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Original Article

Qualitative and Quantitative Determination of Pyrrolizidine Alkaloids of Wheat and Flour Contaminated with *Senecio* in Mazandaran Province Farms

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Abstract

The Pyrrolizidine Alkaloids (PAs) are a group of chemicals found in a variety of plant species throughout the world. These toxic alkaloids are distributed mainly in *Senecio* (Compositae), *Crotalaria* (Legominosae) and *Heliotropium* (Boraginaceae) species. Plants containing these alkaloids cause significant mammalian morbidity and mortality, especially in humans. Upon ingestion, metabolic activation in liver converts the potent compounds into highly reactive electrophiles capable of reacting with cellular macromolecules forming adducts, which can initiate acute or chronic toxicity.

One of these plants is *Senecio vulgaris* which is abundant in wheat farms of Mazandaran province. When wheat (*Triticum* spp) is being harvested, seeds and aerial parts of *Senecio* would also be collected with it. Since the presence of PAs in *Senecio vulgaris* is proven in a previous research, hence in this study, quality and quantity of PAs of wheat and flour contaminated with *Senecio* in Mazandaran province farms have been studied.

The specimens were collected from all flour industries four teen sites and silo of Mazandaran. The Ehrlich reaction test and spectrophotometeric method were used for the qualitative and quanlitative examinations, respectively. The Amount of PAs and their N-oxides calculated on the basis of senecionine. The reaction is specific for alkaloids having an unsaturated basic moiety of Δ^3– pyrroline ring.

In the qualitative test, the existence of PAs was demonstrated in all specimens. Mean amount of the total PAs and their N-oxides in 0.512g of specimens was 0.020 to 0.05 mg (as senecionine). Total PAs in 0.512g of *Senecio vulgaris* was 0.4mg.

LD_{50} of senecionine, fatal toxic dose of PAs and nonfatal toxic dose of PAs are 64.72 ± 2.24 mg/kg, 6–167 mg/kg, and 2–27 mg/kg, respectively. Comparing the amount of PAs in wheat and its flour as well as its toxic dose, the specimens would not scem to produce acute complications of Pas. However, long term exposure to low levels of PAs may cause cumulative damage especially hepatotoxicity. Meanwhile chronic toxicity to humans by diet, including the specimens, is possible.

Keywords: Pyrrolizidine alkaloids, Hepatotoxicity, *senecio*, Wheat, Spectrophotometry.

Introduction

Plants containing hepatotoxic pyrrolizidine alkaloids (PAs) exist in most parts of the world (1) and often cause poisoning of grazing livestock. PAs have also caused human fatalities. Large outbreaks of poisoning have occurred when cereal crops have been contaminated with PA-containing seeds, and poisonings by herbal teas or medicines containing pyrrolizidines continue to be reported from time to time. A number of PA-containing plants have been used for food or medicinal purpose (2).

Pyrrolizidine alkaloids (PAs) also known as senecio alkaloids (3, 4) are found mainly in the families Compositae, Boraginaceae and Legominosae (5, 6). PAs are specific within the *Amsinekia*, *Arnebia*, *Borago*, *Echium*, *Trichodesma*, *Senecio*, *Symphytum*, *Cynoglussum*, *Eupatrium*, *Heliotropium* and *Crotalaria* genera. Many of these genera contain the species most frequently associated

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with human illness (6). These genera are distributed in various climates. More than 200 PAs are identified. These constituents are distributed in 6000 plant species (about 3% of world's flowering plants) (6, 7).

Toxicosis and disturbances have been reported from PAs as a result of: consciousl consumption (herbal medicine for gastrointestinal complaints, infant colic, arthritis, sprains, bruises and local wound care or ingestion of these plants as vegetables) or inadvertentl consumption (contamination of foodstuffs or mistake in the recognition of the plant) (6, 8, 9, 10).

Cultural practices, economic circumstances and medicinal uses of the plants determine the mode of exposure to the PAs (8, 11).

In this study, qualitative and quantitative determination of *Senecio's* pyrrolizidine alkaloids of wheat and flour in Mazandaran province farms have been investigated. *Senecio vulgaris* (common groundsel, fam. Compositae) is abundant in farms and is harvested with wheat (*Triticum* spp. Fam. Graminae) (12, 13, 14).

