Separation and purification of indigotin and indirubin from Folium isatidis extracts using a fast and efficient macroporous resin column followed reversed phase flash chromatography

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A fast and efficient procedure for preparation of indigotin and indirubin from Folium isatidis extracts using macroporous resin followed by reversed phase flash chromatography was investigated in this paper. Nine widely used macroporous resins were used to enrich and separate two alkaloids from Folium isatidis. Finally, D3520 resin was chosen to enrich two alkaloids through static and dynamic adsorption tests. After one run of adsorption and desorption, the contents of indigotin and indirubin were 15.2 and 15.5-fold increased with recovery yields of 87.8% and 89.7%, respectively. The enriched sample was directly subjected to flash chromatography on C18 reversed phase silica gel column, and the separation of indigotin and indirubin was dramatically improved by using ionic liquid as mobile phase modifier for decreasing band tailing, reducing band broadening, and improving resolution. Indigotin and indirubin with purities of more than 96% were produced with recovery yields of 90.1% and 92.0% by C18 reversed phase flash chromatography. The developed procedure boasts production of high-purity product as well as high recoveries, and allows an easy scale-up. This study provides an excellent basis for large-scale preparation of alkaloids from Folium isatidis or other plant extracts.

1. Introduction

Folium isatidis named “Da-qing-ye” in China, is the dried leaf of Folium isatidis. It is a biennial herbaceous plant and has been used for a long time as a traditional Chinese medicine (TMC) [1]. It has antibacterial, antiinflammatory, antipyretic, antiviral, and antitumor properties, and also promotes immunological response [2–4]. According to previous reports, alkaloids, organic acids, nucleosides, lignanoids, and flavonoids have been found in Folium isatidis [5–7]. Indigotin and indirubin are major alkaloids in Folium isatidis [8]. Indigotin and indirubin are structural isomers, and their structures are shown in Fig. 1. Indigotin has been demonstrated to have inhibition of polymorphonuclear neutrophil infiltration and the nonenzymatic antioxidant effects [9]. Indirubin has been originally proven to possess antileukemic activity in treatment of chronic myelocytic leukemia [10], and not only could strengthen the immune system properties but also possess physiological properties including antibiosis, anti-inflammatory and anti-tumor activities etc. [11].

In recent decades, a lot of technologies for the enrichment of bioactive compounds from plant extracts have been investigated, such as membrane filtration [12], ion exchange [13], liquid–liquid extraction [14], supercritical fluid extraction [15], adsorption–desorption [16] and solid-phase extraction [17]. In these methods, adsorption and desorption is a feasible technology for the enrichment of bioactive components from plant extracts. It has been widely used for the separation and purification of bioactive constituents from extracts of medical herbs [18–20]. Macroporous resin is a kind of organic polymer adsorbent, and it has macroreticular structure and large specific surface area. It has many advantages such as high loading capacity, low cost, easy regeneration and adjusted selectivity [21], and has been widely used

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Flash chromatography is a rapid form of preparative column chromatography based on air pressure driven hybrid of medium and short column chromatography for particularly rapid separations. Compared with traditional gravity-fed chromatography, flash chromatography gives a rapid and high resolution chromatography, greatly improved reproducibility, higher recovery yield, and a reduced consumption of organic solvents. It can offer satisfactory separation in a short time under a proper chromatographic condition at a relatively high flow rate with low pressure [22]. Up to now, it has been considered as an inexpensive, fast and efficient separation technique for the separation of natural active components from extracts [23].

As we all know, for basic compounds with polar functional groups, severe band tailing, band broadening and low plate numbers in traditional silica gel chromatogram often occur due to the free silanols [24]. So, the separation and purification of these compounds require reversed-phase packings based on silica particles with low number of silanol residues, and mobile phase with high ionic strength and amine modifiers. In this sense, finding a solvent system which can be used in liquid chromatography efficiently with good separation for these compounds is one of the greatest challenges in this field. Ionic liquids, considered “green” chemicals, are widely used in many chemical fields due to their unique properties. It was found in previous research that ionic liquids could compete with basic compounds for the silanol group on the alkylsilica surface and form weak bilayer electronic structure on reversed phase silica gel [25]. Thus, the application of ionic liquids as mobile phase modifier may be a simple and effective way to separate basic compounds.

