LC-ESI-MS identification of phenolic compounds from Tunisian flaxseeds (*Linum usitatissimum L.*)

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**ABSTRACT**

Flaxseed (*Linum usitatissimum* L.) phenolic compounds are still not completely known, because of the complexity of their chemical composition and the complexity of the matrix in which they are found. Their precise chemical structure is valuable to understand the specific role of each compound on different bioactivities. High pressure liquid chromatography coupled to diode array UV-visible detection and mass spectrometry (MS) techniques (LC-ESI Ion Trap-MS and MS/MS) was used for the identification of phenolic constituents of flaxseed samples cultivated in Tunisia. The results show that lignans are the abundant compounds in flaxseed especially secoisolariciresinol diglucoside and enterolactone. Whilst that, Medioresinol and aryltetralins, are hitherto unreported for the *Linum* species. In the other hand flavonoids, especially apigenin, and herbacetin glycosides have been characterized in the methanolic extract with and without alkaline hydrolysis. Interestingly, oleuropein, complex phenolic compounds of the secoiridoid class, was also identified for the first time in flaxseed.

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1. **INTRODUCTION**

Traditional medicine is an important part of human health care in many developing and developed countries, Although the use of medicinal plants in therapy has been known for centuries in all parts of the world, the demand for herbal medicines has grown massively in recent years. Among plant metabolites, phenolics were shown to exhibit antipathogen, antiherbivore and allelopathic properties (Dave Oomah et al., 1995). Phenolics compounds, secondary metabolites, are widely distributed in plants, and several functions have been attributed to them. Polyphenols are generously distributed as glycosides in fruits, vegetables, flowers and seeds for protection against damage caused by ultraviolet, insect, fungus and pathogen (Caridi et al., 2007). Additionally, these compounds are thought to have benefic effects on human health. They might decrease the risk of hormone dependent cancers, cardiovascular disease and other “welfare” diseases (Tsai et al., 2010).

During the last decade, there has been an increasing interest in the use of *Linum usitatissimum* L. (Linaceae) commonly known as linseed or flaxseed, in the diet in order to improve the nutritional and health status (Lei et al., 2003). In term of polyphenol content, flaxseed are at the top among plant species and their phenolics compounds (*e.g.* lignans) are excellent natural antioxidants and exhibiting oestrogenic activity (Imran et al., 2015; Kasote, 2013). Flaxseed represent a generous source of valuable metabolites such as hydroxycinnamic acids (HCA)
(Beejmohun et al., 2007), flavonols (Struijs et al., 2007) isoﬂavonoids (Mukherjee, 2003) and lignans (Hano et al., 2006; Renouard et al., 2010). The prevailing lignan in flaxseed is secoisolariciresinol diglucoside (SDG; MW = 686.7 Da) (Chimichi et al., 1999; Sicilia et al., 2003), which is often referred to as secosolaricresinol (SECO), the aglycone of SDG (Mazur et al., 1996). In flaxseed, SDG exists in two isomeric forms, (+)-SDG and (-)-SDG, of which (+)-SDG is the major isomer (Eliasson et al., 2003). Other lignans including smaller quantities of matairesinol, isolariciresinol, lariciresinol, demethoxy secoisolariciresinol and pinoresinol have also been identiﬁed in flaxseed in lower quantities (Meagher et al., 1999; Sicilia et al., 2003). Since phenolic compounds play diverse and important roles in plant physiology and food chemistry, our study focused on the determination of diversity of these compounds in flaxseeds. The understanding of the nature of these compounds is crucial for their possible exploitation in drugs and functional foods. High performance liquid chromatography and its coupling to mass spectrometry detection (LC-MS) is becoming important tool for the characterization and identiﬁcation of compounds in crude plant extract. Hence, the aim of this article was the identiﬁcation of phenolic compounds by liquid chromatography coupled to UV-Visible detection and electrospray ionization-tandem.

