Proximate Based Comparative Assessment of Five Medicinal Plants to Meet the Challenges of Malnutrition

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Authors’ contributions

This work was carried out in collaboration between all authors. Authors JH, NR, and LA designed the study, author ALK performed the statistical analysis and wrote the first draft, authors HH, TSR and ZKS wrote the protocol. All authors read and approved the final manuscript.

ABSTRACT

Aims: The present study aimed to assess the nutritional significance of some of the economically important medicinal plants species collected from Pakistan.

Study Design: The study was designed in randomized block design and each analysis was performed with three replicates.

Place and Duration of Study: Kohat University of Science and Technology, Kohat and duration of the study was ten months.

Methodology: Present study was conducted to determine the nutritional importance of medicinal plants viz. Achyranthes aspera, Xanthium strumarium, Albizzia lebbeck, Amaranthus cruentus and Calotropis procera. Proximate compositions of these plant species (moisture, ash, fats, fiber, alcohol soluble extractive (ASE), proteins,

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carbohydrates and energy value) were carried out using standard methods of food analysis.

Results: The mean moisture content ranged from 0.334% for *X. strumarium* to 8.18% for *A. cruentus*. *X. strumarium* was found highest in fats, fibers and ASE. Highest percentage of moisture (8.2%) and ash (15.9%) was found in *A. cruentus*. *C. procera* was identified as a good source of carbohydrates (67.3%) and energy value (301.9 kcal/100g). The ICP-OES analysis of the medicinal plants showed that *A. lebbeck* had significant concentrations of calcium while *A. cruentus* had highest concentration of iron, potassium, magnesium, manganese and phosphorous as compared to other medicinal plants. The heavy metals contents were either absent or negligible.

Conclusion: The present finding suggests that the selected medicinal plants have a promising potential to not only cure ailments but to maintain a healthy life.

Keywords: Medicinal plants; proximate analysis; kjelflex; nutrients.

1. INTRODUCTION

In recent years, there has been a gradual revival of interest in the use of medicinal plants in developing countries because herbal medicines have been reported safe and without any adverse side effect especially when compared with synthetic drugs. Thousands of rural communities still depend mainly on folklore medicine to cure diseases in developing countries. Medicinal plants are cheap for most of the populations around the globe. As a result of proximity, reliability and age long practice, people still depend largely on traditional medicine for their health care [1]. Medicinal plants play significant role in providing primary health care services to rural people and are used by about 80% of the marginal communities around the world [1].

Besides that, every human needs a daily supply of different types of food materials to enable a healthy life. However, the productive value of food depends on the quantity eaten and the extent to which the food is consumed with the required energy, protein, minerals and vitamins [2]. The use of medicinal plants as food alternative traces back to ancient human civilization [3]. Possessing diverse climate conditions, Pakistan occupies a unique position among developing countries having considerable potential within the variety of medicinal plants [4]. Among the 6,000 flowering plants reportedly present in Pakistan, approximately 600 are considered to be medicinally important [5,6].

*Achyranthes aspera* (Amaranthaceae) is an erect herb, used in Ayurveda medicine as an abortifacient [7]. The roots of *A. aspera* are used for contraceptive purposes, inflammatory disorders, malarial fever, hypertension, leprosy, dyspepsia, renal and vesical calculi, and general debility [8,9]. The hypoglycemic effect of *A. aspera* has been reported in diabetic rabbits [10], and in rats [11]. *A. aspera* seeds stimulated immunity and increased resistance to *Aeromonas hydrophila* infection in fish [8]. Additionally, its decoctions are active to induce abortion and abdominal pain. “*Xanthium strumarium*” (Compositae), is a herb, mainly distributed in China and Europe. In traditional Chinese medicine, *X. strumarium* is used for sinusitis, headache, urticaria, emphysema, and arthritis [12]. *Xanthium* is also known for its ability to clear nasal and sinus congestion [12]. The whole plant is known to possess diaphoretic, sedative and diuretic properties [9].

