH	Pent1 – Rha2
3	OCH_3	OH	Pent1 – Pent2
4 (Linarin)	OCH_3	H	Glc1 – Rha2

Figure 4: Structures of flavonoids constituents identified in leaves extract of *Odontonema strictum*

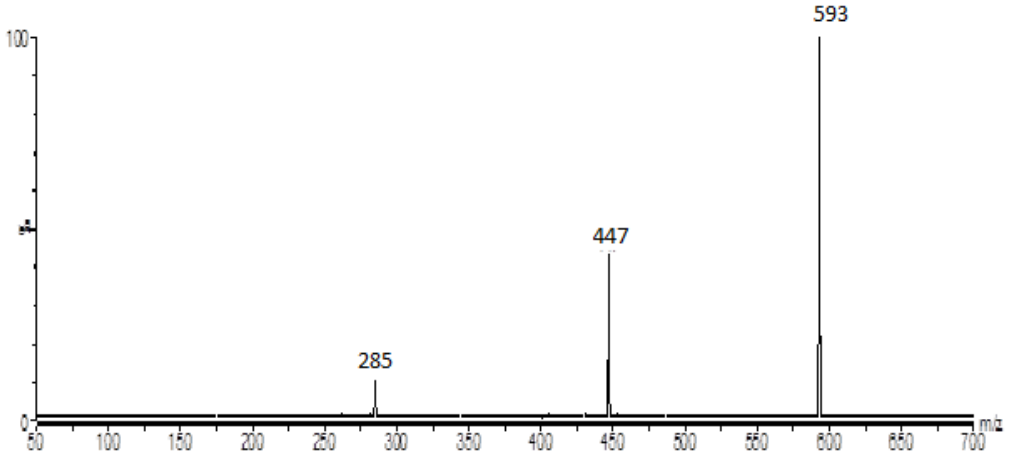


Figure 5: LC-MS/MS analysis of extract OSEB: collision-induced dissociation (CID) mass spectrum of the *m/z* 565 ions (t_R=18.18 min).

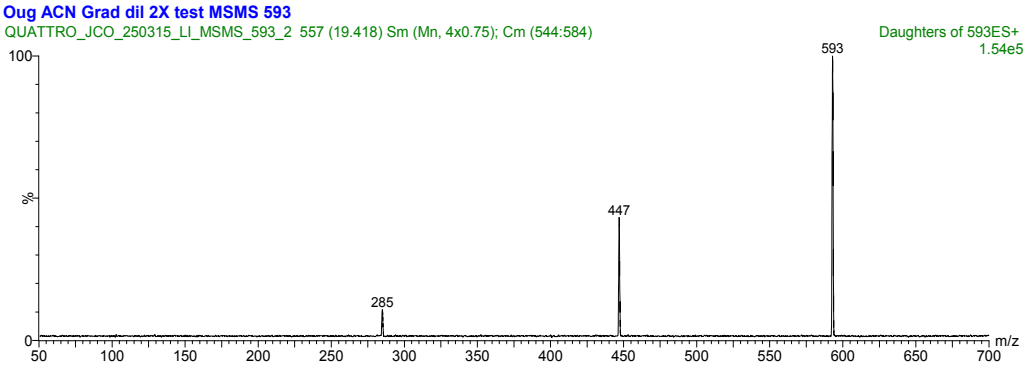


Figure 6: LC-MS/MS analysis of Linarin: collision-induced dissociation (CID) mass spectrum of the *m/z* 565 ions (t_R=19.41 min)

The MSMS spectrum of the protonated second isomer (*m/z* 565, t_R=18.18 min, Figure 5) presents a base peak at *m/z* 301 (Figure 5) arising from the complete loss of both the pentoses. The identification of two pentoses comes from the presence of the intermediate signal at *m/z* 433, corresponding to a single pentose loss. These ions present then the same fragmentation mode as the *m/z* 551 ions (compound **1**), previously investigated. The 14 u mass difference between both ions (m/z 565 and 551) is attributed to the presence of a methoxyl group in the m/z 565 ions (compound 3) instead of a hydroxyl group in the m/z 551 ions. We tentatively propose the structures in Figure 4 for the m/z 551 and 565 ions.

Linarin (**4**), our flavone glycosylated standard, is then analyzed in the same conditions and is characterized by a retention time at 19.41 min. The MSMS fragments of protonated Linarin arise from successive losses of rhamnose

[MH – 146] and hexose [MH – 146 – 162] (Figure 6). The decomposition pathway of protonated Linarin is closely related to the ethanol extract unknown compound 5 and this further reinforces our preliminary identification (Figure 4). Interestingly, the ions detected at *m/z* 593 (t_R=18.16 min) present a CID spectrum different from the spectrum of protonated Linarin, confirming that the corresponding flavonoid is an isomer of Linarin.

CONCLUSION

In this study, several extracts from *Odontonema strictum* leaves were analyzed for their total phenolic content, total flavonoid content and antioxidant activity. A significant relationship between antioxidant capacity, total phenolic content and total flavonoid content was found and the ethyl acetate extract have the highest content (phenolic and flavonoid) and antioxidant activity.

The high-performance liquid chromatography – electrospray ionization tandem mass spectrometry enabled to think of some flavonoid glycosides in the leaves. Yet, these molecules need to be isolated and fully characterized.

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