*Original Research Article*

# Behavioural Reactions of Random-Bred Mice under the Influence of Hypouricemia and *Aegopodium podagraria* L. Preparations

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## ABSTRACT

**Aims:** *Aegopodium podagraria* L. preparations normalize uric acid metabolism and exert organoprotective effects. Still, their efficacy was not determined in combined use with therapeutic doses of allopurinol. This study addressed the changes of uric acid metabolism and CNS in mice undergoing hyperuricemia correction with allopurinol combined with *A. podagraria* extract or tincture.

**Study design:** The mice were randomly distributed to five groups: Group I: intact control; Group II: control for manipulations and hypouricemia (allopurinol, 2.5 mg/kg); Group III: potassium oxonate (PO), 250 mg/kg + allopurinol, 10 mg/kg; Group IV: PO, 250 mg/kg + allopurinol, 10 mg/kg + extract, 1 g/kg; Group V: PO, 250 mg/kg + allopurinol, 10 mg/kg + tincture, 1 ml/kg.

**Place and Duration of Study:** Central Scientific-Research Laboratory, National University of Pharmacy, Kharkiv, Department of Biochemistry, Kharkiv National Medical University, June 2017 – September 2017.

**Methodology:** Beginning from the 15$^{th}$ day psychopharmacological tests were carried out. At day 21, xanthine oxidase activity in the liver and kidney, uricase activity in the liver, uric acid level in blood and brain, GABA, serotonin, aspartic and glutamic acids concentrations in the brain were determined.

**Results:** The extract as well as the tincture did not counteract the influence of allopurinol on xanthine oxidase, liver uric acid level was decreased, and uricemia slightly elevated (especially by the tincture) allowing to suggest the changes of uric acid transport. Such changes were also possible in brain resulting in the increased uric acid level in animals receiving combinations with *A. podagraria* or allopurinol per se. In these groups, GABA brain content was reduced, while aspartic and glutamic acids content was increased. The extract and especially the tincture decreased brain serotonin level (which was elevated by PO and allopurinol). Allopurinol per se and its combinations with *A. podagraria* preparations mildly reduced locomotor activity. Allopurinol and PO increased the duration of stay in the open arms of the elevated plus maze that was eliminated by the tincture, which also normalized the number of mice immediately visiting the open arm. The extract and the tincture decreased depressivity level in the tail suspension test. The tincture restored physical endurance in the weight-loading forced swimming test.

**Conclusion:** The results substantiate the combined use of allopurinol and *A. podagraria* preparations, which do not counteract the main effect of allopurinol and do not cause the negative changes of the CNS (no unfavourable shifts in locomotion and anxiety are induced, while depressivity and physical endurance are partially improved). Further studies are needed to elucidate the mechanisms of *A. podagraria* active components interaction with allopurinol within the brain.



*Keywords: uric acid, potassium oxonate, allopurinol, central nervous system, Aegopodium podagraria L., mice*


## 1. INTRODUCTION

Hyperuricemia is a widely discussed risk factor of cardiovascular diseases, diabetes mellitus and kidney injury [1–3]. At the same time the relationship between purine metabolism and CNS activity is not so obvious. These aspects are now attracting much attention. Evolutionary advantages of the loss of uricase and respectively high uricemia are supposed, being important for the appearance of the intellectually developed primates as well as, in later periods of human society development, for the formation of high achievements up to genius development [4–5] or, at least for higher levels of everyday activities [6]. In this context, disadvantageous effects of the low uric acid level in blood are reported, and the treatment of hyperuricemia should not result in the excessive decrease in this value [3].

Herbal preparations are promising for hyperuricemia counteraction through the different mechanisms (suppression of uric acid synthesis, uricosuric effect or influence on free radical processes associated with urate synthesis as well as on other processes pathogenetically important in hyperuricemia) [7]

Besides, a great body of evidence exists about the favourable psychotropic effects of the herbal drugs [8], which can exert multidirectional effects due to the complex composition. Still, the relationship between antihyperuricemic and psychotropic activity of the herbal preparations is not completely elucidated in the literature.