2. MATERIALS AND METHODS

2.1. Plant Materials

In this study, the detailed polyphenols proﬁle of Tunisian flaxseed (Linum usitatissimum L.) was characterized. Samples were harvested from Beja area, located at 36°43′16.74″N (North) latitude, 9°19′09.8″S (South) longitude, and 356 m (metr) altitude, at maturity stage in June 2016. Three batches replicate samples (50.0 g each) of flaxseeds were ground and transformed into a ﬁne homogeneous powder by crushing with the presence of liquid nitrogen to limit hydration and avoid oxidation (The seeds were ground by a domestic coffee grinder (SEB Prep line). Frozen powdered samples were lyophilized using a LEYBOLD model (LYOVAC GT2). Then, they were stored at -82 °C until further use. Solvents, reagents and phenolic standards Glacial acetic acid, acetonitrile and methanol (HPLC grade) were purchased from Fischer (Fischer scientiﬁc Loughborough, UK). Formic acid was purchased from Merck (Darmstadt, Germany). Hexane was purchased from Sigma Aldrich (USA). Anhydrous methanol (Prolabo, HPLC grade). Ultrapure water was obtained using a Milli-Q water system (Millipore, Bedford, MA). Phenolic standards: secoisolariciresinol, apigenin and herbacetin were obtained from Sigma (Sigma – Aldrich, Germany).

2.2. Sample preparation

2.2.1. Delipidation and methanolic extraction of the crude material

In order to obtain repeatable results, defatting of flaxseed prior to further treatment is crucial. Five grams of freeze-dried flaxseed powders were extracted three times with 50 mL of hexane to remove fatty material. The insoluble residue was freeze-dried for 24 hours. Then the dry powder was extracted three times with 50 mL pure methanol containing 1 % v/v acetic acid (50 mL each). The three liquid methanol extracts were pooled and evaporated under vacuum using a rotary evaporator (45°C). A volume of pure water (50 mL) was added before the complete removal of methanol and evaporation was continued until complete elimination of methanol and drastic reduction of the volume (less than 40 mL). The volume was recovered, frozen at -80°C and lyophilized for 48 hours.

2.2.2. Sample preparation for direct analysis of the acidified methanolic extract

Polyphenols including ﬂavonoids and lignans were extracted from the freeze dried methanolic extract powders after delipidation using acidiﬁed methanol. Precisely weighted aliquots of freeze-dried defatted methanol extract powders (20 mg) were extracted using 2.4 mL of pure MeOH 1% formic acid in an ultrasonic bath for 15 min. Then, the mixture was ﬁltered on PTFE filters (0.45 μm, Uptidentiﬁcher, France). The ﬁltrate was analyzed by LC-ESI-MS in negative ion mode.

2.2.3. Alkaline hydrolysis of the acidiﬁed methanolic extract

Three milliliter of acidiﬁed methanol (1 % formic acid, v/v) was added to a weighted fraction of the freeze-dried methanolic extract (5 mg). After incubation for 15 min, one milliliter was drawn and mixed to 3 mL of 2 M NaOH for alkaline hydrolysis. The mixture was stirred at room temperature for 3 h. Then, the mixture was acidified with 1.5 mL of concentrated acetic acid to obtain a pH = 4.4. Finally, the obtained mixture was adjusted to an exact volume of 20 mL by acidiﬁed methanol (1 % formic acid). Four microliters of the
reaction mixture was filtered and analyzed by LC-ESI-MS. This analysis was dedicated to the monitoring of SDG oligomers that are released into SDG and other glycosides during the process of alkaline hydrolysis.

2.2.4. LC-ESI-MS and MS² for characterization of phenolic compounds in flaxseeds
This analysis was performed using an LC-ESI-MS system with a system of degasification SCM1000 (ThermoQuest, San Jose, CA, USA), an automatic system of injection (ThermoFinnigan, San Jose, CA, USA), a binary pump Series 1100 (Agilent Technologies, Palo Alto, CA, USA) coupled with a photodiode array detector Spectra system UV6000LP (ThermoFinnigan, San Jose, CA, USA). The mass spectrometer was an ion trap LCQ Deca (ThermoFinnigan San Jose, CA, USA) equipped with an electrospray ionization source (SIE) used in the negative mode. The separation was performed on a RP18 Zorbax Eclipse XDB-C18 (2.1 mm x 150 mm, 3.5 µm, Agilent Technologies) equipped with a guard column 4 x 4 mm of the same RP material and thermostated at 30°C. The volume of injection was 2 µL.

