Original Article

PRODUCTION TECHNOLOGY AND ANTITOXIC EFFECTS OF N-METHYLCYTISINE

A.A.Azamatov[1], G.B.Sotimov[2], F.M.Tursunkhodzhaeva[3*]

[1]Pharmacology and Toxicology Department, S.Yu.Yunusov Institute of the Chemistry of Plant Substances, Sciences Academy of the Republic of Uzbekistan, 77, Mirzo Ulugbek st., Tashkent, 100170, Uzbekistan.
azizbek.azamatov@bk.ru
[2] Experimental Technology Laboratory, S.Yu.Yunusov Institute of the Chemistry of Plant Substances, Sciences Academy of the Republic of Uzbekistan, 77, Mirzo Ulugbek st., Tashkent, 100170, Uzbekistan.
dr.sotimov@mail.ru
[3]Pharmacology and Toxicology Department, S.Yu.Yunusov Institute of the Chemistry of Plant Substances, Sciences Academy of the Republic of Uzbekistan, 77, Mirzo Ulugbek st., Tashkent, 100170, Uzbekistan.
ftm40438@gmail.com
Corresponding author: F.M.Tursunkhodzhaeva, S.Yu.Yunusov Institute of the Chemistry of Plant Substances, Sciences Academy of the Republic of Uzbekistan, 77, Mirzo Ulugbek st., Tashkent, 100170, Uzbekistan.
ftm40438@gmail.com

DOI: 10.47750/pnr.2022.13.S07.421

Abstract

Quinolizidine alkaloids attract an interest due to their physiological activity, and wide distribution in plants of the Central Asia. The aerial part of *Thermopsis alterniflora* is used as a raw material in our investigation. Alkaloids have been reported as the main group of biologically active compounds of this plant. A long-term study of the alkaloid sum isolated from *Thermopsis alterniflora* found cytisine and N-methylcytisine in equal amounts. A technology for complex processing of the plant *Thermopsis alterniflora* to produce cytisine, N-methylcytisine, and Flateron (flavonoids sum) has been developed. It was shown that N-methylcytisine in severe and extremely severe acute alcohol intoxication reduces the severity of intoxication, and has antihypoxic and antioxidant effects. It may have potential therapeutic use as antidote remedy.

Keywords: *Thermopsis alterniflora*, N-methylcytisine, antitoxic, antihypoxic, antioxidant.

Introduction

Plants contain various groups of biologically active substances with different physical, chemical properties, and biological properties. For their complete extraction, complex processing of plant raw materials is necessary. This process is also economically beneficial, as resource-saving, low-waste and allows obtain several drugs from one raw material. With the complex use of raw materials, it is necessary to use solvents of different polarity, and the selection of optimal conditions at all stages of the technological process.

Quinolizidine alkaloids attract an interest due to their physiological activity, and wide distribution in plants of the Central Asia. The aerial part of *Thermopsis alterniflora* is used as a raw material in our investigation. Alkaloids have been reported as the main group of biologically active compounds of this plant. The aerial part of *Thermopsis alterniflora* at the beginning of flowering contains 3.35% of combined alkaloids.[1] The separation of the combined bases yielded cytisine, N-methylcytisine, pachycarpine, thermopsine, and alteramine. The use of alkaloid cytisine in medicine is well-known.[2] Also, this plant contains 2.35 % of the sum of flavonoids (relative to the initial air dry material weight). A hypolipidemic drug Flateron has been developed on their base in ICPS.[3]

A mass production of the cytisine substance from *Thermopsis alterniflora* has been established on the basis of the Pilot manufacture of ICPS, the Academy of Sciences of the Republic of Uzbekistan. Every year, more than 100 tons of air-dried plant can be harvested on the territory of Uzbekistan without damage of nature. A long-term study of the alkaloid sum isolated from *Thermopsis alterniflora* found cytisine and N-methylcytisine in equal amounts. Previously, we reported that alkaloids pachycarpine, N-methylcytisine, alteramine, thermopsin, argentamine, dimethamine, argentine remain in the mother liquor after isolation of cytisine nitrate. We found that the mother liquor contains up to 60% N-methylcytisine. A technology for complex processing of the plant *Thermopsis alterniflora* to produce cytisine, N-methylcytisine, and Flateron (flavonoids sum) has been developed. [3,4]

Our primary pharmaco-toxicological study showed that N-methylcytisine has an antitoxic effect surpasses cytisine in acute alcohol poisoning in mice. It shortens the duration of narcotic sleep caused by the introduction of subtoxic doses of ethanol by 3-4 times.[5] The continuation of these works showed that N-methylcytisine in antitoxic activity in severe and extremely severe acute alcohol intoxication significantly exceeds naloxone, an opiate antagonist, aminostigmine, an inverse cholinesterase inhibitor, picamilon, a GABAergic drug from the group of nootropics, cytisine, a respiratory analeptic, and is not inferior to metadoxil - a metabolic therapy drug, which are prescribed in acute alcohol intoxication. [6,7]

Thus, we have previously shown the prospects for the use of N-methylcytisine in severe alcohol intoxication.

