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Article

Anthocyanins Characterization and Antioxidant Activities of *Odontonema strictum* (Nees) Kuntze Flowers

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ABSTRACT

In the last decades, the interest in anthocyanin pigments has enhanced due to their possible use as natural food colorants and mostly as antioxidant agents. Anthocyanins in flowers of *Odontonema strictum* (Nees) Kuntze were characterized by high-performance liquid chromatography combined with electrospray ionization quadrupole-time-of-flight mass spectrometry. A total of five anthocyanins were identified. They were reported from *O. strictum* flowers for the 1st time. *O. strictum* flowers extracts were also investigated for their total phenolic, flavonoid, anthocyanin contents, and antioxidant activities using the 2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity assay. Using the Folin-Ciocalteu's reagent, the highest total phenolic content from flowers was obtained with methanol extract (84.0 ± 3.0 mg AGE/g dry weight). The total anthocyanin content (TAC) showed a similar trend (0.3 ± 0.03 mg cyanidin-3-glucoside/g dry weight) for methanol extract. In line with the total phenolic and TAC, the methanol extract of *O. strictum* flowers presents the highest antioxidant capacity (IC_{50} = 0.28 ± 0.03 mg/mL). The highest total flavonoid content was observed with ethyl acetate extract (29.0 ± 0.6 mg rutin equivalent/g dry weight). This study provides useful information for the production of valuable nutraceuticals from flowers of *O. strictum* (Nees) Kuntze.

Key words: *Odontonema strictum*, Anthocyanins, High-performance liquid chromatography-Electrospray ionization-mass spectrometry, Antioxidants, Functional food.

1. INTRODUCTION

Nowadays, public interest in the health benefits of phytoceuticals for reducing and inhibiting chronic diseases and aging has stimulated the nutritional supplement industry to develop "functional food" and herbal supplements containing these ingredients [1-3]. Anthocyanins, a group of glycosylated phenolic compounds, exist widely in fruits, vegetables, and flowers and are responsible for their bright colors such as blue, orange, and red [1,3,4]. Natural anthocyanins generally include cyanidin, delphinidin, pelargonidin, peonidin, malvidin, and petunidin. The molecular structure of anthocyanins is mainly constituted of an aromatic ring (a), a heterocyclic ring (c), and another type aromatic ring (b), as shown in Figure 1 [1,5]. The main differences of anthocyanins are the types of functional groups (i.e., OH or OCH_3 on positions 3, 5, 7, and 3'-5', as shown in Figure 1) as well as the types of bonded sugar (glucose, rhamnose, arabinose, and xylose..) or acylated acids (caffeic acid, ferulic acid, and *p*-hydroxybenzoic) moieties (on positions 3 and 5, as shown in Figure 1). The recent and renewed interest in anthocyanins is not only due to their bright colors as natural colorant for food additives [4] but also to the fact that they enhance the health-promoting qualities of foods [1]. Recent studies with human cells and rats showed that dietary anthocyanins might have beneficial actions against the initiation and the development of vascular diseases [3,6]. Anthocyanins and their derivatives have antioxidant activities [7], anticancer [8], antidiabetic activities [9], and the potential to prevent memory loss and neurodegenerative diseases [10].

Flowers of *Odontonema strictum*, a plant used in Burkina Faso by the traditional medicine practitioners against hypertension, also contain anthocyanins [6,11]. Many previous studies were focused on the biological properties (antioxidant and antihypertensive activities) and tentative identification of flavonoids from the leaves. However, to the best of our knowledge, the characterization of anthocyanins from *O. strictum* flowers was not yet reported.

The objective of this work was to extract and purify anthocyanins from *O. strictum* flowers and analyze the molecular structure of the major anthocyanins using the high-performance liquid chromatography-mass spectrometry (HPLC-MS). Furthermore, the total phenolic content (TPC) and antioxidant activities of *O. strictum* flowers extracts will be assessed using the Folin-Ciocalteu and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) agent, respectively.

