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Desert legume *Prosopis cineraria* as a novel source of antioxidant flavonoids / isoflavonoids: Biochemical characterization of edible pods for potential functional food development

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ABSTRACT

Flavonoids and isoflavonoids in foods are attracting attention as they are significant antioxidant and phytoestrogenic compounds that are beneficial for human health. In this study, the edible pods of the underutilized desert legume *Prosopis cineraria* from Rajasthan, India were used to extract flavonoids. The pods from semi-arid zone showed the highest flavonoid content (432 mg Rutin hydrate/gm). UV spectrophotometric analysis was also done to characterize flavonoids. The flavonoids and isoflavonoids were further purified from semi-arid zone plants using column chromatography with Amberlite XAD7HP and Sephadex LH-20. LC-MS analysis revealed the presence of medicinally valuable antioxidant flavonoids and isoflavonoids in the pods, viz. vitexin, puerarin, phloridzin, and daidzein. It was seen that the flavonoids/isoflavonoids are present in the selected legume in different forms i.e. pure aglycone, C-glycoside as well as O-glycoside. This finding makes *P. cineraria* an attractive source candidate for extraction of these nutraceuticals with a potential for development into functional food.

1. Introduction

Generation of Reactive Oxygen Species (ROS), mainly due to increasing pollution, side-effects of synthetic drugs and sedentary lifestyles, leads to many human disorders like inflammation and cancer. Epidemiological studies have indicated that antioxidant-rich diets are capable of ROS suppression [1]. This has given rise to a new category of food viz. 'functional food' with health benefits beyond nutrition, when consumed on regular basis [2]. The commercial importance of food-based antioxidants is increasingly being realized, as exemplified by research on various plants including legumes, primarily due to the toxicity of synthetic antioxidants to humans [3]. Interestingly, many of these antioxidants such as flavonoids are stored in the plant cell's vacuole in glycosylated forms, which are acted upon by specific β-glucosidases to make the more active aglycone available to the plant [4]. On a more commercial forefront, **Mai et al.** [5] have recently characterized a β-glucosidase from mangrove sediments, showing high catalytic activity on soy isoflavone glycosides. As dietary nutraceuticals, especially in legume family of plants, the isoflavonoids are significant contributors to human health as they show free radical-scavenging; estrogenic/anti-estrogenic, and pharmacological effects like anti-cancer and cardioprotection [[6,7]].

From emerging research standpoint, traditional plants of the desert ecosystem need deeper exploration from phytochemical/molecular perspective as they are subjected to stresses in the form of extreme temperatures, UV light, etc and are capable of accumulating unique bioactive metabolites in response. The desert legume *Prosopis cineraria* has been used in the indigenous medicinal system as anthelmintic, antibacterial, antifungal, antiviral, anticancer agent [8]. Its edible pods are known to be rich sources of nutrients particularly vitamin C, calcium and iron. The pods have been known to be effective in prevention of protein-calorie malnutrition and iron-calcium deficiency in blood. The seeds of *Prosopis* species have also been recommended as a potential candidate for development into an economic food for humans and animals [9].

Limited studies exist on the edible pods of *P. cineraria*, such studies being most crucial if it is to be targeted for development into a functional food for future. Recently, **Ram et al.** [10] reported that crude ethanolic extracts of the pods (obtained from a local herbal shop) led to elevation in SOD (superoxide dismutase) and catalase activities, as well as reduction in lipid peroxidation in rabbits. They attributed these effects to the bioactives viz. polyphenols, flavonoids, saponins, etc. Recently, our research group used a sequence of chromatography steps and obtained a flavonoid-enriched fraction (FEF) with antioxidant potential