PAs that obtained from the aerial parts of *S. vulgaris* are up to 0.76% and mainly consist of senecionine and seneciphylline (15, 16).

Senecionine is a 12–member macrocyclic diester and formed from retronecine as necine base with senecic acid. Seneciphyline is 13, 19 –didehydro senecionine (11, 15) the structures of these compunds are shown in figure 1. These two hepatotoxic PAs have an allylic ester group Both of these compounds can produce hepatotoxicity, mutagenicity and tratogenicity and furthermore cause morphological changes of lung (6, 9).

Considering the excessive consumption of wheat in forms of bread, macaroni, sweets, in diet and pyrrolizidine alkaloid's complications, the importance of the determination of contamination to PAs in wheat of Mazandaran province and its preparations is of great importance.

a) b)

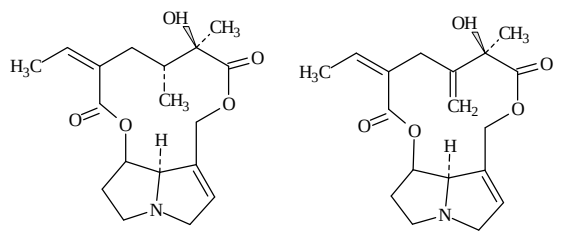


Figure 1: molecular structure of Senecionine (a) and Seneciphylline (b)

Experimental

Materials

Ascorbic acid, sodium nitroprosside, 4-dimethylaminobenzaldehyde, acetic acid, percholoric acid, sodium pyrophosphate, hydrogen peroxide, potasium iodide, methanol, diglyme (diethylene glycol dimethyl ether), ammonia, sodium sulfate, chloroform, acetone, zinc powder were all abtained from Merck chemicals (Germany).

Collection of Samples

In this study, samples were collected from all (four teen) factories and silos in Mazandaran.

Qualitative determination

Ehrlich regent: 5 g of 4–Dimethylamino benzaldehyde was separately dissolved in 3 ml of water, 10 ml of percholoric acid 60% and 60 ml of acetic acid (15).

Powdered plant, wheat or flourplant or wheat or flour (1.5 grams) along with 5% ascorbic acid (40 mg) were mixed with a small amount of sand, separately. Each of them were shaken and after 5 min. filtered and divided into sample and blank. To the sample tube 12 ml of alkaline sodium nitroprosside 5% reagent was added. Next, to both of the tubes Ehrlich reagent was added and they were heated on the water bath for a few minutes. The red color determines the presence of PAs in the sample (17).

Quantitative determination

Oxidation reagent: A 30% Hydrogen peroxide (containing 5 mg/ml sodium pyrophosphate for its stability) solution was prepared. 0.1 ml of this solution was diluted with methanol to 25 ml (this reagent must be freshly prepared.

Modified Erlich reagent: 2 g of 4–Dimethyamino benzaldehyde was dissolved in 100 ml of methanol (containing 10 ml BF_3 14% in methanol) (7).

For the assay of alkaloid, or alkaloid-N–Oxide, the sample should be dry and contains

between 5 and 30 µg of alkaloid (or alkaloid - N-Oxide) in the basic form.

samples:

S. vulgaris powder, or wheat or flour (1.024 g) were macerated in methanol and boiled for 1 h. The extracts were filtered and concentrated to dryness.

Dilute hydrochloric acid was added to the residue and washed with ether to remove fats and chlorophyll. The aqueous phase was divided to two portions. One half was made basic with ammonia, and shaken with four portions of 20 ml of chloroform. The combined chloroform extracts was then dried with sodium sulfate and the total volume was brought to 10ml. Next, Two 3 ml aliquots were evaporated to dryness. The other half of the aqueous liquor was reduced with zinc dust for 0.5 h, filtered, and treated in a similar mannerto that described above.

Absorpion was determined according to the following procedure:

A. 0.5ml (±5%) of oxidation reagent is added to the sample tube and the lower half of the unstoppered tube immersed in a boiling water bath for 20–30 min. After 10 min. methanol was evaporated.