2. MATERIALS AND METHODS

2.1. Plant Material

O. strictum flowers were collected in March 2018 at "*Institut de Recherche en Sciences de la Santé*" experimental station in

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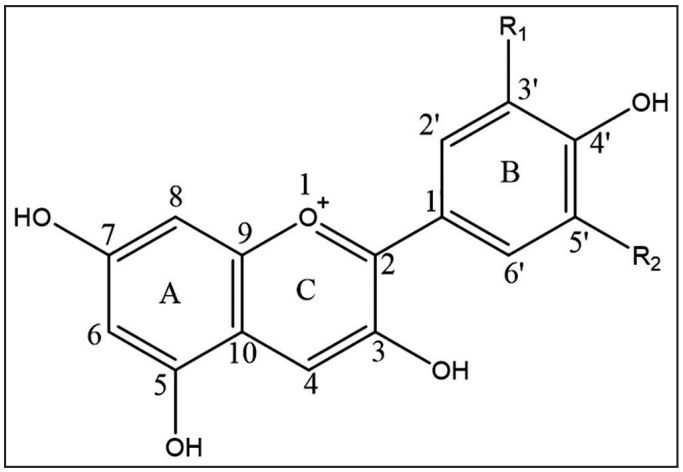

Anthocyanin	R_1	R_2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Malvinidin	OCH_3	OCH_3

Figure 1: General structure of anthocyanins (Zhao *et al*., 2018).

Ouagadougou, Burkina Faso. The identification of the plant was made at Joseph KI-ZERBO University by Dr. Amadé OUEDRAOGO. A voucher specimen under the code N° 6900 was deposited in the Herbarium of the Joseph KI-ZERBO University (Ouagadougou, Burkina Faso). After harvest, *O. strictum* flowers were washed carefully before drying by lyophilization and powdered into a fine powder in a blender.

2.2. Chemicals and Standards

All the solvents used without acetonitrile (HPLC grade) were of analytical grade and purchased from Sigma-Aldrich (Taufkirchen, Germany). Water was purified by a Millipore instrument (MOLSHEIM France). Amberlite XAD-7 was purchased from SIGMA Life Sciences. Gallic acid, DPPH, and Folin-Ciocalteu phenol reagent were obtained from Sigma Chemical Co. (St. Louis, MO). Rutin (analytical grade) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). Buffer salts and all other chemicals were of analytical grade. Samples and standards solutions were filtered before chromatographic analysis using through Millipore membrane of 0.2 µm.

2.3. Crude Extraction

Twenty-five grams of powdered flowers were successively defatted by n-hexane and dichloromethane. After filtration and drying, the marc was extracted until bleaching with ethyl acetate and 0.5% HCl methanol, respectively. The filtrated extracts were concentrated at 40°C using a rotary evaporator (Buchi). The two extracts were subjected to total phenolic, flavonoid, and anthocyanin contents determination, and antioxidant activities assessment.

2.3. XAD-Enriched Extract

For further enrichment of anthocyanins, 5 mL of methanol (0.5% HCl) was enriched using Amberlite XAD-7, non-ionic resin column. Initial washing of Amberlite XAD-7 column was done with 0.5% HCl water to remove free sugars and non-aromatic compounds. This resin adsorbed the aromatic compounds, including anthocyanins, whereas sugars and non-aromatic compounds were eluted by washing with acidified water (0.5% HCl). The adsorbed anthocyanins were eluted by acidified methanol (0.5% HCl). The pooled methanolic eluate was concentrated on rotavapor under vacuum at 40°C to obtain a dried powder.