In this study, the enrichment and purification of the alkaloids from the crude extract of *Folium isatidis* using macroporous resins followed by flash chromatography on C18 reversed phase silica gel column was investigated. In flash chromatography, the potential application of ionic liquids as mobile phase modifier for the separation of indigotin and indirubin was focused. The chemical structures of purified alkaloids were verified by UV and MS. To the best of our knowledge, no report has been published on the enrichment and purification of indigotin and indirubin from *Folium isatidis* extracts by macroporous resin followed by flash chromatography.

2. Materials and methods

2.1. Chemicals and reagents

HPLC grade methanol was purchased from J & K Chemical LTD (Beijing, China). Distilled water was purchased from Wahaha Company (Zhejiang, China) and analytical reagents were obtained from Beijing Chemistry Corporation (Beijing, China). All macroporous resins including ADS-17, ADS-5, DM-130, D3520, NKA-9, HPD826, D4020, AB-8, D101 were obtained from Nankai Hecheng S & T (Tianjin, China) and Bonchem (Hebei, China). Their physical and chemical properties are listed in Table 1. Their polarities ranged from non-polar to strong polar. Prior to use, the resins were pretreated according to the manufacturers’ recommendation to remove the monomers and porogenic agent trapped inside the pores during the synthesis process. The moisture contents of the resins were determined by drying the beads at 100 °C until the mass did not change any more in a drying oven for over 24 h. C18 reversed phase silica gel (30–50 μm) was purchased from Daiso Co., Ltd. (Osaka, Japan). 1-butyl-3-methyl-imidazolium tetrafluoroborate ([bmim]Br), 1-butyl-3-methyl-imidazolium tetrafluoroborate ([bmim][BF4]), 1-butyl-3-methylimidazolium chloride ([bmim]Cl) were purchased from Shanghai Chengjie Chemical Co. Ltd (Shanghai, China).

2.2. Preparation of Folium isatidis extracts

*Folium isatidis* was collected from Heilongjiang province China, and authenticated by Professor Zijun Mao in the Key Laboratory of Forest Plant Ecology, Ministry of Education, Northeast Forestry University, China. Collected material was dried in the shade at room temperature, milled and then stored in dark. Pulverized *Folium isatidis* (7 kg) was extracted with 80% aqueous ethanol (50 l) at room temperature for three times. The extraction solution was concentrated by a rotary evaporator (RE52AA, Shanghai Huxi Instrument Co., China) at 40 °C. The dried crude extracts were obtained, and then distilled in 20% aqueous ethanol to get sample solution. The concentrations of indigotin and indirubin in solution determined by HPLC analysis were 0.091 mg/ml and 0.169 mg/ml, respectively.

2.3. HPLC analysis

Indigotin and indirubin in samples were analyzed by an Agilent 1200 series liquid chromatography system (Agilent, San Jose, CA, USA) equipped with a G1311A quaternary pump, a G1322A degasser, a G1365B MWD UV detector and a G1328B manual injector. Chromatographic separation was carried out on a Luna C18 reversed phase column (250 × 4.6 mm i.d., 5 μm, Phenomenex, USA). The mobile phase was methanol–water (70:30, v/v). Flow rate was 1.0 ml/min, detection wavelength was 289 nm, the column temperature was maintained at 30 °C, and injection volume was 5 μl. The chromatographic peaks were identified by comparing their retention time with those of standards.

2.4. Enrichment of indigotin and indirubin by macroporous resin

2.4.1. Static adsorption and desorption tests for screening of resins

The static adsorption and desorption tests of nine macroporous resins (Table 1) towards indigotin and indirubin were investigated. The adsorption procedure was as follows: pre-weighed hydrated resins (equal to about 1 g dry resin) were introduced into a 100 ml triangular flask, and then 60 ml sample solution prepared in Section 2.2 was added into each flask. The flasks were then continually shaken at 120 rpm for 6 h at constant temperature 25 °C. After adsorption, the residual sample solution and resins were separated, the concentrations of indigotin and indirubin in the residual sample solutions were analyzed by HPLC. The adsorbate-laden resin was desorbed with 90 ml 90% aqueous ethanol. In the desorption process, the flasks were continually shaken (120 rpm) for 8 h at 25 °C. After 8 h desorption, desorption solutions were separated from the resin and analyzed by HPLC. The adsorption capacities and the ratios of desorption of individual resins were calculated using the following equations:

\[ Q_s = (C_0 - C_e)V_s/E \]  
\[ D = C_d \times V_d \left( C_0 - C_e \right) \times 100\% \]
Table 1
Physical properties of the macroporous resins used.