*Albizia lebbeck* L (Mimosaceae) belongs to the family Fabaceae and is a widely available plant in the tropics and subtropics regions with economic importance for industrial and
medicinal uses. Medicinally, A. Lebbek leaves carry anticonvulsant activity [13]. Saponins from the pods and roots have spermicidal activity and the bark can be used for soap when dried [14]. “Calotropis procera” (Asclepiadaceae) is an indigenous Mexican tree that belongs to Anacardiaceae. It is approximately 6 m tall, with twisted limbs while used in traditional Mexican medicine (known locally as chupandia or copaljocote). The bark is employed to treat ailments such as diarrhea, dysentery and cough [15,16]. “Amaranthus cruentus” (Amaranthaceae) is a popularly grown leaf vegetable in tropical regions of the world including Africa, India, Bangladesh, Sri Lanka and the Caribbean. It is also grown as leaf vegetable through South-East Asia and Latin America. The economic and nutritional advantage of the A. cruentus as a leaf vegetable is accentuated by its agronomic superiority over many plant protein sources [17].

The selected medicinal plants are used by the local communities either in fresh or extract form against various illnesses [18]. Evaluating their nutritional significance will help in screening the potential edible medicinal plant. In the present study, it was aimed to determine the proximate and essential nutrients compositions of five medicinal plants such as A. aspera, X. strumarium, A. lebbeck, A. cruentus and C. procera (Table 1).

2. MATERIALS AND METHODS

2.1 Collection of the Plant Samples

The selected medicinal plants samples (20 samples for each plant) were collected from different areas of Islamabad, Pakistan, and identified by a plant taxonomist (Table 1). The voucher specimens were placed at the herbarium of Quaid-i-Azam University, Islamabad, Pakistan. The plant leaves are collected for all the analysis and quantification.

<table>
<thead>
<tr>
<th>Specie name</th>
<th>Family</th>
<th>Voucher number</th>
<th>Collection area</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Achyranthes aspera</td>
<td>Amaranthaceae</td>
<td>126874</td>
<td>Islamabad</td>
<td>33°43'N 73°04'E</td>
</tr>
<tr>
<td>Xanthium strumarium</td>
<td>Asteraceae</td>
<td>126873</td>
<td>Rawalpindi</td>
<td>33°36'0&quot;N 73°02'0&quot;E</td>
</tr>
<tr>
<td>Albizia lebbeck</td>
<td>Fabaceae</td>
<td>126872</td>
<td>Hattar</td>
<td>33°51'1&quot;N 72°51'8&quot;E</td>
</tr>
<tr>
<td>Amaranthus cruentus</td>
<td>Amaranthaceae</td>
<td>126869</td>
<td>Kohat</td>
<td>33.35°N 71.26°E</td>
</tr>
<tr>
<td>Calotropis procera</td>
<td>Apocynaceae</td>
<td>126868</td>
<td>Kohat</td>
<td>33.35°N 71.26°E</td>
</tr>
</tbody>
</table>

2.2 Preparation of the Samples

Plant species evaluated in this study were washed with distilled water and finely ground into powder with the help of grinder (IKA, MF 10) using standard sieve of 1 mm size. Dried plant materials were wrapped in black polyethylene bags and were placed in the laboratory at room temperature. The woody parts and dirt were removed from these plant species before analysis.

2.3 Methods Used for Proximate Analysis

The dried samples analyzed for crude ash, crude proteins, moisture, and crude fats according to the standard procedures [19]. Nitrogen content was determined by Kjeldahl method and crude protein content was calculated by multiplying the nitrogen value with
constant 6.25 [20,19]. The moisture content was determined by keeping the samples in an oven at 105°C for 6 hours. The ash content was estimated [21] by keeping 2 g sample in muffle furnace at 550°C for 3 hours and the crude lipid content was determined by extracting the samples using petroleum ether (boiling point 40-60°C) in a Soxhlet for 6 hours. The calorific value was calculated by multiplying the value of crude proteins, crude fats and carbohydrates by a factor of 4, 9, and 4 [21]. The carbohydrate value was obtained by subtracting the sum of ash, fats, proteins and fiber (in percentage) from 100 [22].