2.4. HPLC-Electrospray Ionization Quadrupole-time-of-flight Mass Spectrometry (ESI-QTOF-MS) Analysis

HPLC-ESI-QTOF-MS analysis was performed on an Agilent 1260 Series system HPLC (Agilent Technologies, USA) equipped with a diode array detector (Dionex Technologies) and electrospray ionization-quadrupole-time-of-flight mass spectrometer (micrOTOFQII, Broker Daltonics, Bremen, Germany) [12,13]. The HPLC column used was a 150 mm × 4.6 i.d., S-3 µm YMC-Park Pro C18 (YMC Co., Ltd. Shimagyo-Ku, Kyoto, Japan). The temperature-programmable column oven was used to maintain the column temperature at 30°C during the HPLC analysis. Anthocyanins were eluted with solution A (5% formic acid acetonitrile /water, 1:1, v/v) and solution B (5% formic acid water). The gradient elution conditions were as follows: From 0 min to 2 min, the volume ration of A-B was at 20%:80%; from 2 min to 15 min, the A-B was increased from 20%:80% to 40%:60%; from 15 min to 17 min, the ratio was increased from 40%:60% to 50%:50%; from 17 min to 27 min, the ratio was maintained at 50%:50%; from 27 min to 40 min, the ratio was increased from 50%:50% to 95%:5%; from 40 min to 50 min, the ratio was maintained at 95%:5%; and from 50 min to 51 min, the A-B was decreased from 95%:5% to 20%:80%. The HPLC column oven temperature was maintained at 35°C. Mass spectra in the m/z range 200–1100 were obtained by electrospray ionization in positive-ion mode. The mass spectrometric conditions were optimized as follows: Capillary and fragmentor potentials 4000 and 220 V, drying gas flow rate 500.0 L min, nebulizer gas pressure 1.5 bar, and gas temperature 220°C, respectively.

2.5. Total Phenolic, Flavonoid, and Anthocyanin Contents

The TPC in *O. strictum* flowers extracts were determined by the Folin-Ciocalteu colorimetric method [11,14] with slight modifications. In brief, 0.1 mL of *O. strictum* flowers extracts and gallic acid solution (standard) was mixed with 0.1 mL of Folin-Ciocalteu reagent previously diluted 10 times with distilled water. After vortexing, the mixture was incubated for 8 min at room temperature, and 1.0 mL of 7.5% saturated sodium carbonate solution was added. The samples were stirred and stored for 30 min in the dark at 37°C. The reading was done at 760 nm spectrophotometrically. The phenolic contents of *O. strictum* flowers extracts were determined using the equation of the calibration curve ($y = 7.3194x + 0.1309$, $R^2 = 0.998$), and the results are expressed in mg of gallic acid equivalents per gram of dry weight.

The determination of total flavonoid contents (TFC) in *O. strictum* flowers extracts was performed as reported previously [11,15] using $AlCl_3$ colorimetric method. Rutin was used as a standard. The quantification was expressed by reporting the absorbance in the calibration curve of the rutin ($2,5608x + 0,0034$, R^2=0.9995). The TFC were expressed as mg rutin equivalents/g dry weight.

The total anthocyanin contents (TAC) assessment was carried out by the pH differential method, as described in the literature [15-17]. Absorbance was measured at 510 and 700 nm in buffers at pH 1.0 and 4.5. The TAC was expressed as milligram cyanidin 3-O-glucoside equivalents/g dry weight and calculated using the formula:

$$TAC\ (mg/L) = \frac{(A \times M \times DF \times 1000)}{(\varepsilon \times l)}$$

$A=(A_{510}-A_{700})_{pH1.0}-(A_{510}-A_{700})_{pH4.5}$; M(molecularweight)=449.2 g/mol; DF=dilution factor; *l*=curvette pathlengh in cm; ε=26,900 L/mol.cm, molar extinction coefficient for cyanidin 3-O-glucoside; 1000:factor to convert g to mg.

All spectrophotometric measurements were performed using an ultraviolet (UV)-Vis spectrophotometer (SHIMADZU, Japan). All the samples were analyzed in triplicate.

2.6. Radical Scavenging Capacity using the DPPH Agent

Radical scavenging capacity of *O. strictum* flowers extracts was measured by the DPPH assay according to a previous report [1] with some modifications [11,15,18,19]. Radical $DPPH^{\bullet}$ absorbs in the visible at λ_{max}=517 nm, but on reduction by an antioxidant compound, its absorption decreases. Briefly, fresh standard solutions (Trolox) of different concentrations from 1 to 5 µg/mL in methanol were firstly prepared. 2 mL of each standard and sample were mixed with 2 mL of 0.01 mM DPPH solution, respectively. The mixtures were incubated in the dark at 37°C for 30 min. The radical scavenging rate of DPPH was calculated as follow:

Radical scavenging rate=$1-(A_s-A_{s0})/A_0$

A_s: Absorbance of the sample; A_{s0}: Absorbance of the samples with 2 mL methanol; A_0: Absorbance of DPPH (0.01 Mm) without the sample.