<table>
<thead>
<tr>
<th>Trade name</th>
<th>Functional group</th>
<th>Surface area (m²/g)</th>
<th>Average pore diameter (nm)</th>
<th>Particle diameter (mm)</th>
<th>Appearance</th>
<th>Polarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NKA-9</td>
<td>Crosslinked-polystyrene</td>
<td>250–290</td>
<td>15.0–16.5</td>
<td>0.3–1.25</td>
<td>Milk white</td>
<td>Polar</td>
</tr>
<tr>
<td>DI01</td>
<td>Crosslinked-polystyrene</td>
<td>400–600</td>
<td>10.0–12.0</td>
<td>0.3–1.25</td>
<td>Milk white</td>
<td>Non-polar</td>
</tr>
<tr>
<td>AB-8</td>
<td>Crosslinked-polystyrene</td>
<td>480–520</td>
<td>13.0–14.0</td>
<td>0.2–0.6</td>
<td>Milk white</td>
<td>Non-polar</td>
</tr>
<tr>
<td>D4020</td>
<td>Crosslinked-polystyrene</td>
<td>540–580</td>
<td>10.0–10.5</td>
<td>0.3–1.25</td>
<td>Milk white</td>
<td>Non-polar</td>
</tr>
<tr>
<td>ADS-17</td>
<td>Ester group</td>
<td>90–150</td>
<td>25.0–30.0</td>
<td>0.3–1.25</td>
<td>Milk white</td>
<td>Non-polar</td>
</tr>
<tr>
<td>ADS-5</td>
<td>Polystyrene</td>
<td>520–600</td>
<td>25.0–30.0</td>
<td>0.3–1.25</td>
<td>Milk white</td>
<td>Non-polar</td>
</tr>
<tr>
<td>DM-130</td>
<td>Crosslinked-polystyrene</td>
<td>500–550</td>
<td>9.0–10.0</td>
<td>0.3–1.25</td>
<td>Milk white</td>
<td>Non-polar</td>
</tr>
<tr>
<td>HPD-826</td>
<td>Crosslinked-polystyrene</td>
<td>500–600</td>
<td>9.0–10.0</td>
<td>0.27–0.34</td>
<td>Milk white</td>
<td>Non-polar</td>
</tr>
<tr>
<td>DI3520</td>
<td>Crosslinked-polystyrene</td>
<td>480–520</td>
<td>8.5–9.0</td>
<td>0.3–1.25</td>
<td>Milk white</td>
<td>Non-polar</td>
</tr>
</tbody>
</table>

where \( Q_e \) was the adsorption capacity at adsorption equilibrium (mg/g dry resin); \( C_0, C_e \), and \( C_i \) were the initial, adsorption equilibrium and desorption concentrations of analyte in the solutions, respectively (mg/ml); \( V_i \) and \( V_d \) were the volume of the initial sample and desorption solution (ml), respectively; \( W \) was the dry weight of resin (g), and \( D \) was the desorption ratio (%).

2.4.2. Dynamic adsorption and desorption tests on the selected DI3520 resin

Dynamic adsorption and desorption experiments were conducted in a glass column (500 × 10 mm) wet-packed with 5.0 g (dry weight) pretreated DI3520 resin. The dynamic breakthrough curves for indigotin and indirubin on DI3520 resin were constructed by dynamic adsorption test to determine feed volume. The bed volume (BV) was 30 ml. During dynamic adsorption process, sample solution prepared in Section 2.2 flowed through the glass column at a constant flow rate of 1 BV/h. During dynamic adsorption, the concentrations of indigotin and indirubin in the aliquots of 1 ml effluents collected at 1/3 BV interval were monitored by HPLC.

The gradient elution tests were carried out as follows: after adsorption equilibration, the adsorbate-laden column was washed with deionized water, and then eluted using 2 BV different concentrations of ethanol (20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%) at a constant flow rate of 3 BV/h, successively. Each part of desorption solutions was analyzed by HPLC.

