Qualitative determination of toxic pyrrolizidine alkaloids in *Trichodesma indicum*: A prevalent ethnomedicine of Northern Pakistan

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ABSTRACT

Aim/Background: *Trichodesma indicum* (L.) Lehm. is a prominent medicinal plant in Pakistan. Various indigenous Pakistani communities use this plant orally to treat various human ailments. The present study is carried out with the aim to investigate the leaves of *T. indicum* for the presence or absence of toxic pyrrolizidine alkaloids (PAs). If these alkaloids are found, then the plant should be used with more precaution.

Materials and Methods: For this, the dried leaf samples of *T. indicum* were collected from Northern Pakistan and brought to Beijing Normal University for further experimentation. Furthermore, to collect literature on the medicinal uses of *T. indicum*, we searched the scientific databases of PubMed, Google Scholar, and Scopus. In the laboratory, *T. indicum* was investigated for PAs by using high-performance liquid chromatography (HPLC)–ultraviolet.

Results: The literature survey shows that the species is used for medicinal purposes throughout Pakistan. The result of HPLC fingerprint analysis showed that leaves were PA positive and out of five PA standards, four were detected, namely, (1) supinine, (2) europine, (3) heliotrine, and (5) echimidine.

Conclusion: To the best of our knowledge, the present study reports, for the first time, the presence of unsaturated PAs in the leaves of *T. indicum*. Given this, we suggest that the plant should be used with more caution and should follow the rule defined by the German Federal Institute for Risk Assessment which requires that the exposure to unsaturated PAs from food should be as low as possible which is a daily intake of 0.007 μg PAs kg$^{-1}$ for human’s body weight.

Introduction

Plants are the basis of both herbal remedies and discovery of modern drugs. Currently, throughout the world, approximately 40,000–70,000 plant species are used as medicines [1]. Around the world, ethnomedicine is used to treat various human ailments and to improve basic health care. According to a recent report, in Chile (70%), Colombia (40%), and throughout the continent of Africa (80%), much of the population relies on herbal medicine [2]. According to recent reports, the estimated value of herbal markets reached about US $100 billion by 2015 [3] and US $107 billion by 2017 [4]. According to a report, approximately 20% of all plants found in the world are used for therapeutic purposes to treat various human ailments [5]. Often, people in developing countries rely more on the traditional medicine possibly due to limited access to modern health services [6]. Indigenous people around the world depend on plants for basic health care and economic value. These benefits are identified based on the experience, need, and observation of elder and native people [7]. Utilization of local medicinal flora by traditional healers or practitioners is often cheap and easily available to people with a low income. The ethnomedicinal uses of plants by the indigenous communities are very important and noteworthy. Ethnomedicine is not only the study of...
herbal medicine as used by local communities but also it provides a basis for drug discovery from new sources [8,9].

As the usage of herbal medicine gained popularity over the past decades, reports of suspected toxicity and adverse events were also described. A meta-analysis of 69 prospective and retrospective studies from different parts of the world involving around 419,000 patients found that nearly 6.7% of all hospitalizations resulted from adverse drug reactions [10]. PAs are also leading plant toxins associated with disease in humans [11]. PAs are widely distributed in plants. In the world, about 3% of all flowering plants contain PAs [12]. Until now, more than 660 PAs have been identified from over 6,000 plants, mainly in Boraginaceae, Asteraceae, and Fabaceae families [13]. In plants, the most naturally occurring unsaturated PAs are known to be hepatotoxic and tumorigenic in humans [14,15]. When human poisoning with PAs occurs, it causes a characteristic disease called hepatic sinusoidal obstruction syndrome (HSOS) which may lead to liver failure [16]. In the past few years, reports on lower levels of dietary exposure via common herbal products and foods such as black, herbal or green teas, PA-producing plant parts, or honey collected from PA-producing plants have caused concerns, especially regarding the potential of carcinogenicity and genotoxicity of unsaturated PAs. These PA-producing plant food products have elevated PA concentrations which pose safety risks [17–19].

