Article

Compounds Characterization of a Mucuna Seed Extract: L-Dopa, Arginine, Stizolamine, and Some Fructooligosaccharides

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Abstract: Human societies demand sustainable alternatives for goods and services. Plants are sustainable sources of important metabolites with beneficial impacts on human health. There are many reported methodologies and commercial suppliers for extract preparations from the plant Mucuna *sp*. They usually claim to be enriched in L-dopa, their distinctive metabolite. However, there are poor characterizations of the metabolite's components in that extracts. Here, we present the metabolite characterization of a Mucuna seed extract, emphasizing the L-dopa identification and quantification. To obtain the extracts, we follow a green and sustainable extraction protocol. The lyophilized extract was subject to liquid chromatography and mass spectrometry to identify its primary metabolites. Additionally, we follow thin-layer chromatography to identify some carbohydrates in the sample. The resultant extract has a 56% L-dopa. Other main components in the extract were arginine, stizolamine, and the fructooligosaccharides sucrose and nystose. The characterized Mucuna extract can be easily standardized as powder presentation and used in several biomedical applications.

Keywords: Mucuna seed; L-Dopa content; Lyophilized extract; Sugars identification; TLC; Mass spectrometry

1. Introduction

The development of human societies has been linked to using plants for food, materials, and medicines [1]. One example of the use of these plants is the legume velvet bean, also known as Mucuna *spp.* [2]. The origins of this plant can be traced to areas of China, Malaysia, and India [3]. Accessions of these plants are now present in tropical regions [4], including the southeast of México [5]. In Central America, farmers use the plant in culture rotation with milpa to recover and improve soil nutrition [6], as green manure and cover crop [7], among related uses. Some uses of Mucuna seeds are feed ingredients in poultry nutrition [8]. The seed shell has been used for wastewater paint treatment [9]. And a methanolic extract of leaves shows broad antimicrobial activity [10]. The most ancient registers on the practical use of Mucuna for improving human health is in Ayurvedic medicine [11]. It has been documented that the use of Mucuna for several pharmacological benefits. Their usefulness includes anti-diabetic [12], aphrodisiac [13], antineoplastic [14], antiepileptic [15], anti-venom [16], antihypertensive [17], anti-neurodegenerative [18], and for improving male fertility [19], among others. These applications are due to their unique metabolite composition [6]. A typical application of Mucuna seed is to alleviate Parkinson's disease [20]. This beneficial effect relies on the L-dopa (levodopa) activity (3,4-Dihydroxyphenylalanine), mainly present in this plant's

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seed [21]. Levodopa is a precursor of the neurotransmitter dopamine, but this last is unable to cross the blood-brain barrier. L-dopa is administered to patients with damaged dopaminergic neurons, beings the treatment by choice for Parkinson's disease [22]. Unfortunately, conform progresses the condition, and after years of therapy, L-dopa becomes less effective and provokes some complications like dyskinesia [23]. Carbipoda, an inhibitor of dopamine carboxylase, is dispensed together with L-dopa to avoid the rapid enzymatic degradation of dopamine [24].

Whole bean, mainly toasted, has been consumed to alleviate or prevent Parkinson's disease [25]. However, treating Parkinson's with seeds has difficulty requiring high seed doses since the content of L-dopa in the grain is less than 10% [26]. In addition, some people present adverse effects to consuming seeds, such as vomiting. One alternative is to concentrate the L-dopa in Mucuna seed extracts [27], which can be more manageable and dried as a powder where L-dopa is demonstrated to be stable [28]. The whole seeds have shown advantages in patients with Parkinson's [29] and animal models versus synthetic L-dopa [30]. Thus, using seed extracts could improve the management of dose quantity. L-dopa in seed extract has been shown to be adequate to alleviate Parkinson's disease at 6 mg/kg [31]. Another study calculates an equivalent daily levodopa dose of 100 mg [32]. The extracting procedures are directed to obtain L-dopa based on their reacting chemical nature to solvents. So, the extraction procedure diminished the presence of most metabolites in the seed yet maintained its advantages over synthetic L-dopa [33]. Many protocols have been developed to extract L-dopa from Mucuna seeds; these range from using water [34] and alcohol, but most of them employ hydro-acid solutions [27]. Some are assisted by microwave or supercritical CO_2 [35].

However, there are few characterizations of the molecules in these extracts beyond L-dopa, and their direct use in animal experiments is customarily done [36]. Notably, there are many commercial suppliers of both seeds in powder and seed extracts with a wide range of levodopa content [37][38]. But these are poorly characterized, or no metabolite composition is reported in these products. This information scarcity makes their appropriate use and the attribution of potential benefits to specific molecules difficult.

In this study, we use a Mexican accession of Mucuna *pruriens sp*. to extract and lyophilize an L-dopa presentation that can be used in biomedical applications. We focus on identifying and quantifying L-dopa and the sample's other significant components. The rest of the manuscript describes our methodological approach, the results obtained, and a brief discussion and conclusions.

2. Materials and Methods

2.1 *Seed material, their production, and L-dopa extract preparation*

Seeds of six cultivars of *M. pruriens* were kindly gifted by Prof. Castillo Caamal [39]. Of these, *M. pruriens var. ceniza* was better adapted and cultivated organically on farmland fertilized with cattle manure without chemicals or pesticides in the community of Tepecoacuilco, State of Guerrero, Mexico (18°18′0″ N, 99°29′0″ W). The cultivation depends on the rain-based season, typically from June to January. The harvest of pods happens when these turn from green to dark. Pods were collected from plants and dried in the sun to liberate the seeds. The clean kernels were stored in containers at room temperature and protected from direct light until their use.