Our efforts are focused on the pharmacological studies of the preparations obtained from the aerial part of *Aegopodium podagraria* L. (goutweed). It is a perennial plant from the *Apiaceae* family, widely used in traditional medicine and consumed as vegetable. The plant is ubiquitous, and the raw material of its aerial part is available for drug manufacturing at respectively low cost. The Latin species name was given to the plant by Linnaeus in accordance with its use in gout; and pharmacological research has confirmed this approach: hypouricemic and uricosuric action of water extract and tincture obtained from *A. podagraria* aerial part was described (together with the beneficial nephroprotective, hepatoprotective, antihyperglycaemic properties) [9–11]. These preparations do not cause a significant shift in the behavioural reactions of the normouricemic animals and exert a moderate favourable influence of anxiety and depression signs, that is dose-dependent and sex specific [12]. There are no data about the influence of *A. podagraria* preparations on the behavioural reactions and the profile of neuromediators in animals with the changes in purine metabolism, including those induced by allopurinol (ALL). The latter is of special importance because of the interest to the herbal drugs use in the combined therapy of hyperuricemia. Besides, ALL is able to change the CNS functional state [13]. Proceeding from the low uric acid level inherent in the intact rodents, it is expedient to use ALL on the model of hyperuricemia. The preliminary results confirm that ALL effect is not blocked after its combined use with *A. podagraria* tincture, and toxicity is not increased in this case [14].

Therefore, the aim of this study was to determine the effects of *A. podagraria* extract and tincture in mice receiving allopurinol, namely the changes in depression and anxiety signs, physical endurance, locomotor activity and exploratory behaviour (and the correspondent changes in brain mediators) as well as state of purine metabolism (uricemia, xanthine oxidase and uricase activity) and

## 2. MATERIAL AND METHODS

### 2.1 Plant material

The aerial parts of *A. podagraria* were collected from the natural population in Kharkiv region (Ukraine) in June. The herbal raw material was dried at room temperature and powdered using a standard grinding mill to obtain the powder with the mean particle size of approximately 2 mm.

The standard technology of *A. podagraria* dry extract and tincture obtaining was in accordance with the requirements of State Pharmacopoeia of Ukraine and was described previously [9,10].

#### 2.1.1 Extract obtaining

The powder obtained as described above was twice extracted with water at 90ºC. The plant material and solvent were taken in 1:10 ratio, the solvent volume was increased according to the swelling index. The extract was filtered under vacuum conditions and concentrated using a rotary evaporator, and a dry solid was obtained (residual water content equalled 5%), corresponding to an average yield of 25%. *A. podagraria* dry extract is a brown powder with a characteristic pleasant odour, sour-bitter to taste.

#### 2.1.2 Tincture obtaining

*A. podagraria* tincture was prepared by double extraction with 70% ethyl alcohol. The plant material (the powder obtained as described above) and solvent were taken in 1:5 ratio, the solvent volume was increased according to the swelling index. The solvent was divided into two parts. The plant material was macerated in 2/3 of the solvent at room temperature for five days being periodically shaked and stirred. The mixture was filtered under vacuum conditions and maceration process was repeated under the same conditions with the rest of the solvent. The obtained liquids were combined into one, kept for two days at 4ºC, filtered and brought to the calculated volume with the solvent. *A. podagraria* tincture is a dark green liquid with a characteristic odour.

## 2.2 Chemicals and reagents

Analytical graded chemicals and reagents were used for this research. Potassium oxonate was sourced from SigmaAldrich (St. Louis, MO, USA), ALL – from Hexal AG (Germany). Commercially-available kits from Spainlab Co. Ltd. and Filisit-Diagnostika Ltd. SME (Ukraine) were used for the measurement of uric acid level in blood plasma and brain tissue, respectively. All other chemicals were of analytical grade.

## 2.3 Animal groups and treatment

Adult male random-bred mice (initial body weight 22–26 grams) were obtained from the Central Scientific-Research Laboratory of National University of Pharmacy. All the experimental protocols were approved and were in accordance with "Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes". The mice were housed in cages with individual ventilation, under a controlled temperature and relative humidity. Food (standard rodent chow, technical conditions UA 15.7-2123600159-001:2007) and water (tap water after boiling and cooling down) were supplied ad libitum. Animal care was in accordance with the Standard Operating Procedures of the Central Scientific-Research Laboratory of National University of Pharmacy.