Afterward, a curve of the percentage of DPPH bleaching activity versus concentrations was established and IC_{50} values were calculated. IC_{50} indicates the concentration of the sample required to scavenge 50% of DPPH free radicals. The lower the IC_{50} value the more powerful the antioxidant activity. All the samples were analyzed in triplicate.

2.7. Statistical Analysis

All analyses were conducted in three replications, and data processed in Microsoft Excel 2016. An ANOVA with a Bonferroni *post hoc* test was performed to determine significant differences. The level of significance was defined as $P \leq 0.5$.

3. RESULTS AND DISCUSSION

3.1. Anthocyanins Preliminary Screening

The extraction and purification method allows one to obtain the exclusively rich-anthocyanins extract. This finding was confirmed from data of acquired chromatograms at different wavelengths and UV-Vis and mass spectra. This method made anthocyanins characterization easier and more effective. In each stage of the experiment, the degradation and hydrolysis events were prevented by working at low temperature (≤40°C) and acidic medium (pH=2).

The preliminary screening of anthocyanins from the methanol extract of *O. strictum* flowers was performed by chemical methods and the high-performance thin-layer chromatography method. A simple test following the pH variation of 1% HCl methanol crude extract was done to detect the presence of anthocyanins in the *O. strictum* flowers. Hence, in the acidic medium, the methanolic crude extract was colored into red and returned into blue in alkaline medium. Besides, visible spots were clearly observed on the HPTLC plate (Figure 2a). That confirmed the chemical revelation tests results, which had detected the presence of anthocyanins in the *O. strictum* flowers.

The UV-Vis spectrum (Figure 2b) of acidified methanol (0.1% HCl) extract of *O. strictum* flowers showed two maxima at 282 nm and 516 nm, revealing the absorption of phenols and the characteristic cyclic nucleus of anthocyanins, respectively [5]. After the addition of aluminum chloride ($AlCl_3$) 5%, the non-bathochromic shifts observed on the maximum visible wavelengths indicated the absence of the vicinal free hydroxyls groups on the anthocyanidin [5]. The A_{acy}/A_{vis} ratio (0.1) of the rich-anthocyanins extract of *O. strictum* flowers indicated the absence of aromatic acylation. However, the A_{440}/A_{vis} ration (0.31) suggested that the anthocyanins of *O. strictum* flowers were -3-*O*-glycosylated and its C-5 were not substituted.

3.2. Anthocyanins Identification

The anthocyanins pigments in the *O. strictum* flowers, red colored [6], were characterized and identified following to their chemical, chromatographic, and spectroscopic (UV-Vis and mass spectroscopic analysis) properties. Figure 3 shows the reverse phase C_{18} HPLC chromatogram corresponding to the 0.1% HCl methanol rich-anthocyanins extract of *O. strictum* flowers. The anthocyanins characterization was performed by HPLC-ESI-QTOF-MS coupled with a diode array detector. Four anthocyanins were detected (**a**, t_R = 09.60 min [2.2%]; **b**, t_R = 10.32 min [1.5%]; **c**, t_R = 35.93 min [17.4%]; **d**, t_R = 49.46 min [26.7%]) on the basis of their UV-Vis spectra and relative retention times.