The mobile phase consisted of solvent A (aqueous formic acid, 0.1% v/v) and solvent B (acetonitrile containing 0.1% formic acid v/v) with a flow rate of 0.2 mL/min; initial, 3% B; 0-3 min, 7% B, linear; 3-18 min, 25% B, 18-30 min, 35% B, 30-40 min, 35% B, linear followed by washing and reconditioning the column to the initial conditions. Helium gas was used as a damping gas. MS/MS spectra were obtained automatically using the Full MS dependent scan option that allows automatic MS/MS acquisition of the main ions detected in the Full MS mode. In this mode, the potential change defining the collision energy was set in the 25-35 % range (arbitrary units) who appropriate for optimizing the production of stable and intense signals corresponding to the main daughter ions. The data were processed using the Xcalibur 1.2 software.

Mass spectrometry data were acquired in the negative mode for all phenolic compounds. Flavonoids in flaxseeds were characterized by means of their retention time, UV-visible spectrum, Full MS and MS/MS spectra revealing the deprotonated molecule and the corresponding daughter ions. Whenever possible, absolute identification was performed by comparison with authentic available standards.

3. RESULTS AND DISCUSSION
For the extraction of phenolic compounds, the choice of a relevant method depends on their molecular structure. Highly hydrophobic phenolic compounds such as tocopherols can be extracted by hexane, in contrast, lignan, exhibiting a higher polarity, can be extracted by polar solvents such as aqueous methanol or ethanol (Sainvitu et al., 2012). As a consequence, findings concerning phenolic compounds in flaxseeds depend on the extraction methods used to recover them. Various organic solvents extractions followed by hydrolysis treatment have been used in several studies to promote the release of phenolic compounds. The phenolic complex in defatted flaxseed are extracted using more polar solvents such as dioxane/ethanol, aqueous ethanol or methanol in combination with heat and mixing (Johnsson et al., 2000). Recently, the phenolic complex was isolated from whole flaxseeds by subcritical water extraction at high temperature in combination with high pressure. For the analysis of secoisolariciresinol, alkaline hydrolysis has been reported as an effective method (Cacace and Mazza, 2006).

3.1. Structural characterization of phenolic compounds in the flaxseed methanolic extracts
3.1.1. Before alkaline hydrolysis
For the analysis of the phenolic compounds (Fig. 1) of the flaxseed methanolic extract, LC-ESI-MS in negative ionization mode was used. Three independent assays were performed for each sample, and no relevant variations were observed regarding the nature of the detected product ions fragments and their relative intensities. The base peak chromatograms of the methanolic extracts before alkaline hydrolysis is shown in Figure 2a. The first approach was to identify the deprotonated molecular ion [M-H]⁻ clearly visible on the full MS spectrum that corresponded to the considered peak on the base peak chromatogram. Generally, the [M-H]⁻ ion corresponds to the most intense signal on the full MS spectrum.

Peak 1 (RT = 2.8 min, Fig. 2a) showed a [M-H]⁻ ion at (m/z = 539). Its MS² spectrum gave product ions at (m/z = 503; the loss of two molecules water -36 Da), 341, 179 (that correspond to a pseudomolecular ion of hexose) and 377, the later due to the loss of a 162 Da (hexose moiety) (Table 1). By comparing its UV (λmax = 280nm) and MS² spectrum and HPLC retention time with those of a previous work on *olea europaea* L. leaves (Bonacci and Tateo, 2011). This peak was identified as oleuropein (Fig. 1). To our knowledge this is the first report on occurrence of oleuropein on *Linum usitatissimum* L.

Peaks 2, 3 and 5 (RT = 11.9, 13.2 and 14.8 min, Fig. 2a)
Figure 1. Some chemical structures of phenolic compounds identified in Flaxseed.
Figure 2. (a) RP18-HPLC base peak chromatogram of the flaxseed methanolic extract before alkaline hydrolysis (b) Reversed phase chromatogram at 280 nm.
Figure 3. ESI-MS/MS spectrum of peak 3 (parent ion at \(m/z\) 549) identified as medioresinol glucoside. The insert shows the UV-Visible absorption spectrum.