The aim of our study is to investigate pharmacological properties, acute toxicity of N-methylcytisine in two animal species with different ways of administration, and to develop an industrial technology for its production from wastes remaining after cytisine production.

Materials and Methods

Investigation of factors influencing the process of extraction of alkaloids from raw materials

At the first stage of the experiments, the factors that affect the process of alkaloids extraction from raw materials were investigated. In extraction of plant raw materials, the correct choice of the extractant plays an important role, therefore, we tried the extraction of raw materials with a number of organic solvents. The extraction of each sample of *Thermopsis alterniflora* was carried out five times under the same conditions. Air-dried raw material samples (0.1 kg each) were loaded into extractors and extracted with various solvents (ethyl alcohol, methyl alcohol, isopropyl alcohol), draining after 6–8 h. The combined extract of each extractor was evaporated and acidified with sulfuric acid. Then the filtrate was alkalized with a sodium hydroxide solution, the alkaloids were extracted from the aqueous part with chloroform, distilled, and dried to constant weight, and the content of the total alkaloids was determined.

Further, to determine the optimal particle size, the raw material (particle sizes 8–10, 5–7, 3–5, 1–3, and 0.5–1 mm) was extracted 0.1 kg each with 80% alcohol until the complete depletion of the raw material. The combined extract of each batch was concentrated and acidified with sulfuric acid. Alkaloids were extracted with chloroform from the aqueous residue after alkalization with sodium hydroxide solution. The content of the total alkaloids was determined.

The temperature plays an important role in extraction of plant substances. The extraction rate increases and the solvent consumption decreases with growth of temperature. Extraction of N-methylcytisine was carried out with 80% ethanol at different temperatures (20–60°C) under the same conditions.

Study the extraction kinetics was investigated to develop a rational regime for the extraction of N-methylcytisine from raw materials. 0.1 kg of the crushed aerial part of *Thermopsis alterniflora* was loaded into six extractors and filled with a measured amount of 80% ethanol. Drains were made sequentially with an interval of 1 hour: from the first extractor - after 1 hour, from the second - after 2 hours, from the third - after 3 hours, and so on. The content of the total alkaloids was determined in six samples of extracts obtained with different infusion times. In

this case, the phase equilibrium was determined at the first contact of the phases.To establish phase equilibrium at the second contact, the experiments were carried out under the following conditions. 0.1 kg of crushed raw materials were extracted in six extractors for 5 h. The extracts were drained and poured with new portions of the solvent. Every 1 hour, the extract from the corresponding extractor was drained to determine the change in the concentration of the total alkaloids.

To determine the duration of the process at the third contact of the phases, the same amount of plant material was extracted twice (5 and 3 h) until equilibrium in the system. Then, the time required to achieve phase equilibrium was specified. Similarly, the phase equilibrium was determined at the fourth and fifth phase contacts.

25 kg of the aerial part of *Thermopsis alterniflora* with a moisture content of 12% is loaded into the extractor and 80% alcohol (150 L) is poured. The extraction is repeated 5 more times, pouring 125 liters of alcohol and insisting for 6-8 hours. The combined alcohol extract (720 L) is evaporated in a vacuum evaporator, and fed from the collector in portions of 15-20 L. Steaming is carried out at a temperature of 40-50 ºC and a vacuum of 0.04-0.08 MPa (0.4-0.8 kgf/cm^2). The obtained 5.2 L of condensed aqueous extract was acidified with sulfuric acid to pH 3-4 and left overnight. The precipitated phenolic compounds were separated and removed from the technological cycle. The acidic solution of the alkaloid sum was alkalized with NaOH to pH 6-7 and extracted 3 times with 2 L of chloroform. The chloroform extract containing thermopsine, pachycarpine and associated neutral and weakly basic substances was evaporated, and this way the said substances were removed from the technological cycle.

Then aqueous alkaline solution was alkalinized and extracted with chloroform. Chloroform was evaporated, and technical N-methylcytisine was crystallized from extraction gasoline. 95.0 g of N-methylcytisine (0.38% by weight of the raw material) was yielded.

