

Nutrient and bioactive compounds from *Neltuma* spp. seeds

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Abstract

In the search for compounds with nutritional and functional value, seeds from several *Neltuma* species were examined for lipid, protein and phenolic components. Lipid contents ranged between 9.3 - 12.5 g/100 g (seed DW), and showed MUFA and PUFA predominating largely. Lipids were also a good source of tocopherols (170 – 603 mg total tocopherols/kg). Proteins accounted for 14.7 - 35.5 g/100 g seed. Essential amino acids (EAA) comprised 26.1 – 40.2 % of the total AA content. Based on the EAA score, proteins from all the species analysed were deficient in leucine, isoleucine and valine, but could meet requirements for histidine, threonine, phenylalanine, tyrosine, and lysine. The qualitative patterns of phenolic compounds were similar each other (predominance of flavonoids, particularly apigenin derivatives). Keeping in mind the examined chemical components, *Neltuma* seeds have potential value as a source of healthy macronutrients and bioactive compounds.

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ABSTRACT

In the search for compounds with nutritional and functional value, seeds from several *Neltuma* species were examined for lipid, protein and phenolic components. Lipid contents ranged between 9.3 - 12.5 g/100 g (seed DW), and showed MUFA and PUFA predominating largely. Lipids were also a good source of tocopherols (170 – 603 mg total tocopherols/kg). Proteins accounted for 14.7 - 35.5 g/100 g seed. Essential amino acids (EAA) comprised 26.1 – 40.2 % of the total AA content. Based on the EAA score, proteins from all the species analysed were deficient in leucine, isoleucine and valine, but could meet requirements for histidine, threonine, phenylalanine, tyrosine, and lysine. The qualitative patterns of phenolic compounds were similar each other (predominance of flavonoids, particularly apigenin derivatives). Keeping in mind the examined chemical components, *Neltuma* seeds have potential value as a source of healthy macronutrients and bioactive compounds.

Keywords Amino acids, Lipid components, Phenolic compounds, *Neltuma* spp., Seeds

Abbreviations AA, amino acid; EAA, essential amino acid; DW, dry weight; FA, fatty acid; MUFA, monounsaturated fatty acids; PUFA; polyunsaturated fatty acids; TT, total tocopherol; TPC, total phenol

content.

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Introduction

Cultivated legumes are widely consumed as food worldwide. They provide a good source of protein and many other important nutritional and functional components (Grela et al., 2017; Bennetau-Pelissero 2019). Several wild legume species have also been investigated as a source of nutrients for humans and livestock (Arinathan et al., 2003; Bhat and Karim 2009). In many cases, they are present in agroforestry systems where besides providing invaluable benefits such as ecosystem services, can contribute sustainable food resources especially in rural communities.

The species of the genus *Neltuma* (ex *Prosopis*, Fabaceae) are important vegetation elements, particularly in arid and semiarid regions of the world. Many of them produce abundant pods which, since ancient times, have provided basic foodstuffs for man as well as forage for wildlife and domestic herbivores. Most of research on composition and nutritional value of *Neltuma* pods has focused on the mesocarp components (Felker et al., 2013). Studies on seed components are largely unaddressed. This could be due to the belief that the seeds have little nutritional benefit. So, in some regions and countries *Neltuma* foodstuffs are obtained by using only the pods, and seeds are discarded. Currently, there are few reports on seed chemical components of *Neltuma* spp. Fatty acid and sterol compositions have been investigated in *N. alpataco* and *N. denudans* from the Patagonian region in Argentina (Mazzuca and Balzaretto 2003). By analysing chemical and nutritional properties of different parts of *N. alba* fruits, it has been found that the seeds contribute the majority of the protein; in addition, they provide significant amounts of total polyphenols (Sciammaro et al., 2016). Interestingly, protein concentrates obtained from *N. alba* seeds have been proposed for formulation of functional foods because of their good amino acid profiles and important biological and functional properties (Cattaneo et al., 2014).

In Argentina, several *Neltuma* species have become widespread (Joseau et al., 2013). They can grow and fruit in marginal areas for conventional crops, under conditions of very low water availability. Our general interest is to contribute information to nutrient databanks of underexploited plant resources. In the present study, four *Neltuma* species were evaluated for unexplored seed components with a focus on nutritional and functional compounds.