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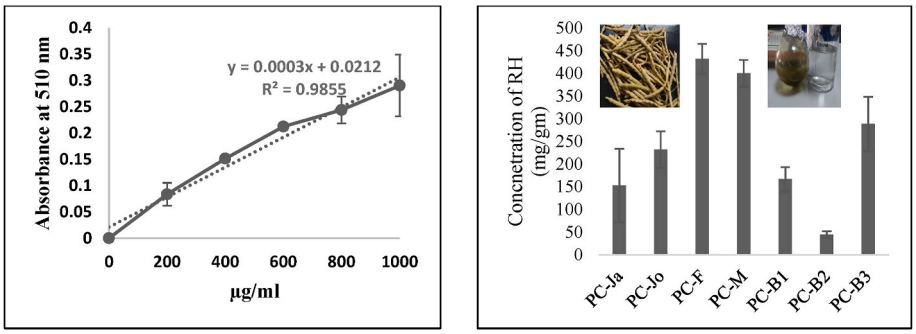


Fig. 1. Analysis of Total Flavonoid Content in *P. cineraria* pods from diverse regions of Rajasthan desert. A. Standard curve plotted using Rutin Hydrate for total flavonoid content analysis. y = 0.0003x+0.0212, R^2 = 0.9855. **B.** A comparison of total flavonoid content (mg RH/g methanolic extract) in different samples of *P. cineraria* collected from selected regions in Rajasthan; data represents mean ± SD, n = 3. Inset shows the pods of *P. cineraria* and the methanolic extract.

from the pods [7]. The current work is aimed at elucidating the region-wise phytochemical diversity in *P. cineraria* pods and purifying and characterizing their flavonoids.

2. Materials and methods

2.1. Collection of plant material

The pods of *Prosopis cineraria* were collected from different regions of Rajasthan (India) belonging to different zones viz. Bikaner and Jodhpur (arid); and, Pilani and Jaipur (semi-arid). The samples from Bikaner region (28.0229° N, 73.3119° E) were abbreviated as PC-B1 (7 days old, young pods), PC-B2 (15 days old, mature pods) and PC-B3 (25 days old, senescent pods); from Jodhpur region (26.2389° N, 73.0243° E) as PC-Jo; from Jaipur region (26.9124° N, 75.7873° E) as PC-Ja; from Pilani region (28.3802° N, 75.6092° E) as PC-M (market procured) and PC-F (collected from field). The pods were shade-dried and were ground to a fine powder in a mixer-grinder. The powder was then stored in airtight container at room temperature until further use.

2.2. Reagents and chemicals

All the organic solvents and acids used for the experiments were of analytical or HPLC grade and were procured from SRL Laboratories and HiMedia. The standards of the flavonoids/isoflavonoids were obtained from Sigma-Aldrich Chemicals Company. The Thin Layer Chromatography (TLC) plates (precoated with aluminum-backed silica gel 60 F_{254}) were procured from Merck (Darmstadt, Germany).

2.3. Extraction and measurement of total flavonoid content in P. cineraria pods

The powdered plant material was defatted using hexane, extracted with 80% methanol thrice and concentrated using rotary evaporator (Aditya Scientific, India). Total flavonoid content was measured by the $AlCl_3$ colorimetric assay [11]. An aliquot (1 mL) of extract or standard solutions of rutin hydrate (200–1000 µg/mL) was added in 10 mL volumetric flask containing 4 mL of distilled water. To the flask was added 0.30 mL 5% $NaNO_2$ followed by addition of 0.3 mL 10% $AlCl_3$ and 2 mL 1 M NaOH after 5 min; finally, the volume was made up to 10 mL with water. The absorbance of the solution was spectrophotometrically measured at 510 nm. Total flavonoid content (TFC) was expressed as mg Rutin Hydrate equivalents (RH).

2.4. Determination of UV–Visible spectra for the flavonoid extracts

All the extracts were dissolved in absolute methanol at a concentration of 1 mg/mL, and absorption spectra were recorded for a wavelength range of 200–800 nm with an interval of 2 nm. Methanol solvent was used as a sample blank. The instrument used was V-630 UV–Visible Spectrophotometer (Jasco Corporation, Japan). The classic book by Harborne on phytochemical methods [12] was also referred while interpreting the results.