Peak **a** (t_R = 09.60 min) was identified as cyanidin-3-*O*-hexoside. The UV-visible spectrum of the peak **a**, with λ_{max} at 280 and 517 nm around, was in agreement with the data reported in the literature [15]. The HPLC/MS (ESI+) spectrum of the peak **a** (Figures 4-7) showed a protonated molecule ion $[M]^+$ at m/z 449.1 corresponding to the molecular formula C_{21} H_{21} O_{11} (Mw=449.17). Compared with the previous reports [1,20], peak **a** ($[M]^+$=m/z 449.1) was tentatively identified as cyanidin-3-*O*-hexoside. Similar process carried out in peak **b** (t_R = 10.32 min) identified as cyanidin-3-*O*-rutinoside (λ_{max} at 280 and 517 nm). The MS (ESI+) spectrum of peak **b** showed a molecular ion at m/z 595.2, which was consistent with the molecular formula C_{27} H_{31} O_{15} (Mw=595.147). This molecular ion suggested that the two sugars are bonded to anthocyanidin. In general, rutinoside was attached to three hydroxyl group of anthocyanin [1]. Compared with the literature [15,21], peak **b** ($[M]^+$=m/z 595.2; M_w=595.147) was identified as cyanidin-3-rutinoside. The aglycone ion at m/z 271 according to the molecule formula C_{15} H_{11} O_5 (M_w=271.24) indicated that the peak **c** was pelargonidin. The fragment at m/z 270.1 would correspond to the loss of proton H. Based on the aglycone ion m/z 271, peak **d** was considered as a pelargonidin derived. The molecular ion at m/z 402.2 and fragment ion at m/z 271 indicated that pelargonidin was linked with a C-5 sugar (xylosyl) $[M-132]^+$. This fragment could correspond to the pelargonidin aglycone, which does not have free orthodiphenol (vicinal free hydroxyls) groups on the nucleus B of the anthocyanin. This result confirms that the peak at m/z 402.2 was identified as pelargonidin 3-*O*-pentoside. Although, peak **d** ($[M]^+$=m/z 285) also contained one methyl group based on the molecular ion at m/z 285 and fragment ion at m/z 271. Thus, peak **d** was also identified as 4'-*O*-methylpelargonidin.

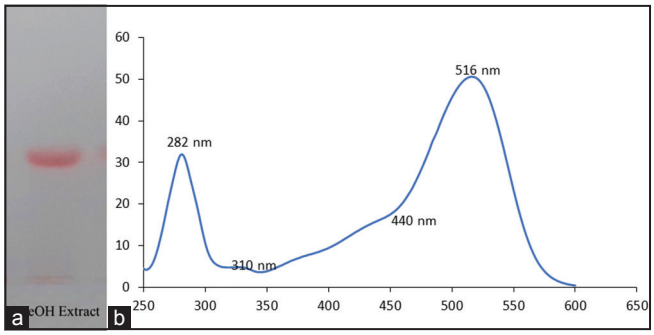

Figure 2: Chromatograms ([a] high-performance liquid chromatography chromatogram; [b] UV-Vis spectrum) of *Odontonema strictum* flowers extract.

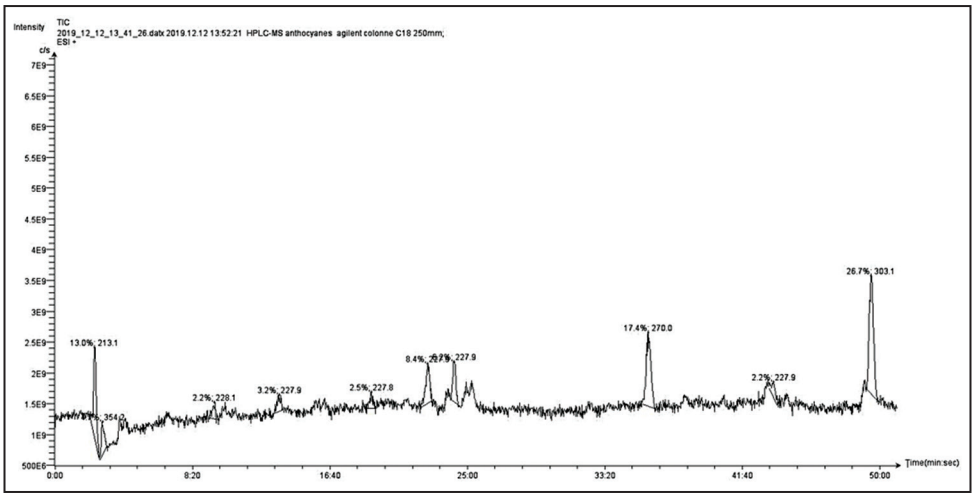

Figure 3: C_{18} reverse-phase high-performance liquid chromatography chromatogram of *Odontonema strictum* flowers extract.