After determining the adsorption and desorption tests based on the above experiments, scaled-up enrichment by macroporous resin was performed under the optimized conditions to obtain enriched sample of indigotin and indirubin for further separation by flash chromatography.

2.5. Separation of alkaloids by reversed phase flash chromatography

Flash chromatography was performed on an automated flash chromatography system (Lisure Science (Suzhou) Co., Ltd., China). The enriched sample by macroporous resin of indigotin and indirubin was dissolved in initial mobile phase. The C18 reversed phase silica gel column (250 mm × 30 mm i.d., about 50 g) was equilibrated with the initial mobile phase. The indigotin and indirubin were separated on C18 reversed phase silica gel column by a gradient elution program: 0−45 min, 55% aqueous methanol containing ionic liquid [bmim]BF\(_4\) at concentration of 5.0 mM; 45−60 min, 70% aqueous methanol containing ionic liquid [bmim]BF\(_4\) at concentration of 5.0 mM; 60−65 min, pure methanol. The flow rate was kept at 30 ml/min. The effluent was fractionated and collected according to the chromatograms at 289 nm and HPLC analysis as described in Section 2.3. Thereby, the effluent only containing indigotin or indirubin was obtained, and evaporated to dryness under reduced pressure at 40 °C to obtain indigotin or indirubin.

2.6. Confirmation of indigotin and indirubin

The chemical structures of purified compounds were confirmed by UV and MS. UV spectra in methanol were measured on a Waters 2996 diode array detector spectrophotometer (Waters, USA). ESI-MS spectra were obtained on an API 3000TM (Applied Biosystems, USA) in negative mode.
3. Results and discussion

3.1. Enrichment by macroporous resin

3.1.1. Static adsorption and desorption tests

The nine resins are classified into the categories of non-polar (D101, D4020 ADS-17, ADS-5, HPD-826, D3520), weak polar (AB-8 and DM-130), and polar (NKA-9) according to their polarities. The adsorption capacities of nine macroporous resin were shown in Fig. 2. As seen from Fig. 2, the adsorption and desorption performances of different resins were distinct. It could be found that the adsorption capacities and desorption ratios were not only correlated with physical and chemical properties of the resins, but also were influenced by the size and chemical features of the adsorbed substance. The physical and chemical properties of the resins directly influenced the adsorption capacities and desorption ratios. The non-polar D4020, D3520, D101 and weak polar DM130 resins exhibited better adsorption capabilities due to their similar polarities with indigotin and indirubin. However, the desorption ratio of D4020, D101 and DM130 resin were significantly lower than D3520. In other words, D3520 resin possessed excellent adsorption and adsorption capacities for indigotin and indirubin. Thus, dynamic adsorption and desorption tests was carried out on D3520 resin.

3.1.2. Dynamic adsorption on D3520 resin

The adsorption effect would decrease, even disappeared with the increase of feed volume, and the solutes would pass through the resin bed. So, it was very important to construct the dynamic breakthrough curve in order to calculate suitable feed volume of sample solution. The dynamic breakthrough curve was established in order to determine the quantity of resin and the suitable feed volume of sample solution. In general, when the concentration in effluent was 10% of the original concentration, the adsorption presumably reached the breakthrough point. According to the volume of effluent liquid and the concentration of two alkaloids herein, the dynamic leakage curves on D3520 were obtained as shown in Fig. 3A. In the present test, the breakthrough points of two alkaloids were not the same. The breakthrough points of indigotin and indirubin on D3520 resin were 4 BV and 3 BV, respectively. In a comprehensive consideration, the feed volume of sample solution on D3520 resin was determined as 3 BV (90 ml).

3.1.3. Dynamic desorption curve on D3520 resin

The dynamic desorption curves were drawn on the basis of the analytic ability of different ethanol concentrations and the indigotin and indirubin concentrations in the desorption solution (Fig. 3B). Dynamic desorption was performed with a gradient elution mode at a flow rate of 3 BV/h. When the ethanol concentration was less than 50%, the two alkaloids were difficult to be desorbed. However, when the ethanol concentration was over 50%, the desorption of two alkaloids increased rapidly and reached higher value at 60–100% aqueous ethanol. Therefore, a gradient procedure with 50% aqueous ethanol and 100% pure ethanol was chosen to enrich two alkaloids. The 50% aqueous ethanol and pure ethanol were used to remove impurities and elute the target compounds.