2.5. Purification of flavonoids and isoflavonoids from the pods of P. cineraria by column chromatography

Flavonoid-enriched fractions (FEF) were obtained by modification of the previously reported process [7]. Briefly, the defatted methanolic extract was subjected to Amberlite XAD7HP chromatography. This protocol was further modified by including an additional purification using Sephadex LH-20 chromatography. The matrix (Sigma Aldrich) was packed into a glass column of 50 cm x 1.8 cm (H X D), equilibrated using 100% methanol. 5 gm of the FEF was dissolved in methanol and loaded. The column was first eluted using 50% methanol, and 20 fractions of 5 mL each were collected. The next elution was performed using 100% methanol. All the fractions were subjected to TLC analysis.

The extracts were dissolved in 100% methanol (1 mg/mL). For developing the TLC plates, a cylindrical glass chamber (10 cm x 11 cm; D x H) was used. 5–10 µg of the methanolic extract was spotted on the TLC plate. The solvent system used was Toluene: Acetic Acid:Acetone:Formic Acid::20:4:2:1. The TLC plates were visualized under UV light at 254 nm and 366 nm. The TLC atlas by Wagner and Bladt [13] was referred while interpreting the results. Images of the developed TLC plates were captured either using a simple camera or TLC Visualizer (CAMAG, Switzerland). The fractions showing the maximum number of spots/-bands were pooled and used for further characterization using HPLC and LC-MS.

2.6. HPLC and LC-MS analysis of the purified flavonoids/isoflavonoids

The methanolic fractions of the extracts were dried thoroughly and dissolved in HPLC grade methanol. The solution was filtered using 0.45 µm Puradisc filters and then subjected to HPLC analysis using LC-2010 CHT (Shimadzu Corporation, Tokyo, Japan) with a solvent delivery system of two intelligent pumps, an intelligent auto injector, and UV-VIS detector. Data collection and integration was carried out using LabSolutions software. Prior to this, the standardization of the flavonoid separation was done by modifying a previously reported method [14]. Briefly, the chromatographic separation was achieved on a C18 column (SpherisorbR, 250 mm H x 4.6 mm i.d., particle size 5 µm, Waters). The optimized mobile phase consisted of aqueous phase A (Milli Q water, pH 3.0), and organic solvent B (Acetonitrile). The HPLC system was equilibrated for a minimum of 1 h by passing the mobile phase through the system at a flow rate of 1 mL/min. For the separation run, low pressure gradient flow mode was followed (at 25 ± 2 °C). Initial concentration of 10% aqueous phase A and 90% acetonitrile were taken. In 33 min, the proportion of solvent B reached 35% and then brought back to 10% in