Figure 4. Proposed fragmentation pattern by ESI-MS\textsuperscript{2} of medioresinol glucoside at [M-H]=549.
Table 1: Tentative identification of phenolic compounds in the Flaxseed methanolic extract

<table>
<thead>
<tr>
<th>Peaks</th>
<th>Tentative Identification</th>
<th>RT(^a)(min)</th>
<th>(\lambda_{\text{max}}) (nm)</th>
<th>[M-H(^-)]</th>
<th>Major MS/MS product ions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Methanolic extract before alkaline hydrolysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Oleuropein</td>
<td>2.8</td>
<td>280</td>
<td>539</td>
<td>503(100); 377(20); 341(5);179(5)</td>
</tr>
<tr>
<td>2</td>
<td>Medioresinol diglucoside</td>
<td>11.3</td>
<td>288</td>
<td>711</td>
<td>693(60);549(20) ; 387(10); 383(100)</td>
</tr>
<tr>
<td>3</td>
<td>Medioresinol glucoside</td>
<td>13.2</td>
<td>287</td>
<td>549</td>
<td>531(100); 387(40); 221(30);207(25)</td>
</tr>
<tr>
<td>4</td>
<td>Prinsepiol glucoside</td>
<td>14.13</td>
<td>291</td>
<td>551</td>
<td>533(100); 389(80);221(25); 209(15); 179(20)</td>
</tr>
<tr>
<td>5</td>
<td>Medioresinol</td>
<td>14.8</td>
<td>284</td>
<td>387</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Dihydrokaempferol hexoside (aromaderrin hexoside)</td>
<td>15.5</td>
<td>449</td>
<td></td>
<td>287(100); 269(25);259(20)</td>
</tr>
<tr>
<td>7</td>
<td>Enterolactone dihexoside Iso1</td>
<td>16.4</td>
<td>270;363</td>
<td>621</td>
<td>561(10) ; 335(100)</td>
</tr>
<tr>
<td>8</td>
<td>Enterolactone dihexoside Iso2</td>
<td>16.8</td>
<td>270;363</td>
<td>621</td>
<td>561(10) ; 335(100)</td>
</tr>
<tr>
<td>9</td>
<td>Apigenin-8-C-hexoside</td>
<td>18.67</td>
<td>272-350</td>
<td>431</td>
<td>341(20);311(100)</td>
</tr>
<tr>
<td>10</td>
<td>Aryltetralins derivative (4'demethyl-6'-methoxypodophyllotoxin)</td>
<td>22.3</td>
<td>310</td>
<td>429</td>
<td>385(50); 249(100); 205(40);179(20)</td>
</tr>
<tr>
<td></td>
<td>Methanolic extract after alkaline hydrolysis</td>
<td></td>
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<tr>
<td>3</td>
<td>Medioresinol glucoside</td>
<td>13.2</td>
<td>298</td>
<td>549</td>
<td>531(100); 387(40); 221(30);207(2)</td>
</tr>
<tr>
<td>11</td>
<td>Herbacetin 3, 8-O-diglucoside</td>
<td>15.5</td>
<td>269;350</td>
<td>625</td>
<td>463(100)</td>
</tr>
<tr>
<td>12</td>
<td>Secoisolariciresinol diglucoside Iso1</td>
<td>16.5</td>
<td>285</td>
<td>685</td>
<td>523(100);361(10)</td>
</tr>
<tr>
<td>13</td>
<td>Secoisolariciresinol diglucoside Iso2</td>
<td>17.5</td>
<td>285</td>
<td>685</td>
<td>523(100);361(10)</td>
</tr>
</tbody>
</table>

\(^a\)RT : Retention time; number in parenthesis correspond to relative abundance of the considered product ions