Animal experiments

Animals were kept under standard vivarium conditions with a natural 12-hour light and shade cycle, at an air temperature of 20±2^{0}C. Animals had unlimited access to food and water. Animal experiments were conducted in accordance with the International Convention for the Protection of Vertebrate Animals used for Experimental and Scientific Purposes (Strasbourg, 1986), and the ethic and protocol of experiments was approved by the Institutional Scientific Board (Protocol No. 1 from January 20, 2022).

The ability of substance to reduce acute alcohol intoxication (AAI) was determined by their effect on the duration of the lateral position of mice caused by a narcotic dose of ethanol. Subcutaneous administration of the testing substance was performed 30 minutes prior to intraperitoneal administration of a 25% ethanol solution at a dose of 4.5 g/kg for rats and 6.0 g/kg for mice.

Acute toxicity of the substance was determined with different ways of administration in mice and rats. Each group contained 10 mice and rats. LD_{50} was determined graphically by the well-known Litchfield-Wilcoxon method.[8]

Cytotoxic hypoxia was induced in mice by a single intraperitoneal injection of sodium nitroprusside at a dose of 20 mg/kg. The criterion in evaluating the antihypoxic effect of the studied substance was the life span of experimental animals. The testing substance was administered at doses of 0.1-5.0 mg/kg subcutaneously 30 minutes before nitroprusside.

Antioxidant activity was determined by the reaction of autoxidation of adrenaline in an alkaline medium in the presence of SOD due to the dismutation of superoxide anion radicals. The investigated substance was introduced to rats one hour before the determination of antioxidant activity. 50 µl of serum and 450 µl of distilled water cooled to 0°C were added to the test tube. 250 µl of ethanol-chloroform mixture was added to eliminate the interfering effect of hemoglobin. Next, the samples were mixed and left to incubate at room temperature for 10 minutes. The resulting suspension was stirred and centrifuged for 10 min. at 6000 g. The supernatant was used to determine SOD. 0.15 ml of adrenaline solution was added to the samples before determination of optical density. The change in optical density was recorded for 3 min. every 30 sec. at a wavelength of 347 nm in a cuvette with

a layer thickness of 1 cm. To calculate the activity, the absorption values of the control and experimental samples were used.

The calculation was carried out according to the formula:

SOD/t=(Ex-E0/Ex) x 100%/50 x F x V x 1000/5- x v x d, where

(Ex-E0/Ex) x 100%/50 - activity unit, 50% inhibition of adrenaline oxidation reaction

V is the total volume of the incubation sample

F - dilution factor (15)

v is the volume of the supernatant used to determine the activity of SOD,

d is the length of the optical path of the cell (1 cm).

Results and discussion

To develop the technology for the production of the N-methylcytisine substance, the technological cycle was studied by stages. The factors influencing the process of extraction of alkaloids from raw materials were studied (Table 1).

The results indicated that the yield of alkaloid sum was the highest in extraction with 80% ethanol, so, this extragent was chosen for further research.

Table 1: Influence of the extractant on the yield of alkaloids

Extractant	Extractant content, %	Yield, % from content in raw material of	
		Alkaloid sum	N-methylcytisine
Ethanol	95	86.5	76.6
	90	87.9	79.3
	80	95.1	85.3
	70	91.4	81.6
Methanol	95	85.5	72.6
	90	86.1	71.3
	80	91.9	82.8
	70	90.1	80.6
Isopropanol	95	83.1	70.2
	90	84.4	72.6
	80	85.8	73.9
	70	84.7	73.3

Investigation of the dependence of alkaloid yield from the particle size of raw material shown, that alkaloids extracted faster when raw material grounded to 0.5–1 mm, but the isolated extract was cloudy and difficult for purification. The best result was noted in particle with size of 3–5 mm, which was sufficient for normal extraction (Table 2).

Table 2: Yield of alkaloids in dependence of raw material grinding

Particle size, mm	Number of draines	Yield of total alkaloids, % from content in raw material
Ungrounded raw material	7	76.2
8-10	7	81.1
5-7	6	82.4
3-5	5	90.3
1-3	4	88.9
0.5-1	4	84.3

The temperature plays an important role in raw material extraction. The extraction rate increase with temperature growth and led to the less solvent consumption. Extraction of cytisine was carried out with 80% ethanol at different temperatures (20-60°C) under the same conditions. The results of the experiments showed that at 60°C the depletion of raw materials occurs faster, but the resulting extract contains more related substances. During alkaloid sum purification from related substances, losses of cytisine occurred. Therefore, extraction at room temperature is the most optimal and does not require additional heating (Table 3).