2.4 Method Used for Nutritional Evaluation

The dried homogenized sample (0.5 g) was taken in Kjeldahl tube (250 mL) and digested with a mixture of sulfuric acid and nitric acid (2:1) at 370°C to a colorless liquid. The resultant liquid was diluted with distilled water up to 100 mL and filtered. The elements were analyzed using Inductively Coupled Plasma Emission Spectrometer (ICP-OES DV 7300, Perkin Elmer, USA) equipped with Perkin Auto-Sampler. The following conditions were optimized: Plasma Flow Rate (15 L/min), Nebulizer Flow Rate (0.8 L/min), RF Power (1500 Watts), Auxilliary Flow Rate (0.2 L/min), Sample Flow Rate (1.25-2.5 L/min), Torch position (-3) for aqueous samples and 15 Sec equilibration.

2.5 Statistical Analysis

All data obtained from experiments were analyzed by Duncan Multiple Range Test (DMRT) using Statistic Analysis System (SAS version, 9.1). Standard error and average values were calculated using Microsoft Excel 2003. Statistical significance was evaluated at $P \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1 Proximate Analysis

Proximate analyses were carried out of the selected medicinal plants to know the nutritional significance of these frequently consumed species in the traditional medicines. These analyses revealed some interesting findings and the results obtained from are presented in Table 2.

Ash value turned out to be high in *A. cruentus* (15.904%), and low in *A. lebbeck* (6.003%) (Table 2). Ash content determined in *A. cruentus* was significantly higher and in conformity with previous findings. The ash content also revealed comparable results with the previously analyzed medicinal plants, *Ammonium sulbulatum* (6.97%) and *Rhazya stricta* (6.21%) reported by [25,20]. The ash content in *A. aspera*, collected from India was 5.3% which are significantly lower than the present findings (12.4%) in the same plant species [23]. Dougall et al. [24] showed that the ash content in *A. aspera*, growing in the African region ranged between 8 to 14%. This is in conformity to our results suggesting that different environmental conditions can drastically impact on the essential plant constituents. The ash content of same amount was found in some green leaves of *Veronia colorata* (15.9%) and *Moringa oleifera* (15.1%) [25]. The results suggested that *A. cruentus* could be good sources of mineral elements.

Crude fibers of these samples varied from 16.480 to 61.811% being lowest in *C. procer* and highest in *X. strumarium* (Table 2). As a nutritive value of food, fibers in the diet are necessary for digestion and for effective elimination of wastes, and can lower the serum
cholesterol, the risk of coronary heart disease, hypertension, constipation, diabetes, colon and breast cancer [26]. Fasuyi and Akindahunsi [17] reported that the crude fiber in A. cruentus, growing in Nigeria, was 8.8% which is significantly lower than what we observed in the A. cruentus (~35%) growing in the study area. Previously, Dougall et al [24] showed the crude fiber in A. aspera was 22.6% which is significantly lower than the same plant. However, the fiber contents of A. aspera was 22.6% is comparable to that growing in India. Intake of such medicinal plants in traditional recipes showed evidence that dietary fiber is associated with enhanced insulin sensitivity and therefore may have a role in the prevention and control of Type 2 diabetes [28]. Thus these medicinal plants can be considered as a valuable source of dietary fiber in human nutrition (Table 2).

Moisture content depends on the environmental conditions such as humidity, temperature, harvest time, and climate as well as storage conditions. Thus it is important for food scientists to be able to reliably measure moisture contents. The results indicated that A. cruentus contained the highest moisture content (8.18%) among the five selected medicinal plants (Table 2). Hussain et al. [25] suggested a strong correlation between moisture contents and fiber, which could be of interest to human health as the fibrous are easily digested and disintegrated. The difference in moisture content between different plants is directly depended to the plant physiological setup and climatic changes.

The results of the fat analysis indicated that X. strumarium (5.511%) and A. lebbeck (3.745%) have higher concentration of fats as compared to the other species (Table 2). The crude fat contents are low compared to reported values (8.3-27.0%) in some vegetables consumed in West Africa [29,30]. The crude fat analysis showed that the selected species are deficient in fats and this makes them good for health.