revealed molecular ions at m/z 711, 549 and 387, respectively (Table 1). The MS/MS spectrum of compound 2 at m/z 711 showed a main product ion at m/z 549, corresponding to the loss of 162 Da which is consistent with the presence of an hexose group in this molecule. The loss of the second hexose group gave a fragments ion at m/z 387. Because no standards were available for comparison, this compound was tentatively identified as Medioresinoldihexoside (peak 2). The MS/MS spectrum of compound 3 molecular ion at (m/z = 549) showed a several main product ions at m/z 531, 387, 221, 207 and 179. The ESI-MS/MS spectrum of this compounds and the corresponding fragmentation pattern is consigned in Figure 3 and was in accordance to the Medioresinolglucosidemolecule. Compound 5 showed a [M-H\(^-\)] ion at m/z 387 (Table 1), by comparing its UV and MS/MS\(^2\) spectra, this compound was identified as Medioresinol, aglyconeMedioresinoldervatives are lignans that have been already described in the literature (Llorent-Martinez et al., 2015). As far as we know, Medioresinol was detected for the first time in flaxseed (Linum usitatissimum L.). Peaks 2, 3 and 5 (RT = 11.3, 13.2 and 14.8 min, Figure 2a) revealed molecular ions at (m/z = 711, 549 and 387), respectively (Table 1). The MS/MS spectrum of peak 2 at (m/z = 711) showed a main product ion at (m/z = 549), corresponding to the loss of 162 Da which is consistent with the presence of an hexose group in this molecule. The loss of the second hexose group gave a fragments ion at (m/z = 387). Because no standards were available for comparison, this compound was tentatively identified as medioresinol diglucoside (peak 2). The MS/MS spectrum of peak 3 molecular ion at (m/z = 549) showed a several main product ions at (m/z = 531, 387, 221, 207 and 179).
The ESI-MS/MS spectrum of this compounds and the corresponding fragmentation pattern is consigned in figure (4) and was in accordance to the medioresinol glucoside molecule. peak 5 showed a [M-H]− ion at (m/z = 387) (Table 1), by comparing its UV and MS/MS² spectra, this compound was identified as medioresinol (aglycone medioresinol derivatives are lignans that have been already described in the literature (Meija et al., 2013; Ozarowski et al., 2013; Llorent-Martinez et al., 2015). As far as we know, medioresinol was detected for the first time in flaxseed (Linum usitatissimum L.) Peak 4 (RT = 14.13 min, Fig. 2) displayed a [M-H]− ion at (m/z = 551) (Table 1). The MS² spectrum gave several productions at (m/z = 533, 389, 221 and 209). The occurrence of fragments at (m/z = 533) and 389 can be associated to the loss of 18 and 162Da corresponding, to the loss of a H₂O molecule and hexoside group, respectively. The aglycone ion ((m/z = 389) was in accordance to the prinsepiol molecule...
formed by the loss of an hexoside group. Thus, this peak was tentatively identified as prinsepiol glucoside. Regarding literature data, this compound has been isolated from the roots of *Valerianapripronophylla* (Piccinelli et al., 2004).

Peak 6 (RT = 15.5 min, Fig. 2a), exhibited an [M–H] ion at (m/z = 449) (Table 1). It suffered the neutral loss of 162 Da (hexoside) in MS², yielding the aglycone at (m/z = 287). The aglycone was characterized as dihydrokaempferol (aromadedrin), due to the MS³ [449 → 287] base peak at (m/z = 259) (Fischer et al., 2011). Hence, this peak was identified as dihydrokaempferol-0-hexoside.

Peaks 7 and 8 (RT = 16.4 and 16.8 min, Fig. 2) revealed both the same molecular ions at (m/z = 621) (Table 1). These peaks were thus identified as two isomers of enterolactone diglucoside that has already been detected in flaxseed and mentioned in the literature (Kasote et al., 2013).

Peak 9 (RT = 18.67 min, Fig. 2) exhibited an [M–H] ion at (m/z = 431), and its MS² spectrum showed typical fragment ions of C-glycosides at (m/z = 311) and 341 (Table 1), corresponding to [M–H-120] and [M–H-90], respectively. This peak showed similar UV absorption maxima with two bands at 272 nm and 350 nm. This type of UV spectra is characteristic of flavonoids. Considering the guidelines for the identification of isomeric mono-C-glycosides flavonoids (Waridel et al., 2001; Ferreres et al., 2011), the peak 9 was identified as a C-8 flavonoid, since the MS² spectrum did not show the loss of water molecules, which is representative of C-6 isomers. Considering bibliographic data, peak 9 was tentatively identified in *Bittuminariabittuminosa* as apigenin-8-C-hexoside (Llorent-Martinez et al., 2015).

Peak 10 (RT = 22.3 min, Fig. 2) revealed molecular ions at (m/z) 429 and its MS² fragmentation gave a fragments ion at (m/z) 385, 249, 205 and 179 (Table 1). The ESI-MS/MS spectra and the proposed fragmentation pattern by ESI-MS² of this peak are consigned in figure 4, which was consistent with aryltetralins derivative (4’demethyl-6-methoxypodophyllotoxin) molecule. The detection and identification of the aryltetralin lignan subclass has been described in *Linumbienne Mill* species by Schmidt et al., 2006.