Materials and methods

Pods from *Neltuma* species (*N. flexuosa*, *N. nigra*, *N. chilensis*, *N. alba*) were obtained from trees of natural populations in the north-western region of Córdoba province (Argentina). The identity of the named species was previously authenticated by using herbarium specimens from the botanical museum CORD (Argentina). From each species, three pod samples (about 1 kg each) - each obtained from at least five individual trees - were used for analytical determinations. Pods were harvested at full maturity stage and, after drying in darkness, the seeds were separated by hand and stored at -20 °C until use. Before proceeding to the different analyses, seeds were ground to a fine powder using a universal cutting mill (Tecno Dalvo, Argentina). Seed dry matter content was determined by oven drying at 80 °C (AOCS 2009).

Lipid analyses

Lipid extraction for total lipid content determination was performed using Soxhlet devices with n-hexane as solvent (AOCS 2009). Fatty acid (FA) composition was evaluated - as methyl esters (FAME) - by gas chromatography (GC) according to protocols for lipid sample preparation and GC conditions previously reported (Cittadini et al., 2021). Identity of FAME was confirmed by means of GC (Clarus 580, Perkin-Elmer, Shelton, USA) - mass spectrometry (MS, Clarus SQ8S) analysis. Separations were performed on a CP Wax 52 CB (Varian, Walnut Creek, USA) fused-silica capillary column using helium (flow rate 1 mL/min) as carrier gas. The GC oven temperature was initially maintained at 180 °C (5 min) and then increased at 2 °C/min to 220 °C. Both injector and detector temperatures were set at 250 °C. The FAME components were identified by mass spectra matching using the Wiley mass spectra search library.

For tocopherol analysis, ground seed samples (20 g each) were extracted with n-hexane (40 mL x three times) at room temperature (22 - 25°C). The solvent was removed using a rotary vacuum evaporator at 40 °C. The lipid samples obtained were analyzed by high performance liquid chromatography (HPLC, Perkin-Elmer, Shelton, USA) following analytical methods used previously (Cittadini et al., 2021). Accurately-weighted lipid samples (1 g each) were diluted with n-hexane to 10 mL. The solutions were filtered through 0.45 µm pore filters. Aliquots of 20 mL of the filtrated solutions were injected into a Supelcosil LC-NH2-NP column (25 cm x 4.6 mm, Supelco, Bellefonte, USA). The mobile phase was n-hexane/ethyl acetate (70/30 V/V) with a flow rate of 1 mL/min. UV detection at 295 nm was performed. Individual tocopherols were identified by comparing their retention times with those of authentic standards (α-, γ- and δ-tocopherols, ICN Biomedicals, Costa Mesa, USA), and were quantified by the external standard method. The linearity of the response was verified by fitting to line results of each one tocopherol individuals (eight standard solutions with known concentrations) covering the concentration range from 2 to 800 ppm, with linearity regression coefficients (R^2) varying between 0.98 and 0.99.

Protein analyses

The Kjeldahl method (total nitrogen x 6.25) was used to quantify the seed total protein content (AOAC 2009). For amino acid (AA) analysis, defatted seed samples (500 mg) were hydrolysed following the standard AOCS (2009) procedure. Hydrolysed samples were filtered using 0.45 µm membrane filters and then submitted to pre-column derivatization. For this purpose, accurately-weighted hydrolysed samples were placed in screw-capped tubes and re-suspended in 500 µL HCl 0.1 M plus 1.8 mL borate buffer, 0.75 mL methanol and 15 µL diethyl ethoxymethylenemalonate (Sigma-Aldrich, St. Louis, USA). The tubes were kept at constant temperature (25 °C) for 24 h. The obtained N-[2,2- bis (ethoxycarbonyl) vinyl] AA derivatives were analysed by reversed-phase HPLC (Perkin-Elmer, Shelton, USA) using a C18 column (Zorbax Eclipse Plus C18, Agilent Technologies, Santa Clara, USA) thermostated at 40 °C. Solvents (A, sodium acetate buffer, 25 mM pH 6; B, methanol/acetonitrile/water, 45/45/10 v/v) were used at a flow rate of 0.9 mL/min, according to the following gradient: 100 % A for the first 18 min, 57 % to 100 % B for the next 5 min, 0 % B from 23.1 to 30 min. Detection of AA derivatives was performed at 280 nm. Identification was carried out by using authentic AA standards (Sigma-Aldrich, St. Louis, USA), and quantification by means of the external standard method. The linearity of the response was verified by fitting to line results of each one amino acid individuals (five standard solutions with known concentrations). Calibration curves from the tested amino acid standards showed linearity regression coefficients (R^2) ranging between 0.95 and 0.99. The contents of the various identified essential AA (EAA) were compared with the FAO/WHO/UNU (2002) reference pattern. Individual EAA scores were determined according to FAO/WHO/UNU using the formula: AA score = mg of AA in 1 g test protein / mg of AA in requirement pattern. Limiting AA were identified as those having a ratio lesser than 1.