The protein content of samples was calculated on the basis of the available nitrogen using Kjeldahl method. The protein content determined for the samples was 1.052% for A. lebbeck, 0.88% for A. aspera, 0.82% for A. cruentus, 0.64% for X. strumarium, and 0.4761% for C. procera (Table 2). The crude protein content was significantly higher in A. aspera growing in Kenya as compared to Oman [24]. Similarly, the protein contents were significantly higher in A. cruentus growing in the Nigerian climate [17]. Contrarily, the protein contents were reported higher in C. procera growing Kurram valley as compared to our findings [21]. The crude protein content was different in different plants growing at different locations. Like the plants growing in Nigeria and Swaziland had significantly different contents of crude protein content. Medicinal plants like Momordica foecide leaves and balsam apple contained 4.6 and 11.29% of crude proteins [31,32]. The same was observed for Amaranthus candatus (20.50%), Piper guineeses (29.78%) and Talinum triangulare (31.0%) [33,34]. The protein content of A. lebbeck is comparable to the peeled cucumber (0.8 g). Availability of such high contents of protein are helpful in maintaining proper growth and development in adults, children, pregnant and lactating mothers which require 34–56, 13–19 and 17–71g of protein daily respectively [35].
Table 2. Proximate analysis of selected medicinal plants

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Moisture (%)</th>
<th>Ash (%)</th>
<th>Fiber (%)</th>
<th>A.S.E (%)</th>
<th>Proteins (%)</th>
<th>Fats (%)</th>
<th>CHO (%)</th>
<th>E.V (Kcal/100g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Achyranthes aspera</td>
<td>1.15 ± 0.03</td>
<td>12.40 ± 0.04</td>
<td>40.10 ± 0.20</td>
<td>8.86 ± 0.03</td>
<td>0.88 ± 0.03</td>
<td>1.08 ± 0.12</td>
<td>45.50 ± 0.20</td>
<td>195.30 ± 0.80</td>
</tr>
<tr>
<td>Xanthium strumarium</td>
<td>0.33 ± 0.01</td>
<td>12.60 ± 0.06</td>
<td>61.80 ± 0.04</td>
<td>16.64 ± 0.01</td>
<td>0.64 ± 0.01</td>
<td>5.51 ± 0.01</td>
<td>19.30 ± 0.09</td>
<td>129.60 ± 0.30</td>
</tr>
<tr>
<td>Albizia lebbeck</td>
<td>6.00 ± 0.01</td>
<td>6.21 ± 0.04</td>
<td>45.90 ± 0.18</td>
<td>7.32 ± 0.07</td>
<td>1.05 ± 0.05</td>
<td>3.70 ± 0.07</td>
<td>43.10 ± 0.19</td>
<td>209.90 ± 0.10</td>
</tr>
<tr>
<td>Amaranthus cruentus</td>
<td>8.18 ± 0.02</td>
<td>15.90 ± 0.03</td>
<td>35.50 ± 0.07</td>
<td>6.73 ± 0.02</td>
<td>0.82 ± 0.02</td>
<td>2.00 ± 0.045</td>
<td>45.7 ± 0.19</td>
<td>204.40 ± 0.30</td>
</tr>
<tr>
<td>Calotropis procera</td>
<td>6.17 ± 0.04</td>
<td>12.30 ± 0.06</td>
<td>16.40 ± 0.06</td>
<td>9.97 ± 0.03</td>
<td>0.47 ± 0.08</td>
<td>3.40 ± 0.02</td>
<td>67.2 ± 0.05</td>
<td>301.90 ± 0.30</td>
</tr>
</tbody>
</table>

CHO = Carbohydrates; E.V = Energy value; ASE = Alcohol soluble extractives; ± shows the standard deviation of means (n=3)

Table 3. Essential nutrients composition in the medicinal plants (ng/g dry weight)