After acclimation, mice were randomly assigned to 5 groups:

Group I: intact control without any manipulations (IC, n=8);

Group II: control for manipulations (M) and hypouricemia (intragastrical administration + allopurinol, 2.5 mg/kg, M +ALL, n=7);

Group III: potassium oxonate, 250 mg/kg + allopurinol, 10 mg/kg, (ALL+PO, n=7);

Group IV: potassium oxonate, 250 mg/kg + allopurinol, 10 mg/kg + *A. podagraria* extract, 1 g/kg (ALL+PO+EXTR, n=7);

Group V: potassium oxonate, 250 mg/kg + allopurinol, 10 mg/kg + *A. podagraria* tincture, 1 ml/kg (ALL+PO+TINCT, n=7);

**Table 1. The experimental groups and treatment**

| Group number | Group, n | Abbreviation | The drugs or solvents given | | |
|---|---|---|---|---|---|
| | | | 1 | 2 | 3 |
| I | intact control, n=8 | IC | – | – | – |
| II | Control for manipulations and hypouricemia, n=7 | M | Drinking water | Allopurinol, 2.5 mg/kg | Drinking water |
| III | Hyperuricemia corrected with allopurinol, n=7 | ALL+PO | Potassium oxonate, 250 mg/kg | Allopurinol, 10 mg/kg | Drinking water |
| IV | Hyperuricemia corrected with allopurinol and *A. podagraria* extract, n=7 | ALL+PO+EXTR | Potassium oxonate, 250 mg/kg | Allopurinol, 10 mg/kg | *A. podagraria* extract, 1 g/kg |
| V | Hyperuricemia corrected with allopurinol and *A. podagraria* tincture, n=7 | ALL+PO+TINCT | Potassium oxonate, 250 mg/kg | Allopurinol, 10 mg/kg | *A. podagraria* tincture, 1 ml/kg |

All studied preparations and chemicals (as well as water in the control groups) were administered intragastrically (in the following order: PO, ALL, *A. podagraria* preparations) twice daily at 6 – 8-h intervals (from 10 p.m. up to 11 p.m. and from 17 p.m. up to 18 p.m.). The amount of fluid that the mice in all groups received was similar. The doses were adjusted weekly, based on the body weight.

The minimally effective dose of ALL (2.5 mg/kg in accordance with the data [15]) was used in the additional control group.

The groups receiving ALL or PO per se were not used, since we addressed the changes of CNS against the background of these agents in the previous study [13] (besides, the use of the additional groups complicate the scheme of the study and prolong the psychopharmacological tests making them less synchronous). In the current work we aimed just at the effect of *A. podagraria* preparations against the background of ALL use in hyperuricemia. Such data seem to be informative for the substantiation of these herbal drugs use in the combined treatment of gout.

Potassium oxonate (PO) was chosen as the classical uricase inhibitor widely applied in pharmacological studies despite the relatively short effect [16]. After its intragastric administration at a dose of 250 mg/kg, the effective increase in uricemia, as well as changes of behavioural reactions, was seen in our previous studies [13]. Therefore, this dose was used in the current study.

For the decrease in uricemia, ALL was administered at a dose of 10 mg/kg intragastrically. The high efficacy of this dose has been confirmed in oxonate-treated mice [10,13].

*A. podagraria* extract and tincture were used at doses 1 g/kg and 1 ml/kg respectively. These doses are the most promising for purine metabolism disorders correction [9,10,14].

Beginning from the 15th day, psychopharmacological tests were carried out. The same mice were used for all of the tests to make correlation analysis of the results possible. The interval between drug administration and the beginning of the study was 1 h for each mouse. All behavioural tests were conducted from 11 a.m. up to 6 p.m. The procedures were conducted in a sound attenuated room. The animals were transported from the housing room to the testing area in their own cages and were allowed to adapt to the new environment for 1 h before testing. The test equipment was thoroughly cleaned using alcohol solution followed by drying before each mouse was tested.