Table 3: Effect of temperature on alkaloids extraction

Extraction temperature,°C	Number of draines	Yield, % from content in raw material of	
		Alkaloid sum	N-methylcytisine
20-30	5	95.2	85.4
30-40	5	95.4	78.3
40-50	5	95.9	72.1
50-60	5	96.3	69.9

Study of the dependence of alkaloids extraction dynamic of time shown in the Table 4. To achieve an equilibrium concentration 5 hours needed at the first contact of the phases, 3 hours at the second, 2 hours at the third, 1 hour at the fourth and fifth contacts of the phases.

Table 4: Dependence of alkaloids extraction dynamic of time

Infusion time, h	Yield of alkaloids, % from content in raw material				
	First contact of phases	Second contact of phases	Third contact of phases	Fourth contact of phases	Fifth contact of phases
1	0.2	0.1	0.15	0.1	0.05
2	0.3	0.2	0.15		
3	0.4	0.3	0.15		
4	0.5				
5	0.6				
6	0.6				

Thus, the extraction of the aerial part of *Thermopsis alterniflora* with a particle size of 3-5 mm should be carried out with 80% ethanol at room temperature five times with a yield of alkaloid sum at least 85% of the content in the raw material.

The IR and UV spectra of obtained N-methylcytisine are shown on Fig. 1 and 2.

Figure 1. IR spectrum of N-methylcytisine

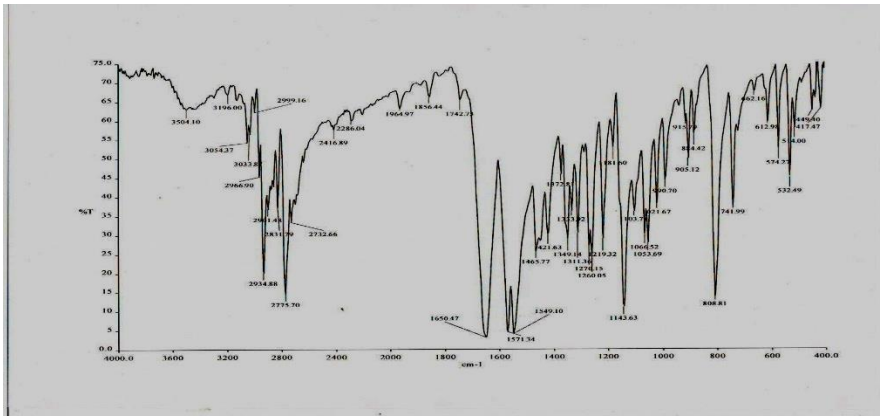
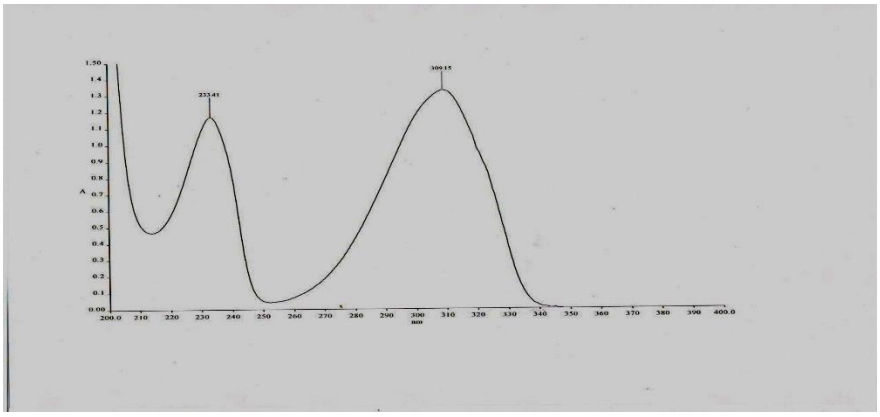


Figure 2. UV spectrum of N-methylcytisine



Toxicity of the obtained sample of N-methylcytisine was studied in white mice and rats. The results of the experiments found that LD_{50} in subcutaneous injection to mice is 40.1 mg/kg, and for rats - 38 mg/kg. The resorptive action of N-methylcytisine shown inhibition of spontaneous motor activity, exophthalm, increased and deep breath, tremor, in subtoxic and lethal doses clonic-tonic convulsions and death from primary respiratory arrest.