<table>
<thead>
<tr>
<th>Medicinal Plants</th>
<th>Ca</th>
<th>B</th>
<th>Fe</th>
<th>K</th>
<th>Mg</th>
<th>Mn</th>
<th>P</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Achyranthes aspera</td>
<td>84.9±0.2c</td>
<td>4.3±0.1a</td>
<td>0.89±0.6c</td>
<td>172.3±10.0c</td>
<td>25.0±0.1b</td>
<td>2.29±0.3b</td>
<td>8.4±0.13c</td>
<td>0.15±0.7b</td>
</tr>
<tr>
<td>Xanthium Strumarium</td>
<td>99.5±0.2b</td>
<td>3.8±0.9b</td>
<td>0.76±0.6b</td>
<td>194.9±0.3b</td>
<td>21.8±1.0c</td>
<td>0.2±0.1c</td>
<td>8.8±0.22c</td>
<td>0.2±0.23a</td>
</tr>
<tr>
<td>Albizia lebbeck</td>
<td>104.5±0.1a</td>
<td>4.5±0.4a</td>
<td>0.9±0.2b</td>
<td>74.07±0.2d</td>
<td>15.5±0.5d</td>
<td>0.1±0.16c</td>
<td>7.6±0.36c</td>
<td>0.06±0.19c</td>
</tr>
<tr>
<td>Amaranthus cruentus</td>
<td>44.7±0.2d</td>
<td>0.43±0.2c</td>
<td>1.07±0.1a</td>
<td>305.6±0.1a</td>
<td>41.8±1.2a</td>
<td>3.15±0.1a</td>
<td>20.8±0.9a</td>
<td>0.27±0.06a</td>
</tr>
<tr>
<td>Calotropis procera</td>
<td>70.7±0.9c</td>
<td>3.34±0.8b</td>
<td>0.96±0.5a</td>
<td>86.9±0.4d</td>
<td>25.2±0.5b</td>
<td>0.53±0.3b</td>
<td>10.0±1.1b</td>
<td>0.19±0.09b</td>
</tr>
</tbody>
</table>

Calcium – Ca; boron – B; iron – Fe; potassium – K; magnesium – Mg; manganese – Mn; phosphorus – P; zinc – Zn. The letter (a,b,c,d) in each column shows significantly different concentrations as evaluated by DMRT (P >0.05).

Table 4. Heavy metal composition in the medicinal plants (ng/g dry weight)

<table>
<thead>
<tr>
<th>Medicinal Plants</th>
<th>Ag</th>
<th>Al</th>
<th>Ba</th>
<th>Cu</th>
<th>Si</th>
<th>Sn</th>
<th>Sr</th>
<th>Ti</th>
<th>W</th>
</tr>
</thead>
<tbody>
<tr>
<td>Achyranthes aspera</td>
<td>0.22±0.4</td>
<td>1.2±0.4a</td>
<td>0.03±0.4</td>
<td>0.08±0.2</td>
<td>3.35±0.9b</td>
<td>0.11±0.9</td>
<td>0.3±0.7</td>
<td>0.02±0.1</td>
<td>0.16±0.3</td>
</tr>
<tr>
<td>Xanthium Strumarium</td>
<td>0.22±0.4</td>
<td>0.86±0.5d</td>
<td>0.04±0.4</td>
<td>0.06±0.6</td>
<td>4.09±0.9a</td>
<td>-</td>
<td>0.36±0.1</td>
<td>0.02±0.1</td>
<td>0.15±0.9</td>
</tr>
<tr>
<td>Albizia lebbeck</td>
<td>0.22±0.5</td>
<td>1.3±0.2a</td>
<td>0.03±0.8</td>
<td>0.72±0.2</td>
<td>3.35±0.1b</td>
<td>-</td>
<td>0.57±0.8</td>
<td>0.004±0.1</td>
<td>0.16±0.1</td>
</tr>
<tr>
<td>Amaranthus cruentus</td>
<td>0.22±0.4</td>
<td>1.06±0.1c</td>
<td>0.03±0.4</td>
<td>0.11±0.1</td>
<td>3.02±0.5c</td>
<td>0.15±0.2</td>
<td>0.38±0.6</td>
<td>0.007±0.1</td>
<td>0.16±0.1</td>
</tr>
<tr>
<td>Calotropis procera</td>
<td>0.22±0.4</td>
<td>1.6±0.6b</td>
<td>0.04±0.3</td>
<td>0.04±0.4</td>
<td>4.64±0.3a</td>
<td>-</td>
<td>0.35±0.8</td>
<td>0.02±0.3</td>
<td>0.16±0.1</td>
</tr>
</tbody>
</table>

Silver – Ag; aluminum – Al; barium – Ba; copper – Cu; silicon – Si; tin – Sn; strontium – Sr; titanium – Ti; tungsten – W. The letter (a,b,c,d) in each column shows significantly different concentrations as evaluated by DMRT (P >0.05). – shows not detected.
Carbohydrates are one of the most important components in many foods, and the digestible carbohydrates are considered as an important source of energy. If we look at the overall percentage of the carbohydrate composition, it was found highest in C. procera (67.294%) and lowest in X. strumarium (19.364%) (Table 2). The C. procera growing in another part of Pakistan i.e. Kurram valley, the carbohydrates contents were lower (62.1%) than the findings in the present study [21]. Although these values were found to be higher than that of the reported values for the leaves of Senna obtusifolia (20%) [36], and Amaranthus incurveus (23.7%) [37], however, they are less than the available carbohydrate content compared to Corchorus tridens (75%) [37]. The recommended carbohydrate dietary allowance values for children, adults, pregnant and lactating mothers are 130, 130, 175 and 210 g respectively [35].