There are many reported methods for L-dopa extractions from Mucuna [27]. Given that we were interested in implementing an organic and sustainable process, we mostly followed the extraction method reported by Polanowska et al. [40]. We slightly modified the protocol by dissolving the equivalent of 100 g milled dried seed in 1L of 0.3% acetic acid and 0.1% citric acid (1:10 w/v); the rest of the protocol was the same as reported. After clearing by centrifugation (OHAUS, Frontier 5816R) the supernatant was freeze-dried in

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a Virtis Freezemobile 12 lyophilizer (ALT Inc. CT. USA). The lyophilized powder was stored in a dark container at 4 °C; we identified this extract product as lot BFLD21-003.

2.2 *Sample preparation for metabolite quantification*

5.0 mg of the freeze-dried powder was dissolved in 1 ml of 1% formic acid and centrifuged at 10000 rpm for 5 minutes to eliminate any insoluble material. The supernatant was passed through an SPE C18 cartridge (ThermoFisher Scientific). This column was pre-activated with 1mL of methanol and equilibrated with 1mL of 1% formic acid. Then the sample (10 ml) was passed, and the eluates were collected. 450 µL of the eluate was taken, and 50 µL of methanol was added. This final solution was filtered using a 0.22 µm PTFE membrane (Sigma-Aldrich), and 1.0 µL was injected into the chromatographic system for metabolite analysis.

2.3 *Sample preparation for L-dopa quantification*

To estimate the quantity of L-dopa in the powder extract, we first took 5.0 mg of the freeze-dried powder and dissolved it in 1 ml methanol with 1% formic acid. Then it passed through an SPE C18 cartridge (ThermoFisher Scientific). The cartridge was pre-activated with 1mL of 50% methanol and equilibrated with 1mL of 1% formic acid. Then the sample was passed by the cartridge and washed with 1mL methanol 40%. The eluates were collected and diluted to 50 mL with 1% formic acid. 200 µL of the final dilution was filtered using a 0.22 µm PTFE membrane (Sigma-Aldrich), and 0.1 µL was injected into the chromatographic system for L-dopa analysis.

2.4 *Analysis of metabolites by liquid chromatography coupled to mass spectrometry (UPLC-ESI-TOF-MS)*

The metabolomic analysis of the extract was carried out in a liquid chromatograph (Acquity UPLC I-Class, Waters, USA). The chromatography equipment was coupled to a high-definition mass spectrometer (Synapt G2-Si, Waters). This last was equipped with an electro-nebulization ionization source, a single quadrupole mass filter, an ion mobility system, a collision cell, and a time-of-flight mass analyzer (ESI-Q-SIM-CID-TOF). We operate the LC-MS system and the analysis and the spectral data acquisition with the Masslynx 4.1 program (Waters, USA).

The chromatographic separation was performed with a column Luna ® Omega C18 100 Å, 1.6 µm (150 x 2.1 mm, Phenomenex). The composition of the mobile phases was 0.1% acetic acid and methanol in a 49:1 ratio using a flow of 200 µL/min at 40 °C and run in an isocratic mode.

For metabolomic analysis, metabolites were carried out in both positive and negative ionization modes. The mass spectrometer was operated with the following parameters: capillary voltage, 3000 V; cone voltage, 40 V; source temperature, 120 °C; cone nitrogen flow, 50 L/h; nebulizer nitrogen pressure, 6.5 bar; nitrogen temperature for desolvation, 350 °C; nitrogen flow for nebulization, 800 L/h. The mass spectra were acquired every second in a continuous format. The spectral range m/z was set from 50 to 1200. Two spectral functions were captured for each LC-MS analysis using argon as a collision gas: low energy at 6V; and an energy gradient from 20 to 60 V. Spectral correction was made by continuously infusing the reference compound leucine-enkephalin: 556.2771 for ESI positive; and 554.2615 for negative ESI. The mass spectrometer was calibrated using NaI (Sigma, USA). The spectral analysis was carried out with the Progenesis Q.I. (Waters) program and was verified manually. The compound identified with a retention time of 3.15 min was confirmed, corresponding with an L-dopa standard (CAS 59-92-7. Sigma Co. USA).

For L-dopa quantification, the mass spectrometer was operated in ESI positive mode, and the mass spectra were acquired every 0.4 seconds in a centroid format. L-dopa was monitored at m/z 198.076 ±0.01 Da.

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2.5 *Estimating the quantity of L-dopa in the extract*

For estimating the quantity of L-dopa in the powder extract, we first make a calibration curve from 50 to 500 pg of commercial L-dopa synthesized from pure L-tyrosine (Sigma Co.) by calculating the area of each calibration point in the mentioned liquid chromatography coupled to mass spectrometry equipment. We extrapolate the quantity of L-dopa in the sample with the calibration curve and their equation.

2.5 *Thin layer chromatography (TLC) analysis*

For TLC analysis, 10 mg of the lyophilized powder extract was dissolved in 90 µL of DMSO (Sigma Co. USA) and 20 µL of 1N HCl. FOS (fructooligosaccharides) and MOS (maltooligosaccharides) standards were purchased from Sigma Co. One and two µL of either sample extract or commercial standards were applied to silica gel TLC plates with aluminum support (10 cm × 10 cm, Aldrich). TLC plates were developed three times with a mobile phase made of butanol/propanol/water (3:12:4, v/v/v). The TLC was done three times; one was visualized with UV light, another developed with ninhydrin to derivatize amino acids like compounds (L-dopa), and another with a solution of aniline/diphenylamine/phosphoric acid reagent in the acetone base to reveal carbohydrates [41].

3. Results

3.1. Obtention of the lyophilized extract

Figure 1 shows the different organs of the Mucuna sp. plant from whose seeds we made the green L-dopa extraction following the green protocol reported in [40]. We obtained a final quantity of 101 g of lyophilized power starting from 500 g of dried, milled seeds (Figure 1C). This quantity represents 20% (w/w). The final lyophilized powder is white with a slightly yellow apparency (Figure 1D). This lyophilized powder was used in the different analytic approaches as described below.