B. Diglyme (1 ml ± 10%) and acetic anhydride (0.1 ml ± 5%) were added respectively. The tube was heated again in the boiling water bath for 1 min ± 10 seconds. It is important to avoid any contamination by water, acids, or hydrogen peroxide.

C. The tube was heated in a water bath at 55-60°C for 4–5 min to develop the color. The cooled solution was diluted with acetone to 4 ml. The absorbance of the sample solution was measured at 565 nm. The blank soluation was prepared by duplicating the above procedure, but omitting the sample.

Concentrated samples could be further diluted with acetone. If samples have to be kept before measurement, they should be stoppered and stored in the dark, and a decrease in absorbance of about 1.4 per hour at room temperature should be allowed for (9).

For estimation of N–Oxides only, the procedure was the same as above, but omitting stage A (9).

Results

For qualitative study and obtaining more exact results, 1.5, 3, 5 and 8 g quantitaties of wheat and flour samples were analyzed. Since the contamination level (weight) of the samples changes the amount of extracted PAs, therefore sample's color would not be the same. For a more appropriate comparison of color, test tubes were given 5+, 4+, 3+, 2+, 1+ and 0 values based on their color intensity.

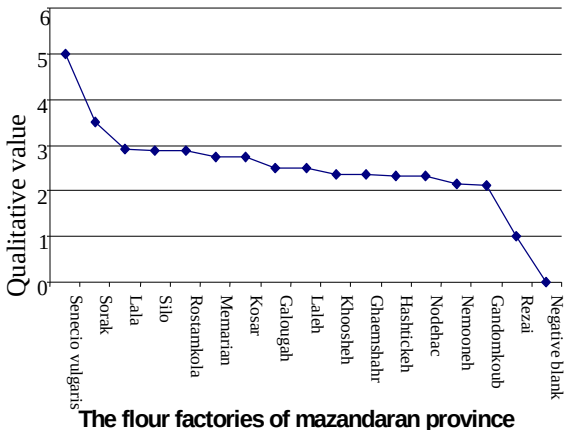
S. vulgaris,which was the positive blank with the highest PAs and had the strongest color was given 5+. Intensity of color, decreases as well as Zero value was related to *Oriza sativa* (fam. Graminae), which was the negative blank without PAs and in comparison with other tubes had the weakest color.

The color of all samples was compared with one of the 6 tubes. To compare the contamination in wheat and flour of all factories and silo, the mean qualitative values of various samples in each factory and silo were calculated. The higher the mean value, the more contaminated the sample examined.

Results of the qualitative studies performed on the wheat are shown in figure 2.

These results only shows the existences of *Senecio's* PAs in samples, and for estimating the amount of PAs and evaluation of their related toxicities, a quantitative study is necessary. In order to perform this study, aspectrophotometric method was used which included oxidation, dehydrogenation and coupling with the Ehrlich reaction stages.

In the oxidation stage, all the solvent was evaporated in 5–10 min and the additional



Figur. 2. Qualitative value of PAs in wheat samples from flour factories of Mazandaran province.

heating time was needed only to remove the excess hydrogen peroxide. Hydrogen proxide containing a small amount of sodium pyrophosphate, consistently gave practically complete oxidation. For successful oxidation, free basic nitrogen is necessary.

The color of modified Ehrlich reagent which contains methanolic boron trifluride (about 14% BF_3) develops at 55-56°C, after 4–5 min. Temperature is important because the color does not reach its maximum below 50°C, and it fades rapidly at high temperatures.

Maximum absorption was in 562–565 nm, with a characteristic inflection at about 330 nm.

The amount of PAs was calculated on the basis of senecionine. Since the isolation of an alkaloid from a given material may result in less than 100% recovery, recovery factor was considered in the calculation for senecionine. Ninety five percent of this compound could be recovered in aqueous solution by four extractions with chloroform.

In addition, *S. vulgaris* contains both PAs and their N–oxides. These can be determined separately.

Recovery factors of PAs and their N–oxides are 0.95 and 0.72 respectively. Slope of estimation curve is 0.06 (9).

The amount of PAs and their N – oxides in the wheat samples are shown in table 1.