Previously, it was well established that intoxication cases due to unsaturated PAs occurred throughout developed as well as underdeveloped countries. Nearly, 10,000 PA-poisoning cases have been documented in many countries, including Great Britain, Germany, Afghanistan, United States, Switzerland, India, China, South Africa, and Jamaica [20]. In 1968 in South Africa, out of 15 children affected, 10 died because of PAs from Crotalaria spp. [21]. In India in 1973, 486 people had the veno-occlusive disease (VOD) because of cereals contaminated with Crotalaria spp. [22]. In Afghanistan between 1970 and 1972, around 7,200 people were diagnosed with VOD because of wheat contaminated with Heliotropium popovii ssp. gillianum [23]. In 1993, 3,906 people suffered from ascites, hepatomegaly, abdominal pain, and alteration of consciousness because of H. lasiocarpum in Tajikistan [24].

In the world, unsaturated PAs are one of the most widespread natural toxins. However, PAs are protoxins as they require metabolic conversion in the liver to show toxic activity [25]. So, the use of Boraginaceae plants or any PA-containing products as a poultice for wounds or external use does not cause any risk [26]. In plants, unsaturated PAs mainly occur as their N-oxides and so these cannot be directly converted to the hydroxy-PAs, but whenever it is ingested orally, bioactivation takes place in the liver by cytochrome P450 monooxygenases and they are reduced to free bases [25,27]. In humans, domestic animals, and wildlife, PAs may cause acute fatal intoxications. Low-level and intermittent ingestion of unsaturated PAs is more difficult to associate with adverse health effects, especially when they become apparent over years after consumption [28]. These low-level exposures of food or herbal products to 1,2-unsaturated PAs can initiate chronic disease and may cause a wide range of cancers, congenital anomalies, cirrhosis, and pulmonary arterial hypertension [29].

**Trichodesma** is comprised of about 40–45 species [30,31] mainly found in Asia, Africa, and western and central Australia [32]. *Trichodesma indicum* (L.) Lehm. (Boraginaceae) is an annual herb with densely hairy branches and pale blue flowers [33]. The present study is focused on a literature survey of ethnomedicine *T. indicum* which is utilized for medicinal purposes in Pakistan. The aim of this study is to investigate the leaves of *T. indicum* for the presence or absence of toxic pyrrolizidine alkaloids (PAs). If these alkaloids are found, then the plant should be used with more precautions.

**Materials and Methods**

**Literature review of ethnomedicinal uses of T. indicum**

We conducted a systematic search of the literature regarding medicinal uses of *T. indicum* in Pakistan. For this, we searched the scientific databases of PubMed, Google Scholar, Web of Science, and Scopus for the search terms: “*Trichodesma indicum* medicinal uses in Pakistan,” “Boraginaceae ethnombotany in Pakistan,” and “Ethnombotany of Pakistan.” All pertinent articles up to April 2018 were reviewed (Table 1).

**Collecting sites of Trichodesma indicum—Northern Pakistan**

Northern Pakistan (Pan-Himalayan regions) lies between 71°E–78°E longitude and 32°N–37°N latitude and occupies a unique bio-geographic position. The Pakistani Pan-Himalayan regions include Azad Kashmir, Chitral, Swat, Dir, Hazara Division, and Gilgit-Baltistan (Fig. 1). The Himalayas are spread...
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Table 1. Searched databases with accompanying range of years and search terms.