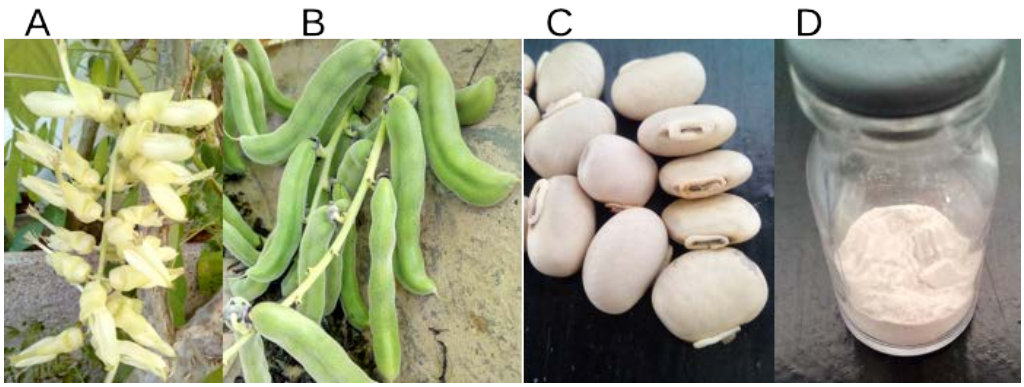

Figure 1. Mucuna pruriens *sp*., and seed extract. Different organs and physiological states of the plant are shown; flowers (A), green pods (B), mature seeds (C), and lyophilized powder extracted from seeds (D).

3.2 Metabolomic analysis of M. pruriens extracts by liquid chromatography coupled to mass spectrometry (UPLC-ESI-TOF-MS)

3.2.1. *Monitoring of metabolites in positive ionization mode (ESI+)*

One first assay we made was to have the whole mass spectrum starting from 1 µL of the sample (see Methods section) in the LC-MS equipment. In the positive mode, we can observe three prominent peaks corresponding to the retention time of 1.52, 3.15, and 6.36 minutes (Figure 2). Further fragmentation of the peaks shows that the 1.52 height

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presents a primary ion $[M+H]^+$ with an *m*/*z* 175.1196, which corresponds to the protonated form of the amino acid arginine ($C_6H_{14}N_4O_2$) (Figure 3).

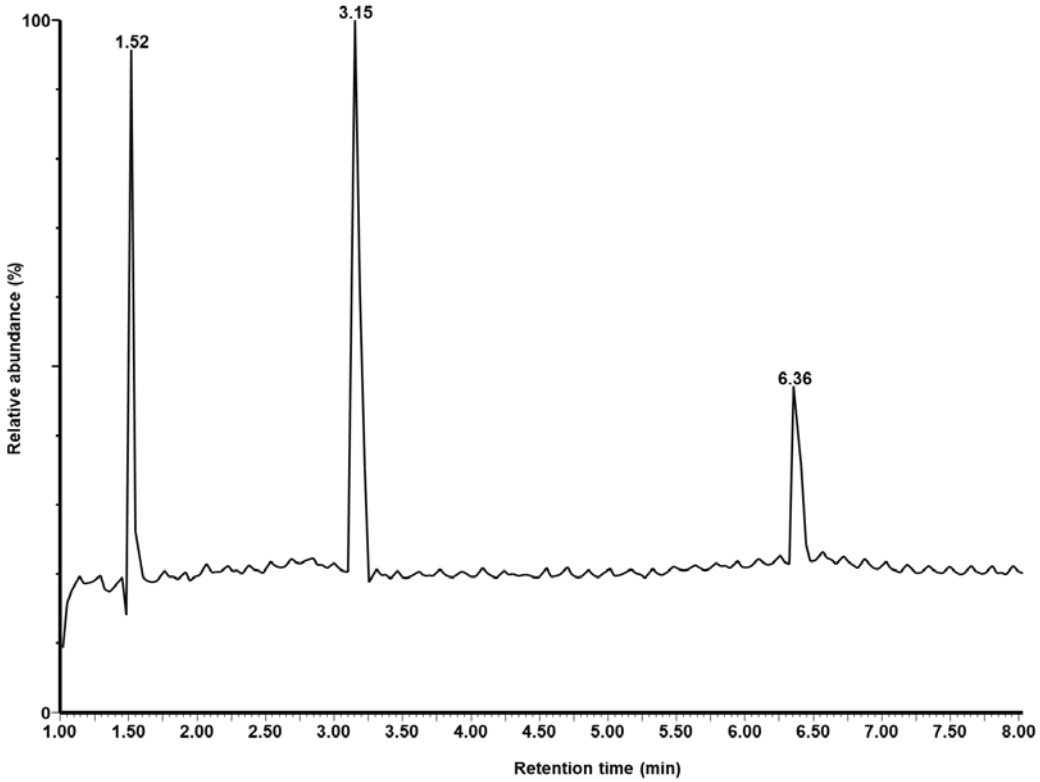

Figure 2. Base peak intensity chromatograms (BPI) of *Mucuna pruriens* extracts in LC-MS in positive ionization mode (ESI+).