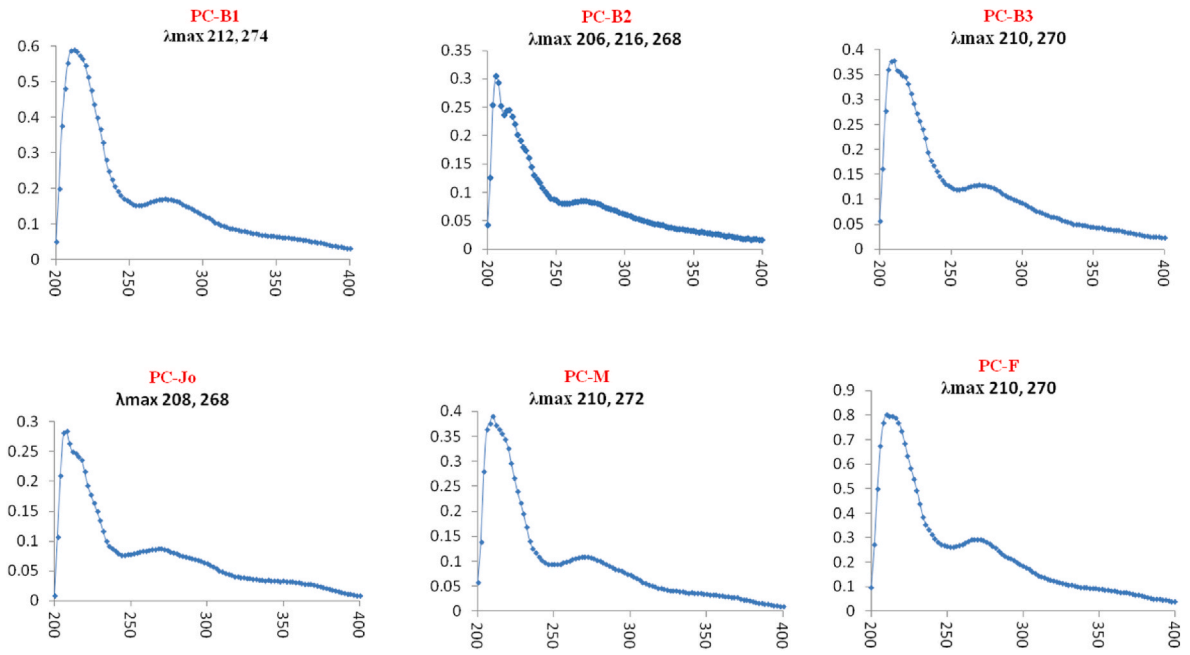


Fig. 2. The UV spectrum of the methanolic extracts of the samples of *P. cineraria* pods collected from selected regions of Rajasthan. PC-B1; PC-B2; PC-B3; PC-Jo; PC-M; PC-F.

the 40th minute. This concentration was maintained for 5 min, and the run was finished.

For MS analysis, a gradient flow separation was performed using acetonitrile and Milli Q water (pH = 3) for a total run time of 50 min. The flow rate for the run was 0.7 mL/min. Absorbance was recorded at 254 nm and 365 nm. The eluted peaks were then subjected to MS analysis using Micromass Q-TOF Micro (Waters, USA) via the electrospray ionisation (ESI) technique (positive ion mode) in methanol. The products formed were analyzed using the Agilent database library MassHunter associated with the instrument. The fragments obtained on the mass spectrum were again analyzed using RIKEN-RESPECT software [15].

3. Results and discussion

3.1. Total flavonoid content in pods/seeds of the P. cineraria from different regions

In this study, the total flavonoid content analysis was carried out in the pods of *P. cineraria* growing in different zones (arid and semi-arid) of Rajasthan, India. Fig. 1A represents the standard curve plotted using Rutin hydrate. The R^2 was 0.9855 for the given graph. The flavonoid content in the test samples was expressed as mg RH equivalent/g dry weight of the extract.

Fig. 1B shows the Total Flavonoid Content (TFC) in pods of *Prosopis cineraria* collected from selected regions of Rajasthan. It was observed that the pods from semi-arid zone (PC-F) had the highest flavonoid content of 432 mg RH/g, while those purchased from Pilani market (semi-arid zone) had 400±29.56 mg RH/gm of extract. In contrast, the sample from arid zone (PC-B2; mature pods) showed the lowest flavonoid content as 44.44 ±7.6 mg RH/g of extract. Interestingly, young pods (Bikaner; arid zone) had 166.66 ± 26.7 mg RH/gm whereas senescent pods showed 288.88± 59.8 mg RH/gm. Pods collected from Jaipur (semi-arid zone) and Jodhpur (arid zone) had 152.88 ±80.95 and 232 ± 40.56 mg RH/gm, respectively. These differences could point at the variable stresses that the plants encounter due to zone-specific climatic variability. For instance, on an average, the semi-arid zone of Rajasthan receives more rainfall (>350 mm annually) compared to the arid zone (<350 mm annually) [16]. However, it is possible that the plants of the arid zone might accumulate other classes of antioxidants in greater amounts viz. non-flavonoid phenolics and carotenoids, which is a subject of further study.