<table>
<thead>
<tr>
<th>Database</th>
<th>Search term</th>
<th>Range of years</th>
<th>Total articles</th>
<th>Selected articles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Web of Science</td>
<td>“Ethnobotany of Pakistan”</td>
<td>2000–2018</td>
<td>195</td>
<td>09</td>
</tr>
<tr>
<td></td>
<td>“Boraginaceae and ethnobotany in Pakistan”</td>
<td>2000–2018</td>
<td>08</td>
<td>02</td>
</tr>
<tr>
<td></td>
<td>“<em>Trichodesma indicum</em> medicinal uses in Pakistan”</td>
<td>2000–2018</td>
<td>05</td>
<td>01</td>
</tr>
<tr>
<td>Google Scholar</td>
<td>“<em>Trichodesma indicum</em> medicinal uses in Pakistan”</td>
<td>2000–2018</td>
<td>245</td>
<td>33</td>
</tr>
<tr>
<td>Scopus</td>
<td>“Ethnobotany of Pakistan”</td>
<td>2008–2018</td>
<td>126</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>“Boraginaceae and ethnobotany in Pakistan”</td>
<td>2000–2018</td>
<td>06</td>
<td>01</td>
</tr>
<tr>
<td></td>
<td>“<em>Trichodesma indicum</em> medicinal uses in Pakistan”</td>
<td>2000–2018</td>
<td>02</td>
<td>00</td>
</tr>
</tbody>
</table>

Figure 1. *Trichodesma indicum* collecting sites, Northern Pakistan.
across three of Pakistan’s provinces. Northern Pakistan encompasses its surrounding valleys, the Nanga Parbat massif, Azad Jammu, and Kashmir [34].

**Plant collection and deposition in herbarium**

As part of the preparation of the revision of family Boraginaceae for the *Flora of Pan-Himalayas*, we carried out comprehensive field investigations in Northern Pakistan, from July 2016 to September 2017 to collect samples of Boraginaceae. In the field, whole plants were pressed in the newspaper for herbarium specimens and for phytochemical analysis, fresh plant material was collected in silica gel. *Trichodesma indicum* was collected and identified at Beijing Normal University. All specimens were deposited at the Beijing Normal University Herbarium with appropriate voucher numbers (Fig. 2).

**Experimental work**

**Authentic standards, chemicals, and reagents**

Five authentic standards of PAs, i.e., (1) supinine, (2) europine, (3) heliotrine, (4) lycopsamine, and (5) echimidine were obtained from ChemFaces (Wuhan, Hubei 430056, P.R. China) and had a purity >98% (see Fig. 3). All reagents were of high-performance liquid chromatography (HPLC) grade. Methanol and acetonitrile were purchased from DikmaPure (USA). Formic acid was the product of Aladdin Industrial Corporation (Shanghai, China). Throughout the experimental process, ultra-pure water from a Milli-Q water purification system was used (Millipore Corporation, Billerica, MA).

**Plant sample preparation for HPLC**

For the HPLC fingerprint analysis, the leaves of *T. indicum* were prepared according to the established methodology [35]. Briefly, 500 mg dried leaves of mature *T. indicum* were weighed and crushed into powder with the help of a FastPrep-24™ Instrument homogenizer (M.P Biomedical, Irvine, CA). The samples were sonicated for 35 minutes with 2.5 ml of methanol immediately followed by centrifugation for 15 minutes at 4,000 × g. The resulting supernatant was transferred to a new tube and the procedure was repeated for a total of three times.

![Figure 2. Trichodesma indicum](image-url)

*Figure 2. Trichodesma indicum* (a) Herbarium specimens; (b and c) field photo.
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Supernatants were pooled and the volume was adjusted to 10 ml with methanol. Samples were mixed thoroughly and 350 μl of sample were filtered with a 13 mm × 0.45 μm FitMax Syringe Filter membrane Nylon (Dikma, USA) before injection.

**Authentic standard preparation**

A stock solution of each PA (1.0 mg/ml) was prepared by weighing and subsequently dissolved in methanol [35,36]. Mixed stock solutions containing heliotrine, supinine, europine, echimidine, and lycopsamine were also prepared at a concentration of 1.0 mg/ml with methanol. Before subjection to HPLC analysis, the solutions of all standards were stored in a refrigerator at 4°C. The standard solutions (300 μl of each) were filtered by a FitMax Syringe Filter membrane Nylon, and an aliquot (10 μl) of each filtrate was subjected to HPLC analysis.