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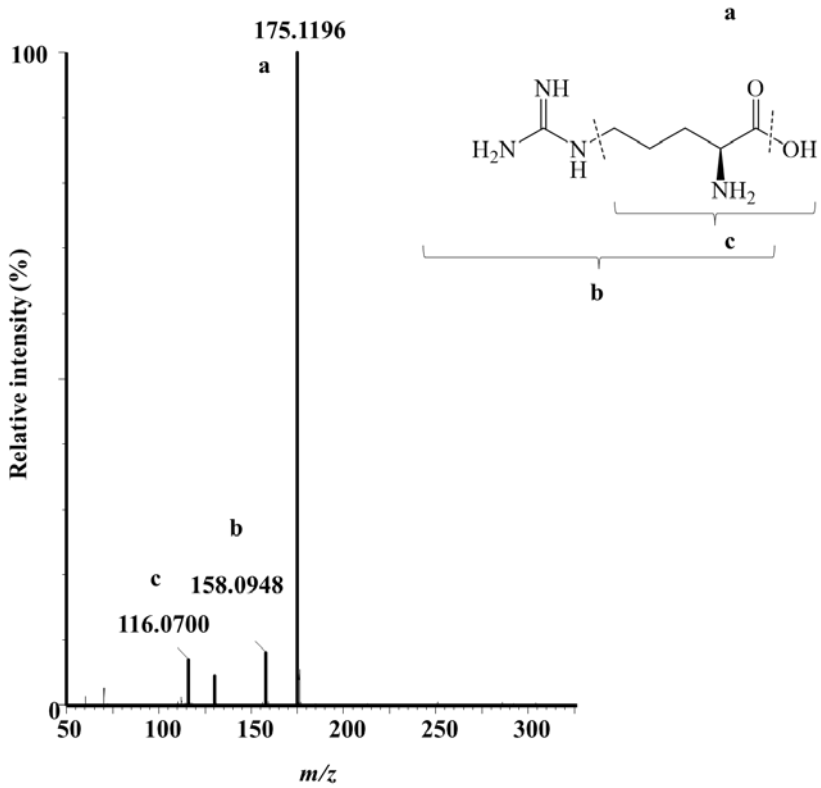

Figure 3. Mass spectrum and proposed structure of the compound at 1.52 min. The complete molecular ion (a) when there is a loss of the hydroxyl group (b) and when there is a loss of three terminal nitrogen atoms (c).

In another way, Figure 4 shows the mass spectrum of the peak corresponding to the retention time of 3.15 min in Figure 2. The mass spectrum shows an ion with an m/z 198.0788 (Figure 4a) corresponding to the protonated form of the molecule $C_9H_{11}NO_4$, identified as L-dopa. We verified this putative identity by comparing its retention time and mass spectrum with a commercial standard of L-dopa (Sigma Co. USA). The most abundant ion (*m*/*z* 152.0694) corresponds to the L-dopa molecule with a loss of its carboxyl group. Additional fragmentations peaks are observed where different functional groups of the molecule are lost (Figure 4).

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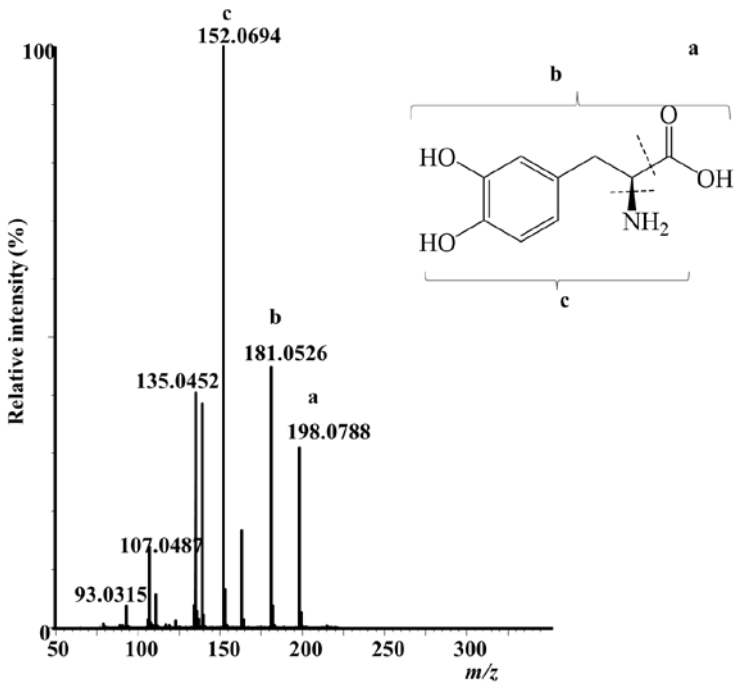

Figure 4. Mass spectrum of L-dopa (peak 3.15 min in Fig. 2). The parent ion (*m/z* 198.0788) (a), when loss an amino group (*m/z* 181.0526) (b), or loss a carboxyl group (*m/z* 152.0694) (c).

Figure 5 shows the mass spectrum of the compound with a retention time of 6.36 min in Figure 2. It reveals a primary ion with an *m/z* of 198.0972. This ion was identified as the protonated form of a compound with the molecular formula $C_7H_{11}N_5O_2$, which corresponds to stizolamine.