## 2.4 Behavioural tests

### 2.4.1 Combined open field

The mice were observed in an open field arena, 22 × 22 × 11 (L × W × H) with floor divided into 16 squares with 16 holes (1.5 cm diameter). After 3 min in a dark cage, the mouse was placed on one of the peripheral squares. During a 3-min test period, the following measures were taken: the number of squares crossed, the number of times the animal reared and made exploratory nose-pokes, the number of faecal boli, urinations, and grooming acts.

### 2.4.2 Elevated plus maze

The elevated plus maze apparatus [17] was made of plastic, glass and wood. It consisted of two brightly-lit open arms (glass surface), 10 × 50 (L × W), two opposed enclosed arms 10 × 10 × 50 (L × W × H), open to the top. The maze was elevated to a height of 1 m. After a 5-min period in a dark cage, the mouse was placed in the centre of the maze, facing one of the open arms. The test period lasted for a 5 min. Traditional anxiety measures were taken, such as the number of entries into the enclosed arms and into the open arms, time spent in the different compartments, latency of entry into the enclosed arm. Besides, the latency of entry into the open arm and the number of times the maze centre was crossed were registered as the widely used additional characteristics of anxiety as described in [12]. Also the number of faecal boli and urinations was registered.

#### 2.4.3 Tail suspension test

The mice were suspended on the support by adhesive tape placed approximately 1 cm from the tip of the tail [17]. The distance from the mouse's nose to the table top was 10 cm. The duration of immobility was recorded for a period of 6 min. The number of faecal boli was also taken into account.

#### 2.4.4 Weight-loaded forced swimming test

The metal load (10% of body weight) was fixed on the tail root of each mice and the animals were placed individually into the pool with water at 22–23 ℃. The pool was filled with 60 cm water, pool ledges equalled 15 cm over the water level, not allowing the rest of the animal on them. Swimming time to exhaustion by the criterion of head dip under water without coming to the water surface for 10 s was recorded [18].

#### 2.4.5 Extrapolation escape task

Cognitive functions were assessed by extrapolation escape task, registering the latency of escape (avoidance through diving) of the animal placed to the cylinder, the edge of which is under water, as described in [19]. The test period was limited to 120 s, and the number of animals capable of solving the task was recorded.

### 2.5 Determination of the biochemical values

After all the psychopharmacological tests were accomplished, the animals were taken out of the experiment under barbiturate-induced anaesthesia (60 min after the last drug administration). The mice were fasted for 12 h before taking final blood samples but they were allowed free access to tap water. Blood was obtained by exsanguination and plasma (the anticoagulant heparin in vitro) was separated immediately by centrifugation. The level of uric acid in blood plasma was measured by the uricase method (commercially available kit). The concentration of uric acid and xanthine oxidase (XO) activity in the liver and kidney were determined (spectrophotometric determination of the synthetized uric acid against the background of uricase blockade), in the liver uricase activity was additionally measured [20,21], the methods and modifications were described previously in details [11].

To evaluate the possible changes in neuroactive mediators and correlate them with the results of the behavioural tests, the levels of GABA, aspartic acid, glutamic acid were determined in brain tissue using the method of high voltage electrophoresis. Uric acid level in brain was determined by using the standard kit as stated above. Serotonin concentration in brain tissue was measured by the procedure derived from the solvent extraction technique and o-phtaldialdehyde methods for the development of fluorophores. The increase in sensitivity was accomplished by volume reduction accompanied with changes of reagent concentration [22].

### 2.6 Statistical analysis

Since the most of data obtained in the study were not normally distributed, medians, 25% and 75% percentiles (upper and lower quartiles) were calculated. Traditionally used means ± standard errors of the mean (SEM) were also shown (M±m). Taking into account a problematical character of multiple comparisons in pharmacology and toxicology [23], Mann-Whitney U test and the Fisher angular transformation were applied to determine the statistical differences between groups. The level of significance was defined as $p<0.05$. To determine the relationship between the individual parameters, the Spearman's correlation coefficient of ρ was used.