**HPLC–UV analysis**

Instrumentation for unsaturated PAs analysis from the leaves of *T. indicum* consisted of a Waters Alliance 2695 liquid chromatography (LC) System equipped with a 2487 Dual Wavelength ultraviolet (UV) Detector (Milford, MA). The HPLC system was comprised of the following modular components: a

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![Chemical structures of the authentic standards.](image-url)

Figure 3. Chemical structures of the authentic standards.
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built-in quaternary pump, auto-injector, four-channel degasser, and auto-sampler with 120 vials (2 ml). The chromatographic analysis was performed on a Zorbax SB-Aq (4.6 × 250 mm, 5 μm particles) column (Agilent, USA) for the separation of the PAs from the leaves of T. indicum.

The LC method for the qualitative analysis of unsaturated PAs was developed for the investigation of PAs in the leaves of T. indicum from Pan-Himalayan regions in Pakistan. We separated unsaturated PAs using the mobile phase which consisted of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The flow rate was set at 0.5 ml/minute with the following solvent gradient elution: 0–15 minutes: 13% A; 15–17 minutes: 50% A; 17–35 minutes: 100% A; 35–40 minutes: 100% A; 40–55 minutes: 13% A. Analysis runtime was 55 minutes with a 15-minute methanol wash in between each sample. The temperature of the column was maintained at room temperature, the wavelength detection was set at 280 nm, and a 20 μl volume of sample was injected each run.

Results

Quantitative review of medicinal uses of T. indicum

To find the medicinal uses of orally ingested T. indicum to treat various human ailments, we searched the literature found on Google Scholar, Web of Science, PubMed, and Scopus up to 2018. The databases were queried with the terms “Trichodesma indicum medicinal uses in Pakistan,” “Boraginaceae ethnobotany in Pakistan,” and “Ethnobotany of Pakistan” in the title, which resulted in 646 studies in the past 18 years (Table 1). Our aim was focused on orally administered T. indicum remedies to treat various human ailments in Pakistan. Therefore, published studies outside of our aim were omitted.

Figure 4. A flow chart of ethnomedicinal information of T. indicum derived from different phases of our literature review.
After reading the title, abstracts, keywords, or a full part of the published articles; we focused on articles pertaining to ethnomedicinal uses of *Trichodesma indicum* to treat various human ailments. This reduced the sampled articles to 35 publications. The literature search strategy design for the ethnomedicinal uses of *T. indicum* for our review is shown in Figure 4.

**Ethnopharmacological use of T. indicum**

*Trichodesma indicum* (L.) Lehm. locally known as Gaaozeban, Handusi booti, Kallri Booti or Handusi is an annual much-branched spreading and densely hairy herb, up to 10–45 cm long, its flowering and fruiting were from March to September. For the investigation of toxic PAs, the samples of *T. indicum* were collected from Muzaffarabad; Azad Kashmir, with an altitude of 763 m. According to the literature surveyed, this species is used for various human ailments. Table 2 presents the literature surveyed of the ethnomedicinal uses of *T. indicum* in Northern Pakistan.

### Separation and determination of PAs in *T. indicum* extracts

The structure of PAs varies in plants, but some chemical properties remain constant. We use these properties to try to achieve equal extraction efficiency of each PA. PAs are alkaloids with the characteristic basic nitrogen. This lends them to the classical methanol extraction method for alkaloids. We find