It was necessary to determine the elution volume of 50% aqueous ethanol for removing impurities and the elution volume of pure ethanol for eluting the target compounds. As seen from Fig. 3C, when the elution volume of 50% aqueous ethanol exceeded 13/3 BV, the concentration of indirubin in effluents increased obviously. So, 13/3 BV 50% aqueous ethanol was used to remove nontarget compounds. Then, most of the target compounds absorbed by D3520 resin were eluted by 16/3 BV pure ethanol.

According to the data, the optimized enrichment process was confirmed as follow: resin type: D3520 resin; feed volume: 3 BV; gradient elution: 13/3 BV 50% aqueous ethanol and 16/3 BV pure ethanol. Under these conditions, scale-up enrichment of two alkaloids by macroporous resin was performed, and enriched sample was obtained. The chromatograms of crude extract and enriched sample were shown in Fig. 4A and B, respectively. Comparison of the chromatograms indicated that after the enrichment on D3520 resin, some impurities were removed and the relative peak areas of indigotin and indirubin accounted for the total peak areas values were increased obviously. The contents of indigotin and indirubin in the enriched product reached 4.73% and 8.99%, which were 15.20 and 15.53-fold to those in crude extract, respectively, and the recovery yields were 87.8% and 89.7%, respectively.
Indigotin and indirubin are basic compounds which may cause severe band tailing, band broadening and low plate numbers in C18 reversed phase chromatography separation. Thus, the separation and purification of indigotin and indirubin required a specific mobile phase with the properties of high ionic strength and amine modifiers. Researches indicated that the ILs could be added into the mobile phase as additive and used as efficient silanol screening agents in the separation of basic compounds. 1-butyl-3-methyl-imidazolium type ILs are the most popular type for the separation of basic compounds [26]. In previous experiment, three 1-butyl-3-methyl-imidazolium-type ionic liquids with different anions (Br\(^-\), BF\(_4^-\) and Cl\(^-\)) were added into mobile phase in order to increase its ionic strength for improving the separation of two alkaloids. It was found that the addition of ionic liquid in mobile phase had great effects on the separation of these basic compounds: decreasing band tailing, reducing band broadening and improving resolution (not shown). This might be due to that imidazolium cations could interact with silanol groups, and compete with the silanol groups on the alkyl phase surface with the polar group of the alkaloids. Based on the screening of the ionic liquids used above, the effect of [bmim]BF\(_4\) was more efficient than others (not shown). It was confirmed that when the low concentrations of ILs were added in aqueous solution, ILs became just regular salts. The specific properties of ILs made by cations and anions with equal amount possessed significantly better silanol blocking activity and peak shape improvements than classical ternary amines. What’s more, the anions could absorb on hydrophobic stationary phase in amount depending on the Hofmeister anion series, BF\(_4^-\) > Br\(^-\) > Cl\(^-\) (the anions of ILs used in this study). And the chaotropic BF\(_4^-\) anion could associate with the cation of basic compounds, forming less polar ion-pair which was more retained by the C18 stationary phase. Therefore, the recovery yields and retention factors of indigotin and indirubin were increased as well as the peak shape since fast hydrophobic interactions were mostly involved in the retention mechanism of the ion-pair [27]. The two basic compounds indigotin and indirubin were weak polar, so a weak polar IL additive with a cosmotropic cation bmim\(^+\) was a good choice for peak shape improvement and reducing retention without increasing the mobile phase organic modifier content [28, 29]. Thus, [bmim]BF\(_4\) was selected as the additive ionic liquid in mobile phase in the following tests.