## 3. RESULTS AND DISCUSSION

### 3.1 The results of the biochemical studies

Lethality cases and toxicity signs were not registered within the terms of the study. There also were no significant differences in body weight dynamics (except for the animals receiving the extract, in which there was a slight decrease compared to the IC value). A significant uricase inhibition was achieved in all animals receiving PO (there were no differences between these groups) confirming the bioavailability of PO and the absence of direct interactions of the herbal preparations studied with it

(Table 2). ALL administration led to an expected decrease in uricemia in all of the treated groups due to XO inhibition (a tendency in mice receiving this drug in a minimal dose and a significant blockade with the results below detection limit after its administration at a dose of 10 mg/kg were registered). In the previous studies [10,11] ALL at the same dose inhibited XO to a lesser extent, and the reason for this may be the prolonged use of this drug two times a day. Uricemia in all of the groups receiving ALL and PO was decreased almost threefold compared with IC value, in mice treated with *A. podagraria* preparations, especially with the tincture, this value was higher than in PO+ALL group. Since liver uric acid level in *A. podagraria*-treated mice was significantly lower (in both groups $P = .08$ compared with the value of PO+ALL group) and in PO+ALL group it did not differ significantly from both control groups, the influence of *A. podagraria* components on the transport systems of uric acid can be supposed. Liver XO activity in all of these three groups was reduced, and it is consistent with the previous data confirming that *A. podagraria* tincture does not interfere with ALL main effect (high doses of ALL were used) [14], and the comparable XO inhibition as well as uric acid level was seen in the kidney of these mice. We have not determined the excretory renal function in this study, still it is known that *A. podagraria* preparations can facilitate uric acid excretion [10,11]. The data concerning the influence of the herbal biologically active substances on the certain uric acid transporters are scarce, though there is evidence that flavonoids (including quercetin and its derivatives which are present in *A. podagraria*) are able to influence the transporters pathogenetically important in uricemia [24,25].

Thus, our data have confirmed that *A. podagraria* extract (1 g/kg intragastrically) and *A. podagraria* tincture (1 ml/kg intragastrically) do not counteract the inhibitory influence of ALL on XO in mice receiving potassium oxonate. Proceeding from the changes in uricemia and liver uric acid level, the influence of *A. podagraria* components (especially those of the tincture) on the transport of uric acid can be supposed.

**Table 2. The effect of allopurinol and *A. podagraria* preparations on the purine metabolism values of potassium oxonate-treated mice, $Q_{50}$ ($Q_{25}$–$Q_{75}$);** M±m, n=6–8

| | IC | M + ALL | PO + ALL | PO + ALL + EXTR | PO + ALL + TINCT |
|---|---|---|---|---|---|
| Uricemia, mMol/l | **0.093 (0.079–0.100)** 0.094±0.008 | **0.070** * **(0.058–0.073)** 0.066±0.009 | **0.030** ***&& **(0.026–0.033)** 0.029±0.002 | **0.040** ***&& **(0.032–0.044)** 0.038±0.004 | **0.035** ***& # **(0.033–0.041)** 0.038±0.003 |
| Uric acid in liver, µM/g tissue | **0.38 (0.31–0.38)** 0.35±0.02 | **0.35 (0.32–0.38)** 0.35±0.03 | **0.35 (0.29–0.36)** 0.33±0.02 | **0.27** *& **(0.23–0.30)** 0.25±0.03 | **0.24** * **(0.16–0.32)** 0.24±0.04 |
| Liver XO activity, ncat/g tissue | **0.39 (0.34–0.41)** 0.39±0.06 | **0.17 (0.15–0.33)** 0.23±0.06 | **0** (BDL) | **0** (BDL) | **0** (BDL) |
| Liver uricase activity, ncat/g tissue | **0.051 (0.047–0.077)** 0.066±0.019 | **0.037 (0.019–0.057)** 0.039±0.013 | **0.016** * **(0–0.020)** 0.013±0.005 | **0 (0–0.037)** 0.017±0.011 | **0.006** **& **(0–0.017)** 0.012±0.006 |
| Uric acid in kidney, µM/g tissue | **0.21 (0.14–0.24)** 0.20±0.03 | **0.18 (0.13–0.21)** 0.17±0.02 | **0.08** **& **(0.06–0.10)** 0.09±0.02 | **0.10** **& **(0.06–0.11)** 0.09±0.02 | **0.10** **& **(0.08–0.11)** 0.10±0.01 |
| Kidney XO activity, ncat/g tissue | **0.25 (0.14–0.28)** 0.22±0.03 | **0.08**** **(0.06–0.09)** 0.08±0.01 | **0** ***&&& **(0–0.005)** 0.007±0.01 | **0.01**** **(0–0.05)** 0.03±0.02 | **0.05** ** **(0.01–0.08)** 0.06±0.03 |