According to the results of the energy values which were, based on the carbohydrates, fats, and protein content, it was revealed that C. procera had significantly highest energy values (301.998 kcal/100g), while the X. strumarium was found to contain the lowest energy value (129.639 kcal/100g). X. strumarium was found to be significantly higher (16.644 %) in extractive potency than the remaining samples ($P \leq 0.05$). In comparative assessment, the energy values of A. aspera (growing in India) were significantly lower than the present one. Significant difference was ($P \leq 0.05$) observed in the alcohol soluble extractives of plant samples. A significant correlation exist between the different medicinal plant species and their proximate properties, suggesting that some species has highly significant percentages of proximate as compared to other as evaluated by SAS DMRT ($P \leq 0.05$). Similarly, some of the results of the proximate parameters are in correlation with that of known vegetable species like corn-cocoyam salad and pealed cucumber. Thus, it suggesting that these can used as alternative for food as well as medicinal, however, further detailed trails are recommended.

### 3.2 Nutritional Evaluation

Nutrients rich foods are vital for proper growth both in adults and children. Keeping in view the importance of essential and trace nutrients, we quantified some of the essential nutrients (calcium (Ca); boron (B); iron (Fe); potassium (K); magnesium (Mg); manganese (Mn); phosphorus (P); zinc (Zn) in the selected medicinal plant species. The ICP-OES analysis showed that Albizia lebbeck had significantly higher concentrations of Ca and B. The Fe, K, Mg, Mn, P and Zn concentrations were significantly higher in Amaranthus cruentus (Table 3). In other medicinal plants, the concentrations of these essential nutrients were significantly lower. Previously, A. aspera was found to have 18.16 and 394 mg of Fe and Ca which is significantly higher than the present study, however, still this is comparable to some edible plants like broccoli.

Additionally, the non-essential heavy metals were also analyzed to assess whether these medicinal plant doesn't harm the human health whilst being used as medicine or food. The ICP-OES analysis were performed to quantify different metals (silver (Ag); aluminum (Al); barium (Ba); copper (Cu); Beryllium (Be); bismuth (Bi); arsenic (As); gold (Au); cadmium (Cd); cobalt (Co); chromium (Cr); lithium (Li); nickel (Ni); lead (Pb); plutonium (Pt); Antimony (Sb); silicon (Si); tin (Sn); strontium (Sr); titanium (Ti); selenium (Se); Thallium (Tl); vanadium (V); tungsten (W)). Some of the results are compiled in Table 4. The analysis suggests that Be, Bi, As, Au, Cd, Co, Cr, Li, Ni, Pb, Pt, Sb, Se, Ti, and V were not detected in the medicinal plant samples. However, Ag, Al, Ba, Cu, Si, Sn, Sr, Ti, and W were found in marginal concentrations in the medicinal plant samples. Most of the metals were either not
present or were in negligible concentration as suggested by the Food and Agriculture Organization.

4. CONCLUSION

All medicinal plants contained high amount of ash, fibers and alcohol soluble extractives. Among these, *X. strumarium* and *A. cruentus* were found to contain adequate and balance nutritious diet. From this trial we found that these medicinal plants can play important role for the maintenance of healthy life and normal body functioning, in addition further investigation is required for exploring the phytochemicals and mineral composition. Moreover, excess amount of intake can be toxic to human as shown by *Achrynthus aspera* (cardiac disorder), *Xanthium strumarium* (poisoning), *Calotropis procera* (mercury poison like effects), therefore, it is essential to further investigate their putative role in human health and nutrition.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support of the Higher Education Commission, and Pakistan Academy of Sciences, Government of Pakistan for the current study under the National Research Program for Universities (NRPU).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES


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