Discussion

PAs toxicity is due to the formation of pyrrole metabolites by liver microsomal oxidation and DNA cross–linking with pyrrolemetabolites. In other hand, pyrrole metabolites, which are potent alkylating agents, react with suitable cellular nucleophiles such as nucleic acids and proteins (10, 11, 18).

In the above mechanism, PAs cause vascular disorders including pulmonary vasculitis, damage to vascular smooth muscle cells, proliferation of endothelium and vascular connective tissue in liver. Such vascular disorders cause pulmonary hypertension and hepatic veno–occlusive disease (VCD). The main pathology of VCD, which is the sign of toxicity, is occlusion of the centriolobular hepatic vein with centriolobular hemorrhagic necrosis, hepatocellular megalocytosis, bileduct hyperplasia and cirrhosis (19, 20).

Among the PAs–containing plants, Heliotropium and Senecio have been found to be responsible for VOD in humans. Clinical manifestation of poisoning in human includes abdominal pain, ascites, hepatomegaly and raised serum transaminase levels (18).

Similarly, occlusive changes in pulmonary arterioles leading to pulmonary hypertension and right ventricular hypertrophy could ultimately lead to congestive heart failure (21).

Table 1. Amount of PAs and their N-oxides present in the wheat samples of Mazandaran province

Source	Number of samples (1.024g)	Mean absorbance of Reduced samples at 565nm	Mean absorbance of unreduced samples at 565nm	Mean wt of alkaloid in the whole of reduced extract (mg)	Mean wt of alkaloid in the whole of unreduced extract (mg)	Mean amount of alkaloid in 0.512g of samples (mg)
Sorak	1	0.182	0.402	45.5	22.33	23.50
Lala	6	0.145	0.330	36.25	18.33	19.3
Silo	2	0.143	0.329	36.75	18.28	19.24
Rostamkola	2	0.140	0.328	35	18.22	19.18
Memarian	1	0.132	0.301	33	16.72	17.60
Kosar	1	0.129	0.298	32.25	16.55	17.42
Galougah	3	0.129	0.298	32.25	16.55	17.42
Laleh	2	0.125	0.290	31.25	16.11	16.96
Khoosheh	2	0.117	0.270	29.25	15	15.79
Ghaemshahr	2	0.112	0.259	28	14.4	15.16
Hashtickeh	3	0.112	0.259	28	14.4	15.16
Nodehac	3	0.107	0.248	26.75	13.78	14.5
Nemooneh	3	0.105	0.243	26.75	13.5	14.21
Gandomkouhb	2	0.104	0.242	26	13.44	14.15
Rezai	1	0.085	0.175	21.25	9.72	10.23
Positive blank (Senecio vulgaris)	1	1.323	2.981	330.75	165.61	174.33
Negative blank (oriza sativa)	1	0	0	0	0	0

*Different results of qualitative and quantitative analysis in wheat and flour are not significant (P > 0.05)

Other toxicities associated with PAs include disturbances of gastrointestinal, pancreatic and renal function (8). It is believed that PAs are natural carcinogens and act as an initiator for damaging the DNA, causing cellular deformation (22, 21).

Also PAs cause teratogenicity and mutagenicity. Among them macrocyclic alkaloids such as sencionine have exhibited the strongest mutagenicity (6, 7).

LD_{50} of senecionine is 64.12±2.24 mg/kg (22). LD_{50} for a pyrrolizidine–rich extract of *Senecio* was found to be 160 mg/kg (23). Toxic doses in nonfatal disease have ranged from 2 to 27 mg alkaloid/kg of body weight and from 6 to 167 mg alkaloid/kg of body weight in fatal cases(19).

The total amount of PAs and their N–oxides in samples of wheat and flour of Mazandaran province have ranged from 0.02–0.05 mg/0.512 g of test sample and 0.4 g / 0.512 g of *S; vulgaris* (Table 1).

As a result of ingesting about 20-50 g of wheat and flour, or 2.5 g of S. vulgaris, one could receive 2 mg of PAs. Based on the consumer's weight, ingestion of wheat and flour preparations in daily dietary could result in chronic toxicity with PAs.

Although wheat and flour specimens can not produce acute complications of Pas, but long term exposure to low levels of PAs may cause a cumulative damage to body organs (6).

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