*BDL – below detection limits; XO – xanthine oxidase.*
** – P < .05 compared to IC values; ** – P < .02 compared to IC values; *** – P < .01 compared to IC values; & – P < .05 compared to M+ALL values; && – P < .02 compared to M+ALL values; &&& – P < .01 compared to M+ALL values;*
*# – P < .05 compared to PO+ALL values.*

It is more difficult to explain the increase in brain uric acid level in mice receiving ALL at a low dose (M+ALL group, Tab. 3). It is known that in hypouricemia as well as in hyperuricemia the level of uric acid in blood serum correlates closely with its level in brain [26]. At the same time, the transport systems for uric acid (including transcellular transport) within the blood-brain barrier are under intensive research now. Mouse urate transporter URAT1 was identified in the cilia and apical surface of ventricular ependymal cells, GLUT9 was observed in ependymal cells, neurons, and brain capillaries, and these transporters provide uric acid transport into the cell. ABCG2 was identified in the choroid plexus epithelium and brain capillaries, but not in ependymal cells, and its dysfunction in the brain capillary endothelial cells is supposed to decrease UA excretion into blood (and these transporters are discussed as a promising target for the local increase of uric acid level in brain in neurodegenerative disorders) [27]. The latter is of special interest in the context of our results. The changes of this transporter in hypouricemia have not been elucidated in the available literature. At the same time, earlier data obtained from the studies of the basolateral membrane of the choroid plexus show that adenosine, guanosine, inosine are able to inhibit the transport systems which mainly provide the excretion of purines from the cerebrospinal fluid into the blood [28]. Under the conditions of XO inhibition, blood and cerebrospinal fluid content of many purines is changed (uric acid precursors level may be logically increased). In particular, adenosine and guanosine concentrations were elevated in the cerebrospinal fluid of mice treated with allopurinol [29]. Thus, the decreased transport of purines out of the brain together with the preserved activity of XO (the enzyme is active in the brain [26], while intragastrically administered ALL at a low dose used in our study could hardly inhibit it in the brain tissue) could led to the elevated level of UA within the brain despite its decreased systemic level. This is indirectly supported by the elimination of the correlation between uricemia and brain uric acid level: coefficients were within the range of –0.11 – +0.10i in all of the groups except for the intact mice ($\rho = -0.74$, $P > .05$). In PO+ALL group brain uric acid level returned to the value of the intact animals (Table 3), that was not seen in mice receiving *A. podagraria* preparations.

The content of the neuroactive amino acids such as GABA, aspartic and glutamic acids was not changed in PO+ALL group (only glutamic acid level showed a slight increment, $P = .06$ compared with the intact control value). Just in the groups in which brain uric acid level was elevated (namely, the groups receiving *A. podagraria* preparations as well as M+ALL group), the reduced level of GABA together with the increased content of aspartic and glutamic acids was seen. This attracts attention in the context of the possible stimulatory effect of uric acid on the behavioural responses, cognitive functions, and motivation [5].

ALL per se at a low dose did not influence on serotonin level in brain, while in mice receiving PO and ALL it was elevated (Table 3). The extract slightly decreased, while the tincture significantly reduced this value. It has been established that purines modulate the effects of neurotransmitters including GABA as well as serotonin [30], and in our study serotonin level increased only in the animals which were treated with PO.

Correlation between GABA and glutamate brain levels was not present in all groups except for M+ALL group in which $\rho$ equalled 0.78 ($P = .07$). Glutamic acid – aspartic acid interrelation was eliminated in M+ALL and PO+ALL groups, the tincture approximated it to the IC group value, while under the influence of the extract the direction of relationship changed. The extract also significantly increased correlations between brain uric acid and glutamic as well as aspartic acid, while the tincture completely eliminated this interrelation.