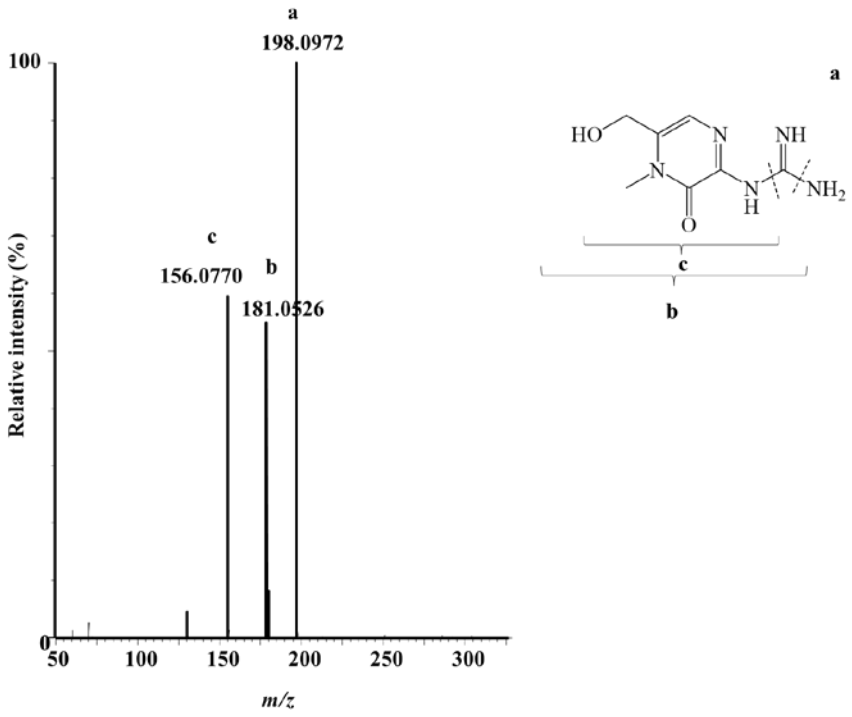

Figure 5. Mass spectrum and proposed structure of stizolamine. The complete molecular ion (a), and when loss of one and two amino groups generating the ions with *m/z* 181.05 (b) and m/z 156.07 (c), respectively.

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3.2.2. *Monitoring of metabolites in negative ionization mode (ESI-)*

We also analyze the extract in negative ionization mode in liquid chromatography coupled to mass spectrometry (UPLC-ESI-TOF-MS). Figure 5 shows the most abundant peak compounds in this analysis. The general profile of metabolites results is very different concerning the positive mode. In a negative mode, two significant peaks appear (figure 6).

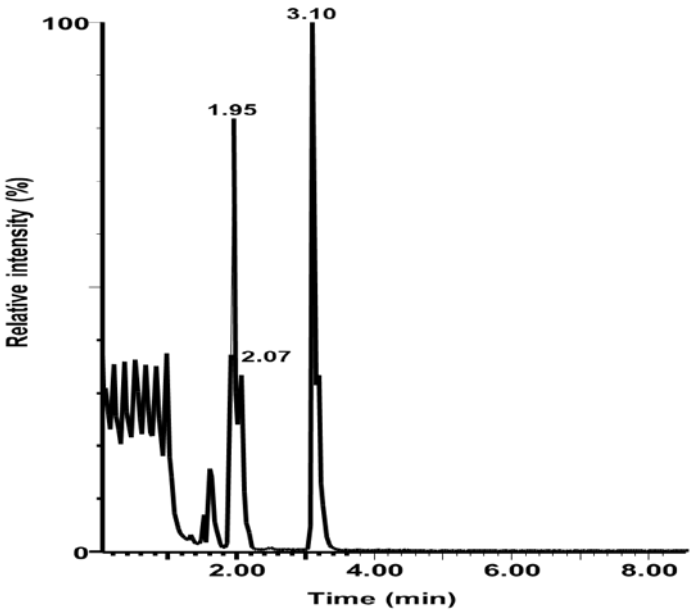

Figure 6. Base peak chromatogram of *Mucuna pruriens* extracts in LC-MS in negative ionization mode (ESI-). Two peaks appear majorly.

Peaks with retention times 1.95 and 2.07 most possibly correspond to the coelution of several oligosaccharides with the molecular formulas $C_{30}H_{52}O_{26}$ (pentamer), $C_{24}H_{42}O_{21}$ (tetramer), $C_{18}H_{32}O_{16}$ (trimer), $C_{12}H_{22}O_{11}$ (dimer) as reveals their respective mass spectra (Figure 7).

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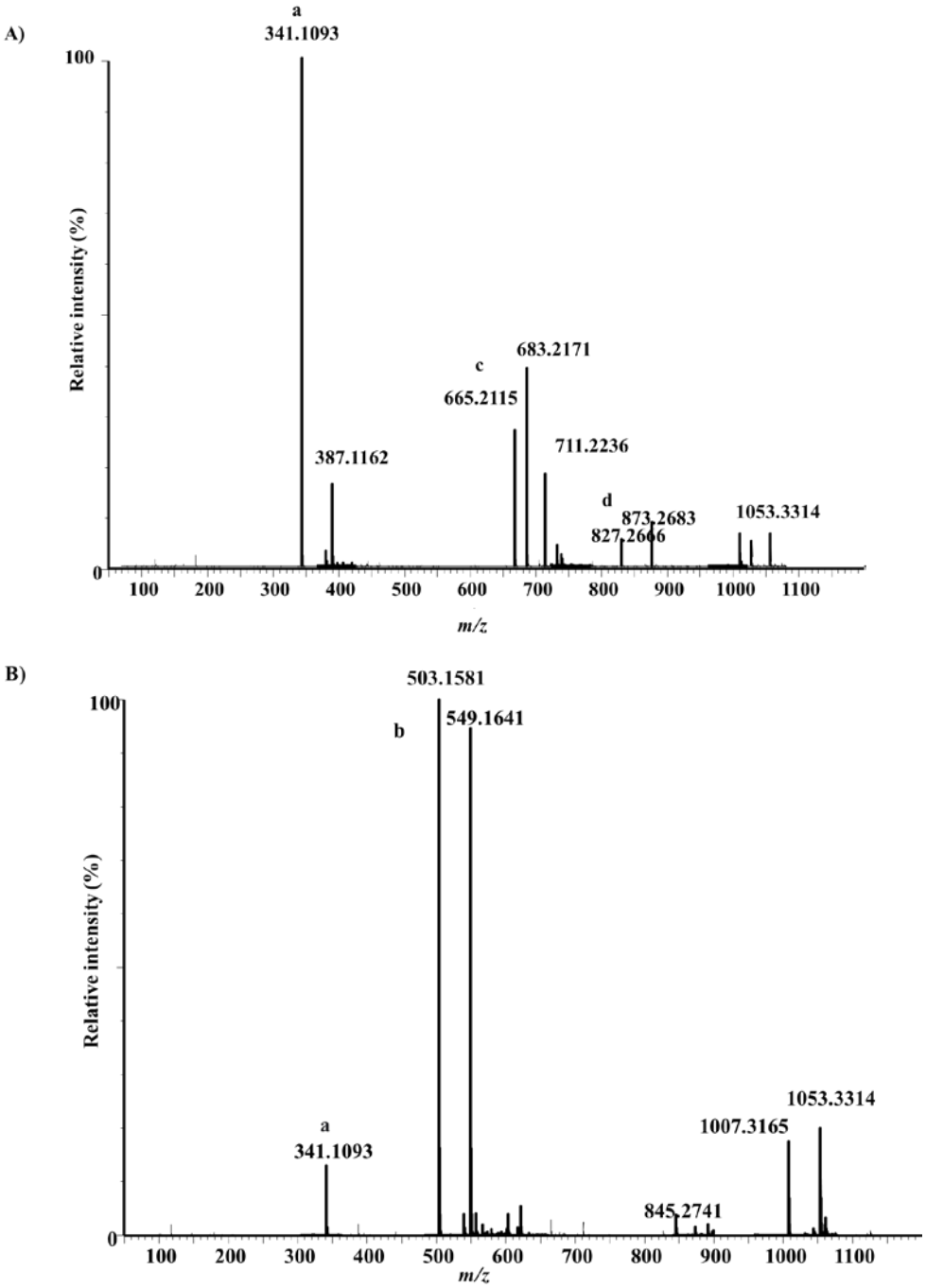

