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Original Article

The Antihypoxic and Sedative Activity of the Dry extract from *Asperula odorata* L.

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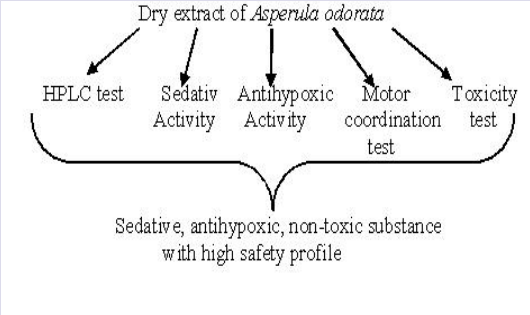
ABSTRACT

Introduction: Sweet Woodruff (*Asperula odorata* L.) is a perennial herb that is spread throughout the territory of Ukraine. This herb is widely used in folk medicine of Ukraine as a sedative medicine at neurosis, neurasthenia, hysteria, depression, applied locally at allergic rash, and it is also used at metritis and colpitis in homeopathy. **Materials and methods:** The air-dried herb of *Asperula odorata* was degreased with chloroform and then treated with the mixture of ethyl acetate-alcohol (8:2) to obtain a dry extract. Sedative activity was studied in the "open field" test. The protective effect of dry extract against hypoxia-induced lethality in mice was evaluated by experimental model of asphyctic hypoxia. HPLC analysis was used to identify the phenolic compounds in the dry extract. **Results:** The dry extract at the dose of 100 mg/kg in the "open field" test caused a significant dose-dependent ($p>0.05$) decreasing of loco motor activity which has evidenced by potent sedative activity. The dry extract at the dose of 100 mg/kg showed a potent anti-hypoxic activity which has been confirmed by increasing mice lifetime by 34.8% in conditions of model hypoxia. The dry extract reveals a sedative activity on the CNS but it doesn't have an adverse effect on skeletal muscle tone and coordination. In the dry extract, 16 phenolic compounds have been identified and quantified. Chlorogenic acid and cynarozide are dominant compounds. **Conclusion:** Our data have provided a rational base for the folkloric use of the dry extract from Sweet Woodruff as a sedative and antihypoxic drug.

Key words: Antihypoxic activity, dry extract, HPLC, Phenolic compounds, Sedative activity, Sweet woodruff.

SUMMARY

The results of the research of sedative and antihypoxic activity of *Asperula odorata* L. dry extract showed a high antihypoxic activity at a dose 100 mg/kg. The dry extract reveals a dose dependent sedative activity on the CNS but it doesn't have an adverse effect on skeletal muscle tone and coordination. By the HPLC method in the dry extract 19 phenolic compounds were revealed, 10 of which were identified. Chlorogenic acid and cynarozide are dominant compounds which provide its pharmacologic activity.



PICTORIAL ABSTRACT

Abbreviations used: CSRL: Central scientific research laboratory, CNS: Central nervous system, HPLC : High performance liquid chromatography.

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INTRODUCTION

The species of the genus *Asperula* L. Family *Rubiaceae* Juss.in the flora of Ukraine are represented by more than 200 species.[1] Plants are not officinal species, but are widely used in folk medicine of Ukraine, which creates a background for their integrated pharmacognosy study. *Sweet Woodruff* (*Asperula odorata* L.) is a perennial herb that is spread throughout the territory of Ukraine. This plant contains different bioligicaly active compounds: in the rhizomes and roots–alizarin and derivatives of anthracene; in the herb–the essential oils, phenol carboxylic acids, coumarins, flavonoids, tannins and saponins.[2-4]Among lipophilic substances mainly iridoids and terpenoids from some species have been studied.[2-4]

The problem of hypoxia attracts the attention of physiologists and clinicians in terms of the mechanisms of various pathological conditions. It is known that hypoxia occurs in most pathological conditions of the human body and plays a key role in the pathogenesis of such diseases as coronary heart disease, myocardial infarction, ischemic stroke and other brain dysfunctions, lungs, liver, kidney and fetal pathology.[5] Histotoxic (tissue) hypoxia is usually the result of an acute poisoning by exogenous toxicants such as cyanides, alcohol, acetone, preventing the utilization of oxygen by blocking the processes of oxidation and reduction of cytochromes. The acute normobaric hypoxia with hypercapnia often occurs in the body in shock, accompanied by a slow circulation of blood, accumulation in the tissues of carbon dioxide in the lungs and microcirculation disorders.[5,6]

An important aspect of the prescription of these drugs is the high cost of modern anti-hypoxic agents, which limits their widespread use in medical practice for a large number of people.