In order to investigate the effect of the ionic liquid concentrations, the concentrations of [bmim]BF\(_4\) ranging from 2.5 to 9.0 mM in the eluent were evaluated for separation. According to previous reports [30], ILs would maintain their supermolecular structure to a great extent when the mole fraction of water (X\(_{\text{water}}\)) was less than 0.4178. Above this concentration of water, the hydrogen-bonding network was broken, and ILs dissociated into free ions. The X\(_{\text{water}}\) calculated in the present study were increased from 0.4902 to 0.6474 with the concentration of [bmim]BF\(_4\) increasing from 2.5 to 9 mM (the mobile phase consisted of 30–45% water).
Therefore, moderate ILs was added into the mobile phase could enhance the ionic strength, which was conductive to the separation of target compounds. The flow rate was kept at 30 ml/min. As shown in Fig. 5A, the addition of [bmim]BF₄ improved significantly the recovery yields of indigotin and indirubin. However, when the concentration of [bmim]BF₄ increased from 5.0 to 9.0 mM, their recovery yields decreased. This effect might be attributed to the following reasons: firstly, the addition of ionic liquids to mobile phase caused the competition between imidazolium cations and the basic compounds for silanol groups on alkylsilica surface, thus resulting in a drastically increase in recovery yields of target compounds. And then as the concentrations of ionic liquids increased to 9.0 mM, the interaction of imidazolium cations with the silanol groups on alkylsilica surface by electrostatic interaction or with the alkyl groups by hydrophobic interaction gradually strengthened, which resulted in an increase in the carbon content of stationary phase, thus decreased slightly in the recovery yields of target compounds [31]. Hence, 5.0 mM [bmim]BF₄ was chosen for the next experiment.

High sample load may distort peak shape and cause an overall decrease in separation efficiency due to column overload. The effect of sample load on separation efficiency was also investigated.
by varying the sample/reversed phase silica gel ratio from 2.5:50 to 1:50. The results in Fig. 5B showed that the recovery yields of indigotin and indirubin increased drastically and then decreased with decreasing sample/reversed phase silica gel ratio from 2.5:50 to 1:50. Although the purities of indigotin and indirubin were more than 95% at the high sample/reversed phase silica gel ratio (2.5:50, 2:50), their recovery yields were lower. As expected, when the sample/silica gel ratio decreased, the separation efficiency significantly improved due to less sample load. So, a sample/reversed phase silica gel of 1:50 was considered as the optimal ratio. The satisfactory separation of two alkaloids was achieved on C18 reverse silica gel column.

Under the optimized conditions, two major peaks were observed in Fig. 6 after 30 min and collected. Finally, 66.2 mg indigotin and 127.5 mg indirubin were obtained by one flash chromatography run. The purities of indigotin and indirubin were more than 96% by HPLC analysis (Fig. 4C and D), and their recovery yields were 90.1% and 92.0%, respectively. In this process, the mobile phase was reusable easily, and high-purity products with high recovery yields were produced. Hence, the developed flash chromatography was suitable for preparation of indigotin and indirubin from *Folium isatidis*.

3.3. Confirmation of indigotin and indirubin

The structures of purified compounds were confirmed by UV and MS spectra. Their UV spectra in methanol were given in Fig. 7A and B. The absorption maxima of indigotin was observed to be 242.7, 284.0 nm and indirubin: 238.0, 290.0, 358.8 nm. The product ion spectra of deprotonated indigotin and indirubin (in negative ion mode) in Fig. 7C and D showed a molecular ion [M−H]− at m/z 261.1 and its abundant product ions at m/z 217.1, and a molecular ion [M−H]− at m/z 261.1 and its abundant product ions at m/z 157.1, respectively. These spectral results were consistent with those from previous publications [4,8].

4. Conclusions

In this study, a fast and efficient procedure using macroporous resin followed by C18 reverse silica gel flash chromatography was developed for enrichment and purification of indigotin and indirubin from *Folium isatidis*. After one run treatment with D3520 resin, the contents of indigotin and indirubin were 15.2-fold and 15.5-fold increased with recovery yields of 87.8% and 89.7%, respectively. Meanwhile, the addition of ionic liquid in the mobile phase of flash chromatography successfully achieved the purification of indigotin and indirubin after one flash chromatography run. Under the optimal conditions of flash chromatography, the purities of indigotin and indirubin were more than 96% with recovery yields of 90.1% and 92.0%, respectively. The results indicated that macroporous resin followed by C18 reverse silica gel flash chromatography was an effective procedure for preparing indigotin and indirubin with advantages of high-purity, high recovery yield, low running costs and easy scale-up production. This study provides a promising strategy for large-scale preparation of alkaloids from *Folium isatidis* or other plant materials.

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