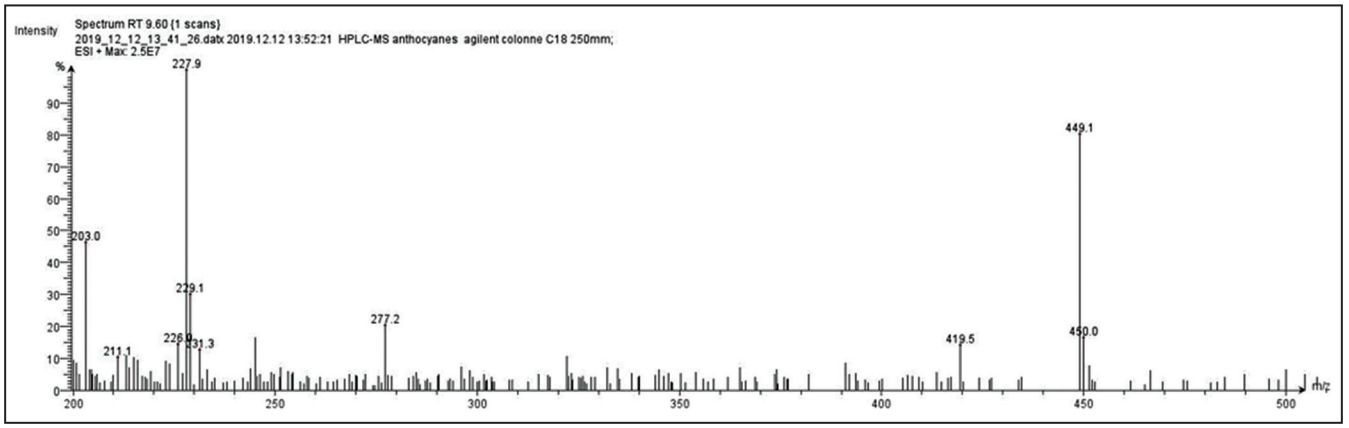

Figure 4: Mass spectrometry spectrum of peak **a**.

Table 1: Total phenolic content, total flavonoid content, and total anthocyanin content

Extract	Total phenolic content (mg AGE/g DW)	Total flavonoid content (mg RuE/g DW)	Total anthocyanin content (mg GG/g DW)
Ethyl acetate	43.4±2.2	29.0±0.6	0.01±0.00
Methanol	84.0±3.0	2.0±0.0	0.3±0.03

3.3. Total Phenolics, Flavonoids, and Anthocyanin Contents

The TPC varied from 84.0 ± 3.0 for methanol extract to 43.4 ± 2.2 mg AGE/g dry weight for ethyl acetate extract of *O. strictum* flowers (Table 1). The TPC of the methanol extract of *O. strictum* flowers showed a value 2 times higher than ethyl acetate extract. The TAC showed a similar trend, varying from 0.3 ± 0.03 for methanol extract to 0.01 mg cyanidin-3-glucoside/g dry weight for ethyl acetate extract of *O. strictum* flowers. Anthocyanins are pigment groups that impart bright and attractive colors to flowers and fruits [1,22]. Now, the level of anthocyanins in the *O. strictum* flowers can explain the intensity in color, which is red. However, the TFC of *O. strictum* flowers showed an opposite trend depending on the two solvents. Indeed, the TFC ranged from 29.0 ± 0.6 for ethyl acetate extract to 2.0 ± 0.0 mg rutin equivalent/g dry weight for the methanol extract of *O. strictum* flowers. The TFC value of ethyl acetate extract was 15 times higher than methanol extract. In contrast, the methanol extract presented the highest total phenolic and TAC.

Table 2: Scavenging of the 1,1-diphenyl-2-picrylhydrazyl radical of *Odontonema strictum* flowers extracts.