Figure 7. Mass spectra of the peaks with retention time 1.95 (A) and 2.07 (B), as in Figure 6, monitored in ESI (-). The deprotonated form $[M-H]^-$ of dimeric sugar $C_{12}H_{22}O_{11}$ (*m*/*z* 341.11), trimer $C_{18}H_{32}O_{16}$ (*m*/*z* 503.15), tetramer $C_{24}H_{42}O_{21}$ (*m*/*z* 665.21) and pentamer $C_{30}H_{52}O_{26}$ (*m*/*z* 827.26) are depicted by (a, b, c and d), respectively. The oligomers form ionic adducts in the presence of formic acid resulting in ions with an increase of +46 *m*/*z* (387.12, 549.16, 683.21, and 873.27), respectively.

Finally, the peak in retention time of 3.10 min in figure 6 corresponds to the deprotonated form $[M-H]^-$ of L-dopa (Fig 8).

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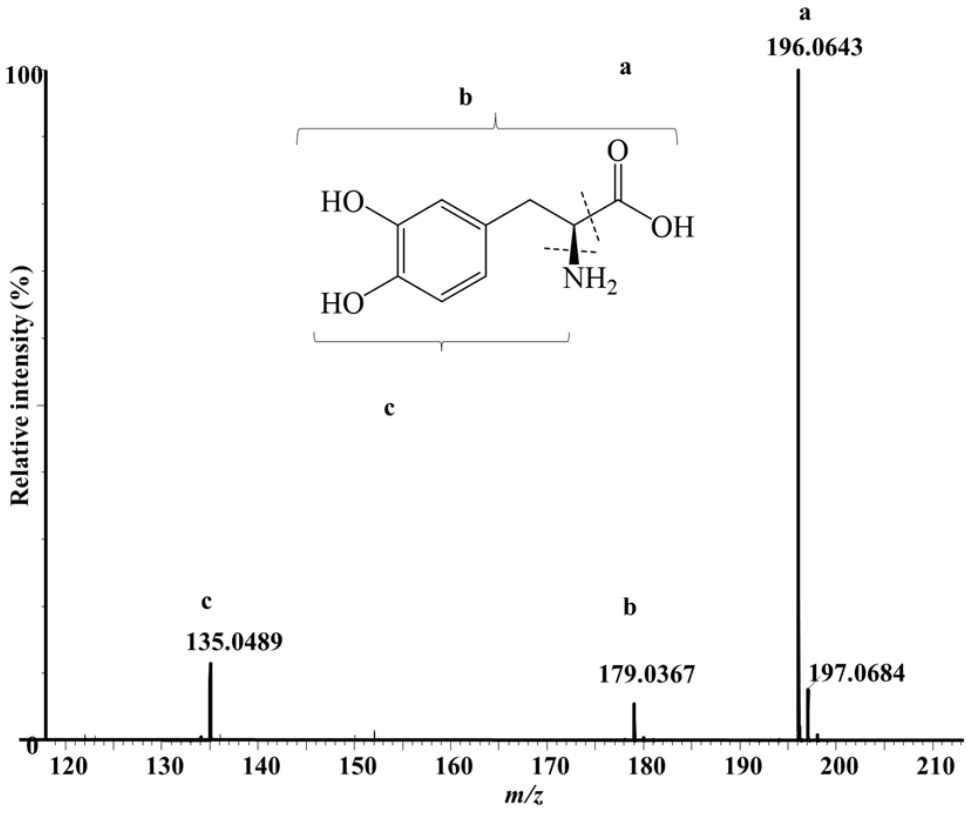

Figure 8. Mass spectrum of L-dopa in negative ionization mode. a) Parent ion (*m/z* 196.0643), b) loss of amino group (*m/z* 179.0367) and c) loss of amino and carboxyl group (*m/z* 135.0489) are depicted in the image.