<table>
<thead>
<tr>
<th>Part used</th>
<th>Medicinal uses</th>
<th>Modes of preparation</th>
<th>Literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves</td>
<td>Flu and cough</td>
<td>Decoction</td>
<td>[37]</td>
</tr>
<tr>
<td></td>
<td>Cough and influenza</td>
<td>Decoction</td>
<td>[38]</td>
</tr>
<tr>
<td></td>
<td>Chest problem</td>
<td>Infusion</td>
<td>[39]</td>
</tr>
<tr>
<td></td>
<td>Dysentery and fever</td>
<td>As a cold drink</td>
<td>[40]</td>
</tr>
<tr>
<td></td>
<td>Fever, diarrhea, and dysentery</td>
<td>Decoction</td>
<td>[41]</td>
</tr>
<tr>
<td></td>
<td>Diarrhea and dysentery</td>
<td>Paste and decoction</td>
<td>[42]</td>
</tr>
<tr>
<td></td>
<td>Fever and dysentery</td>
<td>Decoction</td>
<td>[43]</td>
</tr>
<tr>
<td></td>
<td>Stomach disorder and intestinal worms</td>
<td>Paste</td>
<td>[44]</td>
</tr>
<tr>
<td></td>
<td>Antidote to snakebites</td>
<td>Not mentioned</td>
<td>[45]</td>
</tr>
<tr>
<td></td>
<td>Fever and dysentery</td>
<td>Decoction</td>
<td>[46]</td>
</tr>
<tr>
<td></td>
<td>Medicinal uses</td>
<td>Not mentioned</td>
<td>[47]</td>
</tr>
<tr>
<td></td>
<td>Snakebite</td>
<td>Not mentioned</td>
<td>[48]</td>
</tr>
<tr>
<td></td>
<td>For children dysentery</td>
<td>Cold infusion</td>
<td>[49]</td>
</tr>
<tr>
<td></td>
<td>Diarrhea and dysentery</td>
<td>Paste</td>
<td>[50]</td>
</tr>
<tr>
<td>Whole plant</td>
<td>Urinary disease and dysentery</td>
<td>Not mentioned</td>
<td>[51]</td>
</tr>
<tr>
<td></td>
<td>Fever and diarrhea</td>
<td>Not mentioned</td>
<td>[52]</td>
</tr>
<tr>
<td></td>
<td>Snake bite</td>
<td>Decoction</td>
<td>[53]</td>
</tr>
<tr>
<td></td>
<td>Medicinal uses</td>
<td>Not mentioned</td>
<td>[54]</td>
</tr>
<tr>
<td></td>
<td>Influenza and cough</td>
<td>Decoction</td>
<td>[55]</td>
</tr>
<tr>
<td></td>
<td>Urinary disease and snakebites</td>
<td>Powder</td>
<td>[56]</td>
</tr>
<tr>
<td></td>
<td>Blood purifier and fever</td>
<td>Decoction</td>
<td>[57]</td>
</tr>
<tr>
<td></td>
<td>Abdominal pain, backache, and kidney stone</td>
<td>Paste and decoction</td>
<td>[58]</td>
</tr>
<tr>
<td></td>
<td>Medicinal uses</td>
<td>Not mentioned</td>
<td>[59]</td>
</tr>
<tr>
<td></td>
<td>Dysentery, stomach inflammation, and disorder</td>
<td>Decoction</td>
<td>[60]</td>
</tr>
<tr>
<td></td>
<td>Diarrhea and dysentery</td>
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<td>[61]</td>
</tr>
<tr>
<td></td>
<td>Tumor, snake bite, and urinary disease</td>
<td>Not mentioned</td>
<td>[62]</td>
</tr>
<tr>
<td></td>
<td>Fever, blood purifier, diarrhea, dysentery, and urinary problems</td>
<td>Not mentioned</td>
<td>[63]</td>
</tr>
<tr>
<td></td>
<td>Anodyne, diuretic, and anti-rheumatic</td>
<td>Not mentioned</td>
<td>[64]</td>
</tr>
<tr>
<td></td>
<td>Fever and blood purifier</td>
<td>Decoction</td>
<td>[65]</td>
</tr>
<tr>
<td></td>
<td>Anti-venom</td>
<td>Not mentioned</td>
<td>[66]</td>
</tr>
<tr>
<td></td>
<td>Anti-snake venom</td>
<td>Not mentioned</td>
<td>[67]</td>
</tr>
<tr>
<td>Leaves and flowers</td>
<td>Cough and flu</td>
<td>Decoction</td>
<td>[68]</td>
</tr>
<tr>
<td></td>
<td>Brain tonic, diuretic, emollient, depurative, and snake bite</td>
<td>Decoction</td>
<td>[69]</td>
</tr>
<tr>
<td></td>
<td>Antispasmodic and lipoxygenase inhibitory activity</td>
<td>Not mentioned</td>
<td>[70]</td>
</tr>
<tr>
<td></td>
<td>Vomiting, joint pain, cough, and fever</td>
<td>Decoction</td>
<td>[71]</td>
</tr>
</tbody>
</table>
that sonication with methanol gives the best result for PA extraction from botanical samples [35].