3.2. UV spectral analysis of flavonoid extracts from P. cineraria pods

UV–visible spectral analysis was carried out to indicate the presence of flavonoids in the methanolic extracts obtained from pods of *P. cineraria* from different regions. The typical UV–Vis spectra of flavonoids include two absorbance bands. Flavonoids are composed of two aromatic rings A and B, and a heterocyclic ring C – formed by different biosynthetic pathways. The A ring represents the benzoyl group while the B ring represents the cinnamoyl group. Additionally, the B ring is derived from phenylalanine via the shikimate pathway. The absorbance appearing as band I is shown by ring B, while band II is associated to the ring A. Band II lies in the 210–285 nm range whereas Band I lies in the range of 300–400 nm [17]. Fig. 2 (A-F) represents the UV-spectra of the extracts of *P. cineraria* pods measured from 200 to 400 nm. The insets show the maximum absorption observed in the various samples of *P. cineraria.* As seen from Fig. 2, the methanolic extracts (1 mg/ml) showed the peaks in the range of 206–212 nm and between 268 and 274 nm. Three samples collected from the arid zone (Bikaner region) were of different stages of growth, viz. young (7 days), mature (15 days) and senescent (25 days). The young pods (PC-B1) showed the maximum absorbance peak at 212 and 274 nm with an absorbance of 0.5907 and 0.1709 respectively. The mature pods (PC-B2) showed three peaks at 206, 216, and 268 nm with an absorbance of 0.3049, 0.2445, and 0.848 respectively. In mature pods, the O.D. was recorded at lower values as compared to that of the young pods; however, a 'unique' peak at 216 nm was also observed. In senescent pods (PC-B3), peaks were observed at 210 and 270 nm having absorbance of 0.3778 and 0.1289 respectively. This shows that the flavonoid profile in the pods changes as they mature.

In pods obtained from another arid zone region (Jodhpur), peaks were obtained at 208 nm and 268 nm with an absorbance of 0.2849 and 0.0871 respectively. PC-M showed peaks of 210 and 272 nm with an absorbance of 0.392 and 0.11 respectively, whereas methanolic extracts from PC-F showed maximum absorption at 210 and 268 nm with an absorbance of 0.7847 and 0.2868 respectively. From the values obtained for the absorbance, it is indicated that PC-F showed higher flavonoid

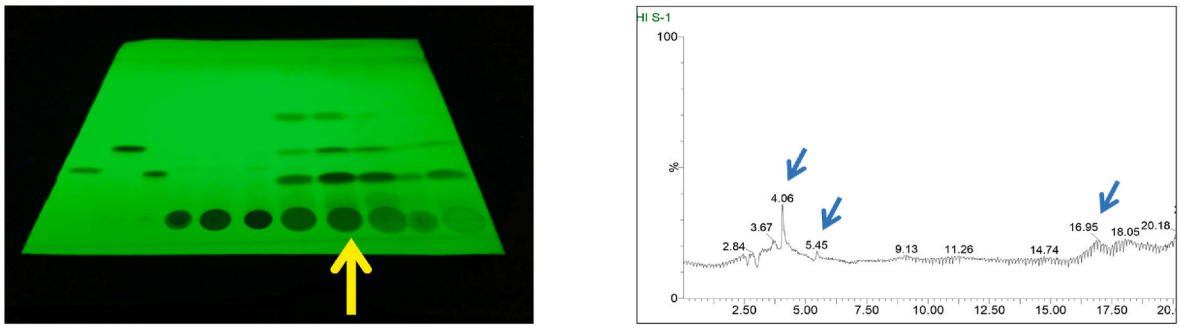
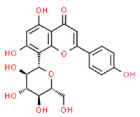
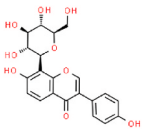
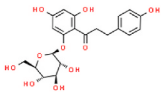
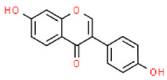


Fig. 3. A. TLC analysis of the flavonoid-rich fractions obtained through Sephadex LH-20 chromatography. The fraction showing maximum band intensity is highlighted. Silica Gel F254 plates were used and flavonoids were illuminated under 254 nm. B. LC-MS chromatogram of the highlighted fraction.