There is limited number of antihypoxic drugs of herbal origin. The most effective drug obtained from leaves of maidenhair tree is Bilobil.[7-9] There by searching and developing effective and safe antihypoxic herbal drugs is important for modern medicine and pharmacy.

Continuing the study of the genus Woodruff (*Asperula* L.), we have developed a technology for producing dry extract from sweet woodruff's *Asperula odorata* L. (*Galium odoratum* (L.) Scop.) herb.

The subject matter of the current research is to investigate the antihypoxic and sedative activity of a dry extract from sweet woodruffherb.

MATERIALS AND METHODS

Obtaining the dry extract

The object of the study was *Asperula odorata* dry extract which was obtained by heating water extraction of exhausted herbal drugs, which remained after the sequential obtaining of lipophilic and phenolic complexes. Purified

extract was evaporated in vacuum to dryness after removal of protein-polysaccharide complex. The yield of the dry extract was 12.03% of the dry herb.

Sedative activity screening

Taking into consideration the fact that the herb of different species of woodruff is used in folk medicine as a sedative medicine at epilepsy, neurasthenia and depression, the influence of dry extract on behavioral reactions in the "open-field" test was studied.[10,11]

Treatment groups

Animals were randomly divided into 3 groups containing 8 animals in each group:

group 1–control control, received distilled water;

group 2–received aqueous extract of *Asperula odorata* herb (50 mg/kg);

group 3 – received aqueous extract of *Asperula odorata* herb (100 mg/kg);

Dose administration

The aqueous solution of dry extract was administered intra gastrically using an oral feeding needle in a preventive mode for a continuous period of five days (the last over 30 minutes before the test). The effect of aqueous solution of dry extract on spontaneous activity was evaluated in mice using special apparatus. The mice were placed individually in the apparatus subsequent to administering and the ambulation was recorded for 3 minutes. The auto-track is an advanced automated Open Field Activity Monitor system using the technology to quantify locomotor activity and trace the animal's path for behavioural analysis. The authomatic system senses motion with a grid of infrared photocells placed around a specified arena. Vertical motion is detected by a second array of photocells placed above the animal. The simultaneous interruption of beams along the horizontal axis provides coordinates that identify animal location. Vertical motion is scored and stored with horizontal position data. Auto track recods these coordinates for later playback and analysis. The investigation of stereotyped motor activity has elucidated the role of various brain mechanisms in the behavior of animals and humans.

Motor coordination screening

Rotarod method was used for screening the motor coordination.[11] This test is used to evaluate the activity of drugs interfering with motor coordination by assessing the ability of mice to remain on an accelerating revolving rod. The rotating rod is divided into four lanes separated by screens. The mouse must walk forward to balance on the rotating center bar. This forced motor activity has subsequently been used by many investigators. Only those animals which demonstrate their ability to remain on the revolving rod for at least 30seconds are used for the test. The length of time that a given animal stays on this rotating rod is a measure of their balance, coordination, physical condition and motor planning.

The animals were tested during the light period and observed in a closed room at constant temperature by the screening methods mentioned above.

Antihypoxic activity screening

Antihypoxic activity of the dry extract (50 and 100 mg/kg) was estimated on model acute hypoxia (normobaric hypoxia) according to the Guidelines of experimental pre-clinical studies of new pharmacological compounds.[12,13,18]

The study was conducted on 44 white male mice weighing 20-25 g divided into 5 groups. Mice were individually weighed and the required dose was calculated for every mouse based on their body weight. Animals of the first and second groups were administered aqueous solution of dry extract at a dose of 50 mg/kg and 100 mg/kg, respectively, in a preventive mode per orally for a continuous period of 6 days and over 30 min before the experimental hypoxia. "Bilobil" (KRKA, Slovenia) was chosen as the reference drug. It was administered to the mice of the fourth group at a dose of 100 mg/kg in the same regime. The animals of the two control groups were administered with the equivalent volume of distilled water.

For modeling acute normobaric hypoxia with hypercapnia, animals were placed in a 200 cm^3germokamer and recorded the time of their death. The longevity of mice was selected as an integral indicator of antihypoxic action.

All animals used in the experiments were kept under standard vivarium conditions of central scientific research laboratory (CSRL) of National university of Pharmacy in accordance with sanitary norms and principles of the European Convention for the Protection of laboratory animals (Strasbourg, 1986).