To obtain the standardized HPLC fingerprint as mobile phases, we used water and acetonitrile with 1% formic acid. For maximum sensitivity, the wavelength was set at 280 nm for detection. In this phytochemical investigation of *T. indicum* from Northern Pakistan, the PAs present were determined and characterized by HPLC-UV. Five PAs namely: (1) supinine, (2) europine, (3) heliotrine, (4) lycopsamine, and (5) echimidine served as an authentic reference. These were used to identify the corresponding PAs in leaves of the *T. indicum*. Using the HPLC conditions discussed in the materials and methods section, the five standards of supinine, europine, heliotrine, lycopsamine, and echimidine were separated with retention times of 4.95, 10.21, 10.87, 37.16, and 46.34 minutes, respectively (Table 3). After identification of the retention time of standards, 20 μl of plant sample was injected and the retention times were compared to those of reference standards. The result of HPLC fingerprint analysis showed that the leaves of *T. indicum* were positive for the PAs supinine, europine, heliotrine, and echimidine. The result of the HPLC chromatogram of *T. indicum* was represented in Figure 5.

**Table 3.** HPLC results of the PAs.

<table>
<thead>
<tr>
<th>No</th>
<th>Reference standard</th>
<th>Retention time (minute)</th>
<th>Formula</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Supinine</td>
<td>4.95</td>
<td>C_{15}H_{25}NO_{4}</td>
<td>283.368</td>
</tr>
<tr>
<td>2</td>
<td>Europine</td>
<td>10.21</td>
<td>C_{16}H_{27}NO_{6}</td>
<td>329.183</td>
</tr>
<tr>
<td>3</td>
<td>Heliotrine</td>
<td>10.87</td>
<td>C_{16}H_{27}NO_{5}</td>
<td>314.196</td>
</tr>
<tr>
<td>4</td>
<td>Lycopsamine</td>
<td>37.16</td>
<td>C_{15}H_{25}NO_{5}</td>
<td>299.173</td>
</tr>
<tr>
<td>5</td>
<td>Echimidine</td>
<td>46.34</td>
<td>C_{20}H_{31}NO_{7}</td>
<td>398.217</td>
</tr>
</tbody>
</table>

**Figure 5.** HPLC-UV chromatograms of *T. indicum* and standard mix.
Discussion

*Trichodesma indicum* is a prominent ethnomedicine of Pakistan. To the best of our knowledge, up to now, no comprehensive study has yet been reported regarding PA determination in *T. indicum*. Our study shows, for the first time reported in the literature, investigation of PAs in *T. indicum*. However, previously it was well established that unsaturated PAs are present in some other *Trichodesma* species such as intermedine, lycopsamine, retronecine, viridiflore, eurpime, and trichodesmine in *T. africanum* [72–74], supinine and senkirkine from *T. ehrenbergii* [75], trichodesmine and inanine from *T. incanum* [76], and supinine from *T. zeylanicum* [77]. Unfortunately, the literature survey of *T. indicum* in our study shows oral ingestion in the form of powder or decoction. According to Mei et al. [78], the ingestion of echimidine consumed as herbal tea, as a medicine or a vegetable has led to several cases of HSOS of the liver in human beings. Moreover, heliotrine, as a heliotridine-type PA, induces mutagenesis, chromosome damage, and is carcinogenic to the liver [79–81].