3.3 Quantification of L-dopa in the Mucuna seed extract

To quantify the presence of L-dopa in the extract, we make a calibration curve with a commercial standard of L-dopa. In Figure 9A, we can observe a point of the calibration curve corresponding to 5000 pg L-dopa. Figure 9B shows the calibration curve for L-dopa. With the calibration curve, we calculate that we have 0.56 mg per mg extract, which corresponds to a 56%, which means a little more than half of the lyophilized mass in the extract corresponds to L-dopa.

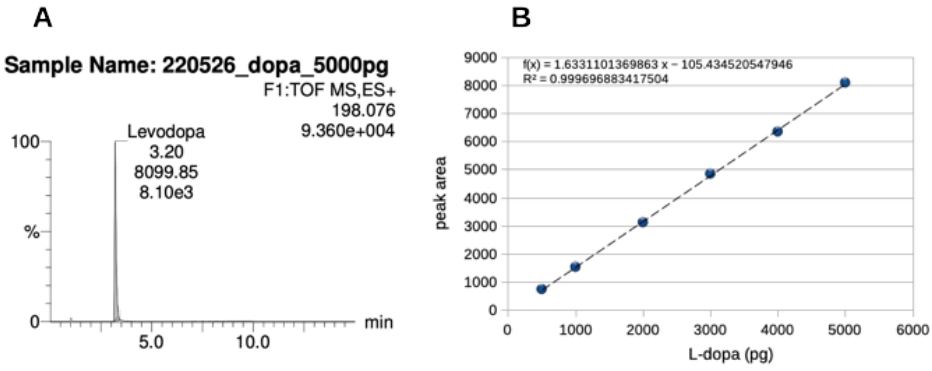

Figure 9. Calibration curve of commercial L-dopa. The left shows a point in the calibration curve (A), whereas B is the result of the complete calibration curve.

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3.4. *Thin Layer Chromatography (TLC) identifies the presence of fructooligosaccharides in the Mucuna seed extract*

The chromatographic analysis of the TLC shows that the sample presents a tiny spot when photographed under normal light (line 2 in Figure 10A). A coincident spot in the upper part of the sample is shared with the commercial standard for L-dopa (line 1). When the sample is exposed to UV light, it displays a strong blue fluorescence, as is mentioned in the case of stizolamine, although no previous image is available (line 2, Figure 10B). This fluorescence is not present in the standard as expected. Ninhydrin, on the other hand (Figure 10C), allows the localization of amino acids like L-dopa in the sample, as in the standard. Finally, when the standard of several maltooligosaccharides (MOS) is run together with the sample, it reveals the presence of oligosaccharides but not like MOS (Figure 10D). When a standard of fructooligosaccharides (FOS) is compared, in an independent run from A-D, the first spot on Figure 10E, line 3 corresponds to sucrose, and there is a second coincident spot corresponding to 1-nystose (N-DP4) though the coloration in the sample is some different, possibly corresponding to one sugar modification. The commercial standard of L-dopa does not present oligosaccharides as expected.

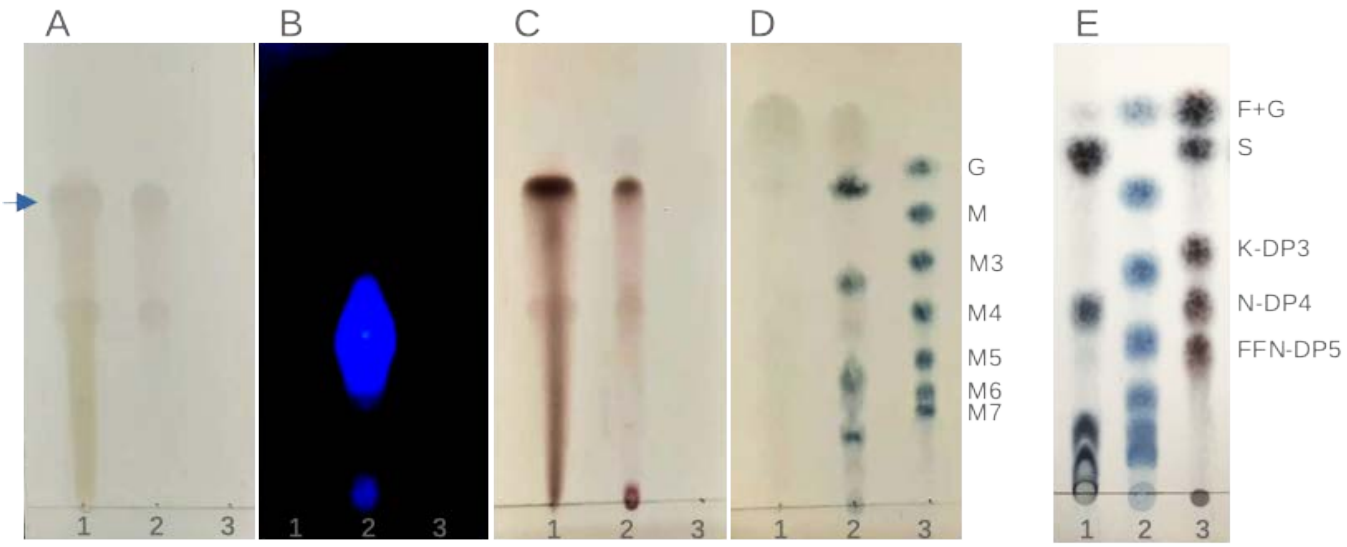

Figure 10. Thin layer chromatography of the Mucuna seed extract. In the same run A-D, line 1 corresponds to 2 µL of L-dopa standard; line 2 to 1 µL of the extracted sample; and line 3 to 1 µL of maltooligosaccharides (MOS) standard mixture. A is revealed under normal light, B is revealed under UV light, C is revealed with ninhydrin, and D with aniline/diphenylamine/phosphoric acid. E is an independent run revealed as in D, line 1 corresponds to 2 µL of the sample; and line 2 to 1 µL of maltooligosaccharides (MOS), line 3 to 1 µL of fructooligosaccharides (FOS) standard mixture. MOS; G, glucose; M, maltose; M3, maltotriose; M4, maltotetraose; M5, maltopentose; M6, maltohexose; M7, maltoheptaose. FOS; F+G, fructose plus glucose; S, sucrose; K, 1-kestose (DP3); N, 1-nystose (DP4); FFN, 1-fructofuranosyl-nystose (DP5).