Extract	Ethyl acetate	methanol	Trolox (standard)
IC_{50} (mg/mL)	3.07±0.002	0.28±0.03	0.012±0.00

3.4. Antioxidant Activity

The nitrogen-centered DPPH radical with an unpaired electron is generally used as an indicator to evaluate the radical scavenging capacity of antioxidants [1,23]. Table 2 presented the values of radical scavenging activities of *O. strictum* flowers extracts using the radical DPPH$^{\bullet}$method. A lower IC_{50} value indicates a higher antioxidant capacity and vice versa. The IC_{50} values varied from 3.07 ± 0.002 mg/mL for ethyl acetate extract to 0.28 ± 0.03 mg/mL for methanol extract. In line with the total phenolic and TAC, the methanol extract of *O. strictum* flowers presents the highest antioxidant capacity compared to the ethyl acetate extract. The antioxidant activities measured by the bleaching of the radical

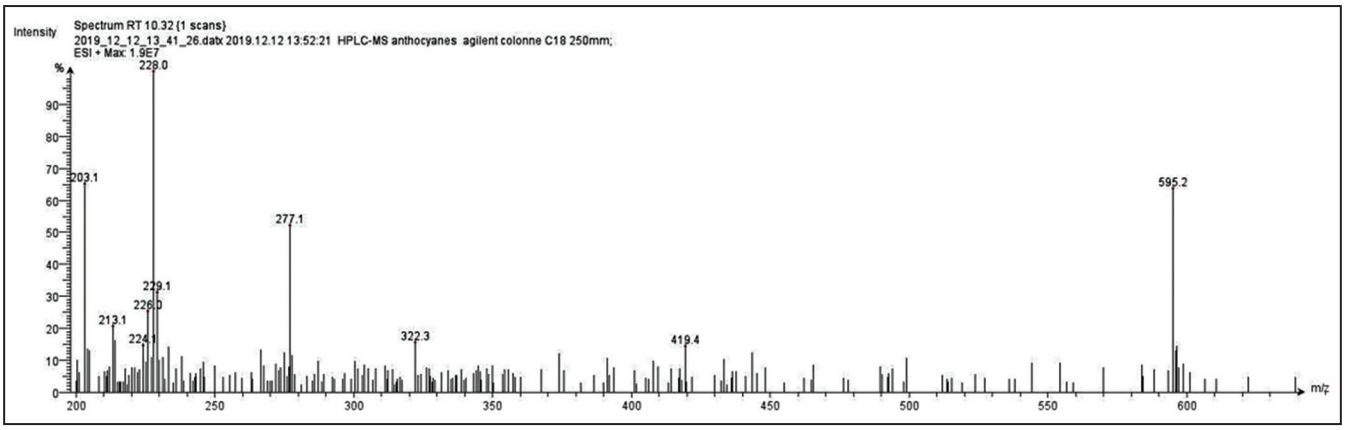

Figure 5: Mass spectrometry spectrum of peak **b**.