Exposure to unsaturated PAs has been regarded as one of the major causes responsible for the development of HSOS [82]. Individuals who consume PA-containing herbal extracts can experience intrinsic liver injury [83]. Patients with HSOS present typical symptoms of abdominal distension and ascites, pain, jaundice, malaise, hepatomegaly, and body weight increase due to ascites and edema caused by fluid accumulation [84]. Currently, misidentified herbal products are a major clinical challenge as they may harm the health of consumers. In China alone, up until 2008, 41 cases with HSOS were reported [85]. Recently in China, a 54-year-old female was diagnosed with HSOS because of the consumption of the traditional herb *Gynura segetum* Merr., which contains hepatotoxic unsaturated PAs senecionine and seneciphylline [86,87].

Potential poisonings by unsaturated PA may have occurred as isolated incidences in the past, but with increased trade and popularity of herbal medicine in industrialized countries, the potential for intoxication increases. This is perpetuated even further by the "green wave" of people seeking natural remedies [79]. Given the risks and disease reports of unsaturated PA consumption by humans and livestock around the globe, the European Medicines Agency has implemented a limit of 1 μg/day for herbal medicine products with the objective of transitioning down to 0.007 μg of 1, two-unsaturated PA/kg body weight (i.e., 0.14 μg PA per day for 20 kg children and 0.35 μg PA per day for 50 kg adult) [88, 89]. The German Federal Institute for Risk Assessment (BfR) also suggests a maximum daily intake of 0.007 μg PA per kg body weight based on a 10,000 margin of exposure (MOE) [88]. This MOE corresponds to the limit of a maximum intake of 0.024 μg PA/PA NO-oxides per kg body weight per day. While in Germany it is specified that for a long-term exposure to PA over 6 weeks, one should be very cautious [90, 91]. In all, the Federal Institute of Risk Assessment (Bundesinstitut für Risikobewertung, BfR, Germany) and UK Committee on Toxicity policies and statements demonstrate that the exposure to PAs from consumable products should be as low as possible with limits at 0.007 μg of PAs/kg of body weight per day [92, 93].

From the literature survey, it is confirmed that *T. indicum* is a prominent ethnomedicine in Pakistan. To the best of our knowledge, this study identified PAs in the leaves of *T. indicum* from the Pan-Himalayan region in Pakistan for the first time. The selected plants were found to be positive for hepatotoxic PAs like supinine, europine, heliotrine, and echimidine. According to the International Program on Chemical Safety, unsaturated PA toxicity depends not only on dose and exposure time but also on age and gender. Males seem to react more than females and children and fetuses are even more sensitive [94]. Therefore, we recommend that this species should be used with more precaution, especially when one considers herbal remedies for children. Previously, it was well established that PAs are an intrinsic component of Boraginaceae and its medicinal use might increase the risk of liver toxicity. Moreover, the information currently available on the use of *T. indicum* is not sufficient to know the risks or any associated chronic health problems caused by unsaturated PAs in this plant. However, recently, the increasing evidence of unsaturated PAs in milk, honey, meat, herbal drugs, grain, and green tea warrants monitoring for PAs to ensure that the intake level does not exceed safety limits.

Limitations and Conclusions

In the laboratory, HPLC fingerprint analysis, besides the four known peaks of PAs, obtained data shows that there were several unknown unsaturated PAs or other compounds between
13–25 and 35 retention times but with no authentic standard availability, we cannot determine their identity. Several other authentic standards of PAs like intermedine, echinatine, laisiarpine, and monocrotaline could not be detected well, using the aforementioned HPLC conditions. The present study has revealed new findings of significance to local people regarding T. indicum. The literature survey shows that the plant is often used for medicinal purposes throughout Pakistan. The HPLC fingerprint analysis shows that besides ethnomedicinal uses it is also a source of toxic PAs heliotrine and echimidine. The study shows the need to at least find the qualitative determination of these alkaloids for recognition of their potential health risks. Secondly, survey results revealed that nearly all local people either use or collect this species for their traditional therapeutic value. Given this, we suggest that the plant should be used with more precaution and especially kept away from children, and also should not be used in large quantities and not for consecutive days.

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Conflicts of Interest

The authors declare no conflict of interest.

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