It has been shown that acute alcohol intoxication is characterized by decrease of the permeability of erythrocyte membranes and the level of transferrin, while the level of proteins carbonyl derivatives, content of glutathione in erythrocytes and ceruloplasmine in blood plasma increase. [9]

In hemic hypoxia caused by sodium nitrite N-methylcytisine bromhydrate at a dose of 0.1 mg/kg subcutaneously increased the reserve time of animal survival for 31.4%, and at cytotoxic hypoxia caused by sodium nitroprusside – for 40.9 and 44.7% at doses of 0.5 and 1.0 mg/kg, accordingly (Table 5).

Table 5: Mice survival at cytotoxic hypoxia caused by sodium nitroprusside (n=10)

No.	**Substance**	**Dose, mg/kg**	**Survival time, min.***	**Increasing of reserve time of survival, %**
1	Sodium nitroprusside	20	10.5±0.8	-
2	Succinic acid	100	12.2±1.3	16.1
3	Cytisine	0.1	12.8±1.0	21.9
		0.5	12.4±1.2	18.0
		1.0	11.5±1.1	9.5
		2.0	10.8±0.8	2.8
4	N-methylcytisine	0.1	12.5±1.2	19.0
		0.5	14.8±1.0	**40.9**
		1.0	15.2±0.9	**44.7**
		5.0	12.4±0.7	18.0

Note:*P=0.05 in comparison to control group

The study of the antioxidant effect of N-methylcytisine by changes in SOD activity during adrenaline autoxidation showed that N-methylcytisine in the first minute of the reaction increases SOD activity by 15.9 times compared with the control, then the SOD activity slightly decreased, for 2 minutes - 6.3 times, and for 3 minutes - 5.8 times.

The result of investigation suggests that N-methylcytisine increases the efficiency of biological oxidation and can positively influence the outcome of acute ethanol poisoning by elimination the consequences of oxidative stress.

Conclusion

Thus, N-methylcytisine reduces the severity of acute alcohol intoxication, the anesthesia time, and has an antihypoxic and antioxidant effects. This preparation may have potential therapeutic value as antidote remedy.

Acknowledgement

These experiments were supported financially by the budget fundamental projects “Screening, pharmacological study, structure-activity, investigation of biological action mechanisms” and “Creating technologies for the production of medicinal substances and their drug forms from local plant raw materials, as well as improving the existing ones, including developing improved technologies for the complex processing of raw materials in order to effectively use the biologically active substances contained in plants” given by Sciences Academy, Republic of Uzbekistan.

References

1. Shaimardanov RA, Iskandarov S, Yunusov SY. A study of the alkaloids of *Thermopsis alterniflora*. *Chem Nat Compd* 1971; 7: 160–4.

2. Karnieg T, Wang X. Cytisine for smoking cessation. CMAJ. 2018 May 14; 190(19): E596.

3. Satimov GB, Mamatkhanov AU, Kotenko LD, Madrakhimov ShN, Faizieva SKh *et al.* A Total Flavonoid Extract from *Thermopsis altherniflora*: Development of the Production Technology, Creation of a Medicinal Preparation, and Evaluation of Hypolipidemic Activity. *Pharm Chem J* 2003; 37: 203–6.

4. Mamatkhanova MA, Sotimov GB, Vinogradova VI, Kotenko LD, Mamatkhanov AU. Development of a technology for obtaining cytisine and flateron from the aerial part of *Thermopsis alterniflora*. Chem and Chem Tech 2016; 4:40-4.

5. Azamatov AA, Tursunkhojaeva FM, Rezhepov, Rakhimov ShB. Study of the effect of cytisine derivatives on the course of acute alcohol intoxication. Reports of the Academy of Sciences of the Republic of Uzbekistan 2013;6: 39-1.

6. Azamatov AA, Rejepov J, Tursunkhodjayeva FM, Rakhimov Sh B, Vinogradova VI. Antixypoxant, anti-narcotic activity and acute toxicity of N-methylcytisine. European science review 2018; 5-6:123-6.

7. Azamatov AA, Rejepov J, Tursunkhodjayeva FM. Antitoxic effects of two new N-benzyl derivatives of cytisine in acute and chronic alcohol intoxication. European science review 2018;11-12: 46-8.

8. Litchfield Jr JT, Wilcoxon F. A simplified method of evaluating dose-effect experiments. J. Pharmacol. and Exp. Ther. 1949, 96 (2): 99-13.

9. Terekhin NA, Zhidko EV, Terekhin GA, Goryacheva OG. Oxidative modification of proteins and indicators of antioxidant protection in acute alcohol intoxication. Medical alphabet 2017;4(28):53-54.