Statistical analysis was performed using the methods of variation statistics. Reliability of intergroup differences was determined by Student t-test.[14]

Phytochemical screening

HPLC analysis has been used to identify the phenolic compounds in the dry extract.[15,16]

The dry extract was dissolved in 90% methanol and filtered through a teflon membrane filter having a pore size of 0.45 mcm in the vial for analysis. Identification of phenolic compounds was performed by retention time and spectral characteristics of the standards.

Analysis was performed on the chromatograph Agilent Technologies (model 1100) equipped with a flowing vacuum degasser G1379A, 4-channel gradient pump low pressure G13111A, G1313A automatic injector, column oven

Table 1: The influence of dry extract from Sweet Woodruff herb on the factors of behavioral and emotional reactions in mice in the "open field" test (M ± m)

Index (3 min.)	Intact control, n=8	Dry extract	
		50 mg/kg n=8	100 mg/kg n=8
Locomotor activity	29.50 ± 2.65	26.88 ± 4.19	24.50 ± 2.90
Orient–research activity			2
• holes	39.63 ± 4.17	31.88 ± 2.75	32.75 ± 4.34
• racks	11.00 ± 2.13	8.88 ± 2.10	7.00 ± 1.68
• sum	50.63 ± 5.78	40.75 ± 2.93	39.75 ± 4.42
Vegetative maintenance of emotional responses:			
• boluses	1.13 ± 0.48	1.00 ± 0.76	0.75 ± 0.41
• urination	0.13 ± 0.13	0.00 ± 0.00	0.00 ± 0.00
• grooming	0.88 ± 0.29	1.13 ± 0.30	0.88 ± 0.23
• sum	2.14 ± 0.44	2.13 ± 0.83	1.63 ± 0.49
The sum of all types of activities	82.25 ± 7.99	69.75 ± 6.82	65.88 ± 6.95

Table 2: The influence of dry extract on muscle tonus and coordination in terms of rotarod method

Group	Fallen to30 sec.	Fallen to 1 min.	Fallen to3 min.
Control, n=5	2/40.0%	4/40.0%	4/80.00%
Dry extractfrom A. odorata 100 mg/kg, n=12	1/8.33%	7/58.33%	10/83.33%

Note.- the absolute number of animals is in the numerator, in the dominator–%

Table 3: Study of antyhypoxic activity of sweet woodruff dry extract and «Bilobil» on the model of normobaric hypoxichypoxia with hypercapnia in mice (M ± m)

Group of animals	*n*	Lifetime	Group of animals	n	Lifetime
Control	8	22.3 ± 0.45	Control	12	17.7 ± 0.62
Dry extract A. odorata, 50 mg/kg	7	27.60 ± 1.78*	«Bilobil»	10	20.3 ± 0.57*
Dry extract A. odorata, 100 mg/kg	7	30.38 ± 2.20*			

Notes: * – probable deviations in comparison with the control group, p<0,05;
n–number of animals in a group

G13116A, diode detector G1316A; chromatographic column size 2,1×150 mm filled with sorbent grained 3.5 microns ZORBAX-SB C-18.

Chromatographic conditions: flow rate of mobile phase is 0.25 ml/min; operating pressure of 240-300 kPa eluent; column oven temperature of 35°C; 2 ul sample volume; as mobile phase was used a mixture solution of 0.1% phosphoric acid and methanol with increasing concentration of the latter in a mixture from 10% to 100%. Detection parameters: scale measuring 1.0; scan time of 0.5 s; options removing spectrum - each peak 190-600 nm; wavelength nm: 280, 313, 350, 371, 254.

RESULTS AND DISSCUSION

A set of screening tests was conducted over a period of six days to assess the effects of a dry extract of *Asperula odorata* on the central nervous system at a dose of 50 and 100 mg/kg per orally. The data in the Table 1 demonstrates that in the open field test, there is a significant trend (p>0.05) of decreasing the locomotor activity at a dose of 100 mg/kg. The results indicate on an expressed sedative activity.