Phenolic compound analyses

Phenolic compounds were obtained from 10 g of ground seed samples previously defatted at room temperature (n-hexane 50 mL x three times). Defatted samples (5 g) were extracted thrice with methanol (80 % V/V, 50 mL every time). The extracts were combined and washed twice (50 mL every time) with n-hexane. The hydro-alcoholic phase was recovered, filtrated through 0.45 µm pore filters, concentrated under vacuum below 50 °C to a final volume of 1 mL, and further used for both total phenol content (TPC) and HPLC-ESI-MS/MS analyses according to procedures reported elsewhere (Bodoira et al., 2022).

The composition of phenolic extracts was analyzed by means of HPLC-ESI-MS/MS using an Agilent 1200 HPLC Series system (Agilent Technologies, Santa Clara, USA). The chromatographic separations were achieved on a Kromasil (Bohus, Sweden) reversed-phase C18 column (5 µm, 250 mm x 4.60 mm i.d.) under analytical conditions used previously (Bodoira et al., 2022). The MS detector was programmed to perform a MS/MS scan of the most abundant ions, using collision energy of 13.0 eV. The identification of phenolic compounds was based on their retention times (Rt), elution order, and comparison of UV-Vis spectra and mass spectrometry data reported in the literature (Picariello et al., 2017; Singh et al., 2017; Cittadini et al., 2021). The Compass version 3.1 software and DataAnalysis version 4.1 software were used for data

acquisition and processing, respectively.

Statistical analysis

Statistical differences between species in the analysed chemical parameters were estimated from ANOVA test at the 5 % level ($P \leq 0.05$) of significance. Whenever ANOVA indicated a significant difference, a pair-wise comparison of means by least significant difference (LSD) was carried out. Statistical analyses were performed using the InfoStat program (InfoStat version 2018, National University of Córdoba, Argentina).

Results and discussion

Lipid content and composition

The seeds of the analysed *Neltuma* species had similar lipid content (11.2 % in average) (Table 1). Overall, it results higher than those found in *Prosopis farcta* (3.2 %, Lajnet et al., 2015) and *N. alpataco* (7.0 %, Mazzuca and Balzaretti, 2003), and similar to those reported in *N. caldenia* (10.7 %) and *N. argentina* (14.7 %) (Lamarque et al., 1994). By way of comparison with other wild edible legumes, the lipid contents we found compare well with those from seeds of *Canavalia* spp. (1.4 – 12.1 %), *Cassia* spp. (3.8 – 7.0 %) and *Mucuna* spp. (6.7 – 14.4 %), as well as with data from several tree legume species recently investigated which showed seed oil yields ranging from 2.1 to 14.9 % (Grygier et al., 2023). On the other hand, seed lipid contents from *Neltuma* result meaningfully higher than those from some cultivated legumes, such as peas (*Pisum sativum*), lentils (*Lens culinaris*) and beans (*Phaseolus vulgaris*) which usually contain about 2 g lipid per 100 g seed (Padhi et al., 2017).

Fatty acid (FA) composition is summarized in Table 1. In accordance with data from wild and cultivated legumes worldwide, palmitic, oleic and linoleic acids were present as major FAs (Arinathan et al., 2003; Bhat and Karim, 2009; Padhi et al., 2017; Grygier et al., 2023). All *Neltuma* species evaluated presented the same FA components but with some differences in concentrations, especially regarding unsaturated FA levels. *Neltuma alba* and *N. chilensis* seeds had very similar composition; with the exception of palmitoleic and arachidic acids, no significant differences were found between their FA profiles. Meanwhile, *N. nigra* and *N. flexuosa* seeds showed significantly higher amounts of oleic and linoleic acids, respectively, whereas the remaining FA percentages were practically unchanged with respect to the two species first named. In general, the concentrations of the main FAs from the *Neltuma* species we studied are similar to those found in other species of the genus. As a difference, it could mention lower linoleic acid percentages in *N. alba*, *N. chilensis* and *N. nigra* (about 41, 39 and 32 %, respectively) with respect to those observed in *N. argentina* (51.1 %) and *N. alpataco* (56.1 %) seed oils (Lamarque et al., 1994; Mazzuca and Balzaretti 2003).

Based on the lipid fraction, a comparison of the FA composition between the *Neltuma* species studied and some cultivated legumes reveals that the former have lesser PUFA (linoleic and linolenic acids) contents than the later (Padhi et al., 2017). However, when FA concentrations are calculated on the basis of dry seed weight, the PUFA content in *Neltuma* accounts for 4.0 – 6.3 g/100 g seed, which represent amounts substantially higher than those present in varieties of peas, lentils and beans (1.05, 0.86 and 1.34 g PUFA/100 g seed, respectively), and similar to those found in chickpeas (5.03 g PUFA/100 g seed) (Padhi et al., 2017).