Table 1
MS^2 analysis of *Prosopis cineraria* flavonoids. The $[M+H]^+$ molecules of the flavonoid/isoflavonoids, their retention time, and structures. All the structures are derived from the ChemSpider database.

S. No.	Retention time (min)	Theoretical MW	[Typical MS^2 Ions] (*m/z*)	Formula	Tentative Identification	Structure	Category
1.	4.06	432.4	323.98, 297.01	$C_{21}H_{20}O_{10}$	Vitexin (Apigenin- 8 C-glycoside)		Flavone
2.		416.4	350.952, 297.01, 281.01	$C_{21}H_{20}O_9$	Puerarin (daidzein-8-C-glucoside		Isoflavonoid
3.	5.453	436.4	437.988	$C_{21}H_{24}O_{10}$	Phloridzin (phloretin-2′-O-glucoside)		Bicyclic flavonoid
4.	16.95	254.24	199.06, 151.07, 126.01	$C_{15}H_{10}O_4$	Daidzein		Isoflavonoid

content as compared to that of the PC-M, which was also reflected from Fig. 1B. Therefore, a simple UV-spectral analysis employed here indicates the presence of flavonoids as well as regionwise variation, and can be used for a quick characterization of such phytochemicals.

3.3. Chromatographic purification of flavonoids and isoflavonoids from pods of the semi-arid zone

For the purpose of flavonoid purification, the hexane-defatted methanolic extracts obtained from the pods of *P. cineraria* (from semi-arid zone) were first subjected to column chromatography using Amberlite XAD7HP matrix for flavonoid enrichment. Flavonoid enrichment was verified by TLC, the details of which have been reported earlier by us [7]. . Fig. 3A shows the results of TLC analysis of the different fractions collected after elution. As observed, the combination of column chromatography used by us were successful in giving selected bands corresponding to flavonoids. The intense bands at 254 nm indicated the presence of UV active compounds; flavonoid presence further verified by spraying with natural product reagent specific for flavonoids (results not shown) [13]. As the eluted fraction no. 8 for *P. cineraria* (P8) showed the presence of a maximum intensity of bands as observed under illumination at 254 nm, it was taken forward for HPLC and LC-MS analysis.

3.4. Characterization of purified flavonoids by LC-MS

The chemical composition of the purified flavonoid-enriched fractions obtained from Sephadex LH 20 was also analyzed using LC-MS. Fig. 3B represents the chromatogram obtained under low pressure gradient flow method, and it depicts the peaks obtained at 254 nm. The prominent peaks were then subjected to ESI-MS in full scan mode analyses in order to identify the protonated ions. A constant collision energy value was applied to each constituent in order to obtain mass spectra with various fragmentation patterns so as to retain as much structural information as it was possible. The different *m/z* values were analyzed using the RIKEN RESPECT database.

Table 1 shows the most frequent ions which characterize the fragmentation of the compounds present in the fraction eluted out during the Sephadex LH20 column chromatography. As a technique, ESI-MS provides sufficient information regarding the aglycone structure and the glycan sequence. The protonated flavonol glycosides generate the protonated ions upon fragmentation whose pattern depends on the type of the glycosyl bonds present in the parent flavonoids. There are reports indicating that relative abundance of the aglycone ion deriving from a homolytic and heterolytic cleavage, respectively, is correlated with the position of the glycosylation of the flavonoids/isoflavonoids conjugates [18].