### 3.1.2. After alkaline hydrolysis

The particular phenolic findings in flaxseed depend on the extraction performed to analyze them. Various organic solvents followed by hydrolysis treatments have been used in several studies to lead the release of phenolic compounds. For the analysis of SDG, alkaline hydrolysis with sodium hydroxide have been reported as effective method (Cacace et al., 2006; Coran et al., 2004). However, the identification of some compounds is so far from being concluded since phenolics such as pinoresinol, matairesinol have been found after solvent extraction followed by acid and enzymatic hydrolysis (Milder et al., 2004). Additionally, some studies have shown that the extraction of flaxseed meal with methanol-ammonia results in a decreasing of the content of soluble esterified phenolic acids and insoluble bound phenolic acids by 20 an 29%, respectively (Varga and Dioso, 1994).

Two secoisolaricresinol diglucosides were detected in the flaxseed extract after alkaline hydrolysis. Peaks 12 and 13 (RT = 16.5 and 17.5 min, Fig. 5) revealed molecular ions at (m/z) 685 (Table 1). These peaks were thus identified as two isomers of secoisolaricresinol diglucoside. The contents of secoisolaricresinol diglucoside oligomers and their hydrolysates in the flaxseed extract and the hydrolytic solutions were determined by Li et al., 2008.

The chromatogram of the Flaxseed methanolic extract after the alkaline hydrolysis (Fig. 5) also showed the presence of one peak (peak 11) with UV-visible spectrum characteristic of flavonols (λₘₐₓ 269 and 350 nm). Thus, peak 11 (RT = 15.54 min, Fig. 2) revealed molecular ions at (m/z) 625 (Table 1). This peak was tentatively identified as herbacetin 3,8-O-diglucoside. In previous studies concerning flaxseed, it has been reported that the secoisolaricresinol diglucoside oligomers is partly conjugated to flavonoids, such as herbacetin diglucoside (HDG), via linear ester-linked complex (Strujs et al., 2007).

Our results confirm previous works. Indeed, herbacetin 3,8-O-diglucoside and two isomers of secoisolaricresinol diglucoside were recently identified by Ramsay et al., 2017 by HPLC-UV and ¹H NMR analysis in different development stages and detected from samples with alkaline treatment. This approach revealed the different constituents of the lignan macromolecule during the developmental stages of the seeds.

The signals corresponding to hydroxycinnamic acid are not visible in the NMR spectra of extracts with and without alkaline treatment in the maturity stage, indicating that these molecules do not accumulate in a free form and are probably linked via ester binding to the lignan macromolecule (Sainvitu et al. 2012).

### 4. CONCLUSION

The particular phenolic findings in flaxseed depend on the extraction performed to analyze them. Various organic solvents followed by hydrolysis treatments have been used in several studies to lead the release of phenolic compounds.
In this study, a detailed methanolic extracts is presented using HPLC-ESI-MS^n method, than 13 compounds were detected. To our knowledge, all of the compounds (except secoisolariciresinol diglucoside, dihydro kaempferol hexoside, enterolactone and herbacetin 3, 8-O-diglucoside) were identified for the first time on the aerial part of Linum usitatissimum L. These compounds corresponded to flavonoids, lignans subclass, and as a most noteworthy finding the cytotoxic lignin podophyllotoxin whish has found its place in pharmacotherapy. It is used topically in the treatment of condyloma (Gross, 2001). Podophyllotoxin also forms a starting compound for the production of three important clinically applied anticancer drugs (Imbert, 1998). Moreover, other compounds in flaxseed, e.g. medioreisol and prinsepioi may also contribute to positive health effects. Thus, knowledge about the oligomeric structure and its components is of importance from a nutritional and biochemical point of view and is crucial for a complete understanding and possible exploitation. Overall, our results exemplify the usefulness of the RP-LC-ESI-MS to complete the current knowledge about flaxseed photochemical. Obviously, more studies should be carried out to elucidate the relationship between the structural features of the identified compounds and their bioactivity.

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