To the best of our knowledge, there are no published studies on tocopherol composition of *Neltuma* spp. Lipids from the studied species contained 170.9 – 603.5 mg/kg of total tocopherols (TT) which were composed mostly of α -tocopherol (167.5 – 544.6 mg/kg lipid) and minor amounts of γ -tocopherol (3.3 – 58.9 mg/kg lipid) (Table 1). On a DW basis of whole seeds, the range for tocopherol contents from all the species evaluated (from 2.35 mg TT/100 g in *N. chilensis* seeds to 7.21 mg TT/100 g in *N. flexuosa* seeds) is similar to that observed in edible pulses (2.2 – 5.7 mg TT/100 g) such as beans, lentils and peas (Padhi et al., 2017).

Protein content and composition

Protein content from *Neltuma* spp. accounted for 14.7 – 35.5 % of the seed dry matter content (Table 2); the highest one was observed in *N. flexuosa*. The values recorded are in line with those reported in other species of the genus such as *P. argentina* (26.9 %) and *P. caldenia* (32.2 %) (Lamarque et al., 1994). Moreover, they

resemble the values found in some tribal pulses (12.9 – 24.7 %, Arinathan et al., 2003), and in cultivated peas (*Lathyrus* spp.) and lentils (*Vicia* spp.) (17.7 – 25.6 % and 20.1 – 32.0 %, respectively) (Pastor-Cavada et al., 2011 a, b).

Amino acid (AA) composition is shown in Table 2. Except for isoleucine - which was below the detection limit we used - and leucine - not quantified in *N. nigra* seeds -, the species analysed showed the protein AA profiles usually reported in cultivated legume species (Pastor-Cavada et al., 2011 a, b; Grela et al., 2017; Bennetau-Pelissero 2019). The percentage of essential AA (EAA), which is considered an indicator of protein nutritional quality, ranged between 26 – 40 % of the total AA concentration. The values found in *N. alba* and *N. chilensis* were similar to those present in whole-wheat flour (about 27 %), which is a widespread and valuable source of proteins. Meanwhile, the values from *N. nigra* and *N. flexuosa* compared well with those of cultivated beans such as *Lathyrus* and *Vicia* species (39 – 42 %) (Pastor-Cavada et al., 2011 a, b).

So far, there is very little information on EAA contents in *Neltuma* seeds. A study on seed protein isolates from *N. alba* showed all EAA (Cattaneo et al., 2014), in quantities above the established requirements for human adults (FAO/UNU/WHO 2002). Besides the absence of isoleucine, proteins from all the species we studied were deficient in leucine and valine, but could meet requirements for histidine, threonine, phenylalanine + tyrosine, and lysine. Seed proteins from *N. alba* and *N. flexuosa* could also satisfy methionine requirements (Table 2). Worthy of mention are the relatively high amounts of histidine, threonine and lysine, particularly in *N. nigra* and *N. flexuosa* (12.7 – 13.2, 6.84 – 9.15, and 10.5 – 11.2 g/100 g protein, respectively). These contents are substantially higher than those present in several *Lathyrus* and *Vicia* species which showed histidine, threonine and lysine contents ranging between 2.2 – 3.3, 4.0 – 6.2 and 6.4 – 8.0 g/100 g protein, respectively (Pastor-Cavada et al., 2011 a, b). While histidine has been associated with factors that improve the so-called metabolic syndrome in humans (Moro et al., 2020), threonine plays a vital role in the modulation of nutritional metabolism, macromolecular biosynthesis, and gut homeostasis (Tang et al., 2021). The requirement for lysine is also a matter of interest as it is limiting in cereals, especially wheat. So, the high contents of these AA in *N. nigra* and *N. flexuosa* highlight the potential of their seed meals to improve the AA profile of cereal flour-based foods.

In line with findings reported from several seed proteins - including those of legumes and cereals - the most abundant non-EAA were those from the two pairs namely aspartic acid + asparagine and glutamic acid + glutamine. Interestingly, the aminograms also revealed higher amounts of arginine than those usually present in edible pulses (Margier et al., 2018). Arginine is considered a semi-essential AA involved in multiple metabolic processes and biological functions (Morris 2006).