Table4: Phenolic compounds of sweet woodruff dry extract

Compound	Hold time, min	Content, mg/100 g
Caffeic acid derivative*	9.47	348.8
Chlorogenic acid	13.12	1675.1
Caffeic acid derivative*	13.49	481.0
Quercytinglycoside*	14.68	23.6
p-Coumaric acid derivative*	15.57	26.2
Kempherol glycoside*	16.18	45.7
Caffeic acid derivative*	16.29	56.7
o-Coumaric acid	16.87	9.4
p-Coumaric acid	17.27	13.1
Not identified	18.28	48.1
Not identified	18.63	65.7
Luteolin glycoside	18.9	451.7
Caffeic acid derivative*	18.96	195.0
Rutin	19.9	124.5
4,5- Dicofeilchinic acid	20.34	186.0
Luteolin-7-O-glycoside	20.76	1409.9
Kempherol-3-O-glycoside	21.42	86.5
Diosmetin-7-O-glycoside	21.71	54.0
Quercetin	23.31	6.1

Note. * –has not been fully identified compound.

It was shown that the dry extract displays a marked inhibiting effect on all types of activities without noticeably affecting, if not reducing the accompanying vegetative indices of emotional responses.

The motor coordination effect was evaluated by the Rotharod method as an ability of mice to remain on an constant revolving rod. The results presented in Table 2 obtained to testify for an absence of an adverse effect of the extract under study on either skeletal mass tonicity or motor coordination. Furthermore, the woodruff dry extract seemed to enhance the riding performance of the test mice, whereby the percentage of fall incidence within 30 sec. was reduced by 31%, which can be indicative of the extract's selectivity on CNS.

The data presented in Table 3 indicated to an increased life span in mice after the administration of woodruff dry extract at a dose of 100 mg/kg under acute normobaric hypoxia (p<0.05). Statistically, the dry extract extended the life span of the laboratory animals by 34.8 %, thus exceeding the results of the control group which were administered a reference drug, "Bilobil", showing a 14.6 % increase under the same conditions. Thus, an antihypoxic effect of woodruff dry extract proved to be twice as high as that of the reference drug "Bilobil".

Toxicity screening

To determine the acute toxicity, we used42 nonlinear white laboratory mice of both sexes weighting 20–22 g, aged 2.0–2.6 month. That was determined by the statistics and the duration of experiment. Study was carried out on the laboratory animals which were divided into 7 groups: Group 1 (n=6) - control - animals which received per orally distilled water; 2-3 groups (n=6) - animals, which received aqueous suspension of dry extract at doses that correspond to different classes of toxicity: 50 mg/kg, 500 mg/kg and 5000 mg/kg in volume 0.8 ml each. Observations were conducted for 14 days. Assessment of acute toxicity was performed by clinical toxicity test, including animals general condition, the functional state of the skin and count the number of died animals.[17-19]

Absence of mortality in mice suggests that LD_{50} of the studied extract exceeds the maximum dose that was used in the experiment–LD_{50}>5000 mg/kg. This value of LD_{50}let us designate the studied complexes to the V class of toxicity–practically non-toxic substances according to the classification by SidorovK.K.[20]

Qualitative phytochemical screening

As a result of research in the dry extract 19 phenolic compounds were revealed (Table 4), 10 of which were identified.

Dry extract contains 5307.1 mg/100 g (5.31 %) of phenolic compounds from which hydroxycinnamic acids are 2991.3 mg/100 g and flavonoids are 2315.8 mg/100 g.

Chlorogenic acid is a predominant compound - 1675.1 mg/100 g, among the hydroxycinnamic acids, and flavonoids were found derivatives of luteolin, kaempferol, diosmetin, quercetin, cynarozide (luteolin 7- O- glucoside) prevails-1409.9 mg/100 g, the presence of which in large quantities is characteristic only for dry extract while in herbal drugsrutin dominates. There is a direct relationship between chemical composition of dry extract and its antihypoxic activity.[21]

CONCLUSION

In this study, the sedative and antihypoxic activity of dry extract from Sweet Woodruff herb have been determined. The study is focused on the comparative analysis between the pharmacological activity and chemical constituents. By the method of HPLC 19 phenolic compounds were identified and quantified: chlorogenic, *o*-coumaric, *p*-coumaric, 4,5- caffeoylquinicacid, quercetin , rutin, kaempferol -3 -O- glucoside, diosmetin-7 -O- glucoside and luteolin -7 -O- glucoside. Chlorogenic acid and cynarozide dominate in the dry extract. The dry extract at a dose 100 mg/kg showed a high antihypoxic activity, which is almost twice higher than that for the reference drug "Bilobil". The dry extract reveals a dose dependent sedative activity on the CNS but it doesn't have an adverse effect on skeletal muscle tone and coordination. According to the classification of Sidorovthe dry extract belongs to the Vclass of toxicity -practically non-toxic substances. So it is a perspective substance for development to antihypoxic drugs with high safety profile.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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