4. Discussion

Mucuna seed extracts are commercially available for human consumption as food supplements and are widely used in many experiments in animal models. These experimental approaches look for evidence of the different qualities of the plants. In most of these experimentations, however, there is no characterization of the extracts. There is just L-dopa quantification, although their content does not always correspond with the declared on the label [38]. In this work, we decided to investigate the component

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resultants on the Mucuna seed extract to be more precise on the potential effects it could result in experimentations. We were sure our extraction methodology was directed to extract L-dopa, but inevitably there are co-extracted additional compounds. So, our first approach was looking for L-dopa with a technique that permits us to differentiate it from other molecules. In this way, we start with analyzing metabolites by liquid chromatography coupled with mass spectrometry (UPLC), followed by ionization of metabolites by electrospray (ESI), that hit the detector in a way that discriminates ions of the same *m*/*z* value with different initial energies (TOF-MS). These analyses were done in both positive and negative modes. The positive mode revealed three well-defined peaks that their further fragmentation revealed to be the amino acid arginine, L-dopa and Stizolamine. Our quantification of L-dopa confirms we have an appropriate extraction method since a little more than half of the mass weight in the sample corresponds to L-dopa (56% w/w). In addition, we found in old literature that in addition to L-dopa, stizolamine was also reported in *Stizolobium* [42] [43]. *Stizolobium* is managed as synonymous with *Mucuna* of the Fabaceae plants family [44]. It is important to emphasize that L-dopa and Stizolamine are molecules already registered as present in Mucuna plants [45]. A straightforward way to differentiate Stizolamine from L-dopa is that the former is said to present a strong fluorescence in color blue under UV light, although not pictured has been shown. In our case, we confirmed the presence of a compound with blue fluorescence in our sample. And this is not present in the standard, as shown in Figure 9B. Stizolamine pertains to the class compounds of pyrazines and has associated biological functions as pollinator pheromones [46] and is of some medical importance [47]. The third abundant component in the positive mode was arginine. It is intriguing the high quantity of this amino acid. The literature reported a structural analog to arginine, canavanine, in the family-related Canavalia plants [48]. The Arg-tRNA can charge canavanine instead arginine. This substitution disrupts the metabolism as a defensive way for this plant to depredators [49]. Both molecules (arginine and canavanine) present very relative molecular weights, differing in just two mass units. Deep analysis shows us that the equipment already detects canavanine, but just at the noise level. So, we are sure that we are visualizing arginine. Arginine is the most basic charged amino acid encoded by six codons and is in high quantities in dietary nuts [50]. Arginine was also the most abundant amino acid among the principal storage proteins of the megagametophyte of the Loblolly pine tree and was hypothesized as essential for nutrition and seedling growth [51]. In addition to glutamine, arginine has been proposed as a crucial immune nutrient for improving health [52]. A metastudy with ten randomized control trials concluded that arginine supplements (1.5 – 5 g/day) could be recommended for mild to severe erectile dysfunction [53]. Then it is possible that arginine can contribute to promoting male fertility with this plant.

Another important fraction component in the Mucuna seed extract, revealed in negative mode, is a mixture of oligosaccharides. The UPLC-ESI-TOF-MS analysis in negative mode presents the peak at 1.95, whose mass spectrum predicts the dimer $C_{12}H_{22}O_{11}$, which probably corresponds to sucrose or cellobiose; the TLC analysis confirms that it is sucrose (a dimer consisting of glucose and fructose). The MS analysis predicts higher oligomers, but by TLC, we can ensure only the $C_{24}H_{42}O_{21}$ corresponds very probably to 1-nystose, consisting of glucose and three fructose. The TLC analysis revealed the presence of at least two higher fructose oligomers, but we could not identify them with our standards (Figure 10D, line 1 bottom). Further characterization is needed to ensure the identity of these complex oligosaccharides. Alternatively, further purification is required to eliminate these oligosaccharides in the sample.

There is scarce metabolite analysis reported for Mucuna extracts. GC-MS analysis of seeds methanolic extracts revealed the presence of 5 major compounds: Pentadecanoic acid, 14-methyl-, methyl ester, Dodecanoic acid, 9,12-Octadecadienoic acid (Z, Z)-, methyl ester, 9,12- Octadecadienoic acid and 2-Myristynoyl-glycinamide [54]. There is possible that the methanolic extraction rends more volatile compounds in this extract regarding

our extracting approach. In another study, using whole seeds and a combination of chromatographic and NMR techniques, authors report the presence of d-chiro-inositol and its two galactose derivatives in *Mucuna pruriens* [55]. Inositol and galactose derivatives may be present in our seeds but diluted in our seed extract.

With this study, we want to contribute to the molecular characterization of an extract of this important plant. The more complete a description of a botanical product more information is available for the consumer and health professionals. Deep knowledge of the components in the product is essential because there are many commercial presentations of Mucuna with poor or no components characterization. There is a remarkable opportunity to have phytochemical products well characterized chemically to be more confident of the attributed health properties.

5. Conclusions

Here we reported a simple preparation of a lyophilized Mucuna seed extract enriched in 56% of L-dopa (w/w). In addition to L-dopa, arginine, stizolamine, and some fructooligosaccharides are also present. Neither of these components has detrimental effects on health.

Supplementary Materials: no

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