Phenolic compounds

Firstly, it is worth noting the wide variation between the *Neltuma* species in the total content of seed phenolic compounds (Table 3); *N. nigra* presented the highest concentration while *N. alba* showed the lowest abundance. In cultivated legume seeds, the total phenolic content may also vary widely (Singh et al., 2017). By way of comparison, total phenolics in *N. nigra* (about 96 mg/100 g seed) may account for a similar amount to those found in cowpeas (about 100 mg/100 g) and pigeon pea seeds (78 – 120 mg/100 g), but occur in lower concentration than reported in chickpea and lentil varieties (Singh et al., 2017).

A total of 39 phenolic compounds were detected in the examined *Neltuma* seeds (Table 3). With the exception of compound 14, which was assigned tentatively as ferulic acid derivative, the remaining components were identified as flavonoids and structurally related substances. Apigenin derivatives were found to show the highest contents in all *Neltuma* species we studied. Apigenin 8-C and 6-C glucosides – commonly named vitexin and isovitexin – were by far the most abundant compounds reaching concentrations near to 40 mg/100 g (DW) in *N. nigra* and *N. flexuosa* seeds. Such concentrations are within the ranges found in some cultivated legumes such as the mung bean (17 – 62.4 and 22.6 – 73.6 mg/100 g, for vitexin and isovitexin, respectively), which is known as a major source of polyphenols (Hou et al., 2019). As a matter of interest, the protective effects of these compounds in several oxidative stress-related diseases are widely recognised, and have been reviewed in a recent published review (Babaei et al., 2020).

Another relatively abundant polyphenolic compounds in *Neltumaseeds* were the apigenin 6-C-glc-8-C-arab and 6-C-arab-8-C-glc isomers, which are usually known as schaftoside and isoschaftoside, respectively. They reached concentrations close to 4 mg/100 g in *P. nigraseeds*. All *Neltuma* spp. examined also contained minor amounts of further glycosylated derivatives (hexosides) of both compounds (peaks number 3 and 4). Schaftoside and isoschaftoside have been found in high amounts (41 - 61 and 337 - 518 mg/100 g, respectively) in the seed germ flour from some *Neltuma* species and in *Ceratoniasiliqua* seeds (Picariello et al., 2017). Interestingly, all these compounds have been described as potent α -glucosidase inhibitors (Zhao et al., 2019) thus suggesting that seeds from all the named plant materials could be used as a source of bio-actives contributing to modulate carbohydrate digestion in humans.

Peaks number 6 and 7 were identified as apigenin 6,8-C-di-glucosides. The occurrence of two peaks with identical [M-H]⁻ signal and major fragment ions suggests the possibility of stereoisomers already described and sometime named as vicanin (Picariello et al., 2017). Both peaks were relatively abundant in *N. flexuosa* but scarce in the other species. Nevertheless, the amounts found in *N. flexuosa* seeds (about 1.2 mg/100 g of both substances) were lesser to those reported in the seed germ flour (18 - 51 mg/100 g) from other *Neltuma* species (Picariello et al., 2017).

Kaempferol aglycone was almost absent in the materials studied but some derivatives - particularly kaempferol hexoside - were abundant in *N. nigra* seeds. The presence of these compounds is worthy of mention due to their wide range of bioactive and pharmacological properties including antioxidant, anti-cancer, anti-inflammatory, and cardioprotective effects (Imran et al., 2019). Other common flavonoids - such as luteolin, quercetin, chrysin, chrysoeriol and isorhamnetin - were generally present - mostly as glycosylated forms - in the examined *Neltuma* seeds, but always in small quantities.

Conclusions

Seeds from the studied *Neltuma* spp. presented valuable lipid contents showing a well-balanced fatty acid composition (low percentage of saturated fatty acids, moderate or high MUFA and PUFA concentrations) and good amount of tocopherols. Proteins accounted for about 15 - 35 % of dry seeds, and included 26 - 40 % of essential amino acids. Based on their AA scores, they were deficient in leucine, isoleucine and valine. Proteins from *N. nigra* and *N. flexuosa* presented meaningful quantities of the EAA histidine, threonine and lysine. In general, the qualitative patterns of phenolic compounds were similar each other (predominance of apigenin derivatives), although they differed markedly in the amounts of the individual components. Overall, seeds from the examined *Neltuma* species represent an undervalued source of healthy macronutrients and functional components. Moreover, as a matter of interest, these species can produce fertile hybrids (Joseau et al., 2013), so they may be a source of great genetic diversity. This in turn opens up the possibility of searching for interspecific hybrids with improved composition of target seed metabolites.

Conflict of interest The authors have declared no conflict of interests.

Compliance with ethics requirements This article does not contain any studies with human and animal subjects.

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