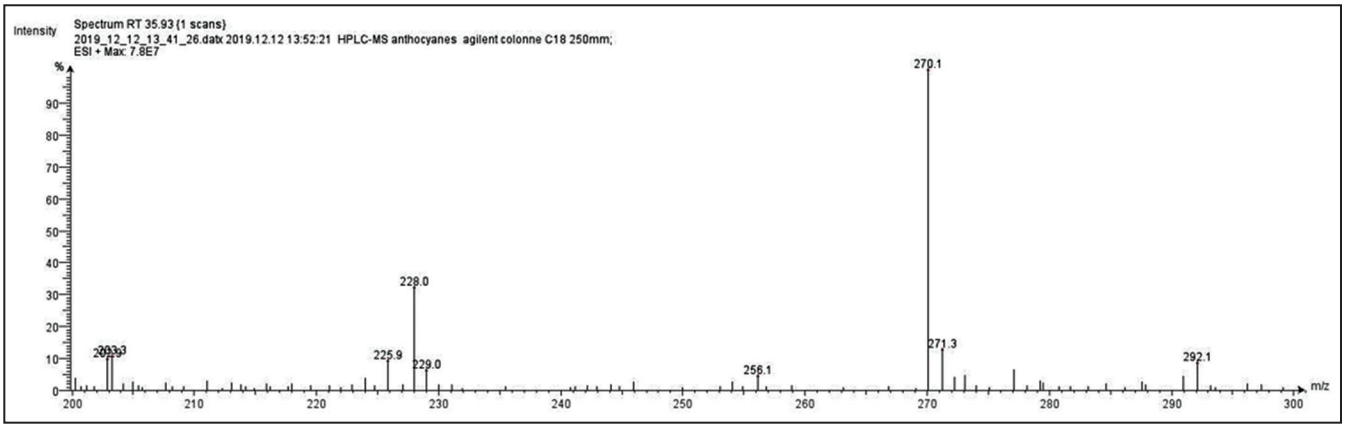

Figure 6: Mass spectrometry spectrum of peak **c**.

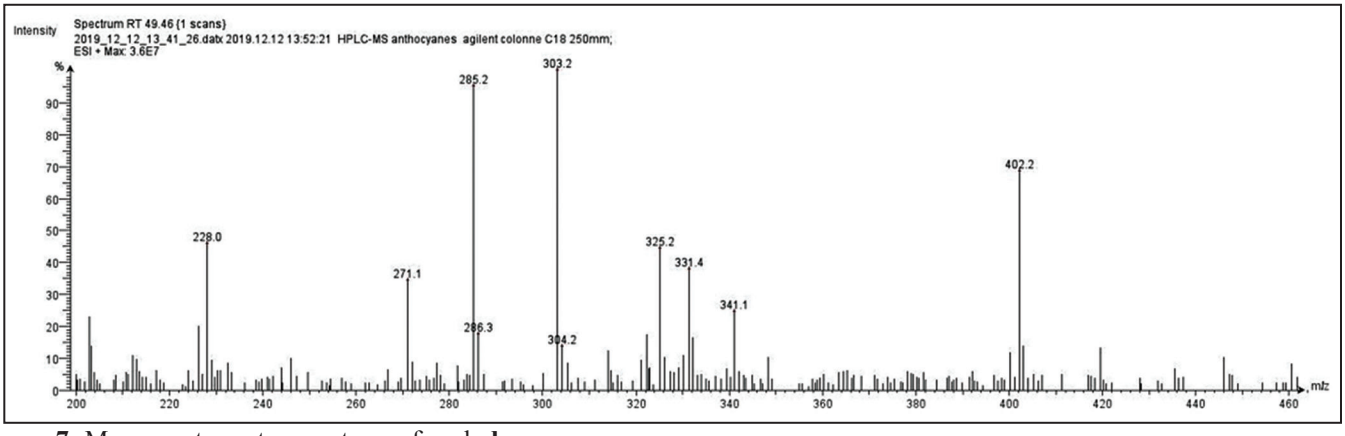

Figure 7: Mass spectrometry spectrum of peak **d**.

method are in agreement with the results of total phenolic and TAC. Thus, the scavenging activity of the plant extracts may be attributed to the presence of phenolic compounds [11,24]. The previous studies reported a strong correlation between phenolic content and antioxidant capacity in plant extracts [1,11,15]. In general, phenolic compounds' antioxidant activity depends on the structure and substitution pattern of hydroxyl groups [11,25]. The total phenolic and TAC, as well as antioxidant property of *O. strictum* flowers extracts, are reported for the 1st time.

4. CONCLUSION

In this study, the identification of anthocyanins and antioxidant activity of *Odontonema strictum* flowers extracts were carried out. Five anthocyanins were identified and quantitated by HPLC-ESI-QTOF-MS analysis for the 1st time. Moreover, the contents of total phenolic, total flavonoid, and total anthocyanin in the flowers extracts of *O. strictum* were determined, and their antioxidant activities were assessed. A positive and significant correlation existed between antioxidant activity and phenolic contents, revealing that phenolic compounds were the dominant antioxidant components. Therefore, *O. strictum* flowers may be more suitable for the production of nutraceuticals with antioxidant capacities.

5. COMPETING INTERESTS

The authors declare that they have no competing interest.

6. ACKNOWLEDGMENTS

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