Major peak 1 (with R_t of 4.06 min) showed fragments with *m/z* values of 323.98, 297.01 corresponding to flavonoid vitexin, and 350.95, 297.01, and 281.01 corresponding to C-glycosylated isoflavonoid puerarin. The second major peak (with R_t of 5.453 min) showed the presence of phloridzin, an O-glucoside of phloretin, which belongs to a family of bicyclic flavonoid, dihydrochalcone. m/z was observed as 437.988, which represents the $[M+2H]^+$ since the molecular weight of phloridzin is 436.13. The third major peak at 16.95 min elucidated the fragments of *m/z* corresponding to 199.06, 151.07, and

126.01 which are characteristic of daidzein, an isoflavonoid aglycone. These three ions are generated by a parental ion with m/z of 254.25.

Earlier, **Liu et al.** [19] had solvent-extracted a few bioactive components (including a flavonoid glycoside) from processed *P. cineraria* pods and elucidated their roles as potent antioxidant and anti-inflammatory agents. However, in phytochemical studies, it is a fact that the choice of the extraction and purification method determines the metabolites the researchers finally obtain, and no one method can be considered universal to elucidate the huge natural product diversity *in planta*. The flavonoids identified in the current study possess significant antioxidant and anti-inflammatory activities and a few of these have also been reported in other legumes like soybean [20] as well as some non-legumes including but not limited to Amaranthaceae [[21,22]], Rosacecae [23] and Poaceae [24]. However, these compounds have never been reported before from the raw, unprocessed pods of *P. cineraria*, the plant chosen in the current study.

4. Conclusions and future perspectives

To the best of our knowledge, the current study is the first attempt at region-wise flavonoid analysis in *Prosopis cineraria* pods. Previous reports on this desert legume were limited either to non-edible aerial parts or carried out from single geographical location. Considering the market for health-benefiting functional foods today, it would be highly lucrative to scientifically validate the nutraceutical potential of the *P. cineraria* pods (also good source of protein). Subsequently, for indigenous human populations, this effort might also offset an overdependence on soybean (often imported from abroad in Indian markets) which is the major source of isoflavonoids in the world today [[25,26]]. An important fact is that extensive research on flavonoid/isoflavonoid metabolic pathways in soybean has enabled the scientists to genetically engineer this legume for enhanced production of isoflavonoids for enriched health benefits [20]. MS analysis of the purified extracts from *P. cineraria* pods showed the presence of vitexin (apigenin- 8 C-glycoside), puerarin (daidzein-8-C-glucoside), phloridzin (phloretin-2′-O-glucoside), and daidzein, which belong to different subcategories of flavonoids such as flavones, bicyclic flavonoids, and isoflavonoids. With emerging extraction technologies that include extraction with various solvents, ultrasound-assisted extraction, supercritical fluid extraction, microwave-assisted extraction, enzyme-assisted extraction and several others, we now have advanced techniques to efficiently and sustainably extract the flavonoids from most natural sources [27]. The desert legume studied in the present work can also be subjected to various extraction protocols to maximize yield of the beneficial flavonoids.

Hu et al. [28] identified a novel flavonoid O-glycosyltransferase (NpUGT6) from the aquatic lotus plants which pointed to a newer source of the enzyme in addition to the already known terrestrial plants. This finding correlated with the abundant presence of the flavonoid quercetin and its glycosides in lotus. Investigating under-explored or unconventional sources of bioactives may be a promising strategy to augment the availability of bioactives as nutraceutical ingredients [29]. The Danish Diet, Cancer, and Health cohort study [30] pointed that a moderate habitual intake of flavonoids was inversely correlated with all-cause cardiovascular- and cancer-related mortality, especially in smokers and high alcohol consumers. Morin, a flavonoid found in mulberry figs and oriental medicinal herbs, is known for its antioxidant properties, and possible protection in Alzheimer's Disease and cancers, was studied for its interaction with Glycogen synthase kinase 3beta (GSK3beta) through crystal structure analysis [31]. It was suggested that this flavonoid could be considered a functional food for chronic diseases such as Alzheimer's disease. Revisiting the adage of '*food as medicine*', unraveling the medicinal potentials of lesser-researched plants will open a treasure trove of functional foods!

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Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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