Thus, *A. podagraria* tincture significantly decreased brain serotonin level, the other studied brain biochemical values did not show changes that could be attributed to *A. podagraria* components influence.

**Table 3. The effect of allopurinol and *A. podagraria* preparations on the brain biochemical values of potassium oxonate-treated mice, $Q_{50}$ ($Q_{25}$–$Q_{75}$);** M±m, n=5–7

| | | IC | M + ALL | PO + ALL | PO + ALL + EXTR | PO + ALL + TINCT |
|---|---|---|---|---|---|---|
| Content, μM/g tissue | Glutamic acid | **10.7 (10.0–11.8)** 10.9±0.43 | **21.4** *** **(21.1–22.2)** 21.4±0.55 | **12.0** &&& **(11.9–12.1)** 12.0±0.11 | **20.1** ***### **(19.0–20.8)** 20.3±0.68 | **22.3** ***### **(21.1–22.9)** 22.9±1.35 |
| | GABA | **1.77 (1.59–1.92)** 1.77±0.09 | **0.71** *** **(0.64–0.76)** 0.71±0.05 | **1.69** &&& **(1.69–1.77)** 1.69±0.06 | **0.77** ***### **(0.72–0.84)** 0.78±0.04 | **0.71** ***### **(0.48–0.87)** 0.69±0.10 |
| | Aspartic acid | **3.82 (3.38–4.06)** 3.69±0.18 | **5.91** *** **(5.74–6.05)** 5.90±0.10 | **3.65** &&& **(3.12–3.79)** 3.64±0.31 | **5.27** ***&### **(4.93–5.55)** 5.29±0.20 | **6.11** ***### & **(5.76–6.31)** 5.96±0.20 |
| | Uric acid | **0.178 (0.173–0.180)** 0.176±0.003 | **0.295** *** **(0.290–0.301)** 0.299±0.007 | **0.191** ** &&& **(0.187–0.205)** 0.197±0.007 | **0.312** ***&### **(0.307–0.339)** 0.322±0.009 | **0.303** ***### **(0.287–0.314)** 0.303±0.010 |
| Serotonin level, nM/ tissue | | **118 (115–121)** 118±2,45 | **115 (112–117)** 115±1.55 | **135** ***&&& **(133–137)** 135±1.44 | **130** ***&&&## **(129–131)** 130±0.81 | **109** ** &&### **(106–110)** 108±1.32 |
| Spearman's correlation coefficients of ρ between the individual biochemical parameters | | | | | | |
| Serotonin – uricemia | | **–0.95** *P* = .05 | **–0.99** *P* < .001 | **+0.10** NS | **–0.03** NS | **+0.15** NS |
| Serotonin – brain uric acid | | **+0.10** NS | **+0.09** NS | **1.0** | **+0.41** NS | **+0.71** *P* = .1 |
| Glutamic acid – aspartic acid | | **+0.68** *P* = .09 | **+0.09** NS | **–0.20** NS | **–0.89** *P* < .02 | **+0.82** *P* < .05 |
| Glutamic acid – brain uric acid | | **+0.50** NS | **–0.71** NS | **+0.60** NS | **–0.99** *P* < .001 | **–0.14** NS |
| Aspartic acid – brain uric acid | | **+0.70** NS | **+0.60** NS | **+0.60** NS | **+0.92** *P* < .01 | **–0.09** NS |

*NS – not significant.*
*** – p<0.02 compared to IC values; *** – p<0.01 compared to IC values;*
*& – p<0.05 compared to M +ALL values; && – p<0.02 compared to M +ALL values; &&& – p<0.01 compared to M +ALL values;*
*# – p<0.05 compared to PO+ALL values; ## – p<0.02 compared to PO+ALL values; ### – p<0.01 compared to PO+ALL values*

## 3.2 The results of the combined open field test

ALL administration to the mice receiving potassium oxonate led to the less significant decrease in locomotor activity compared with the treatment with ALL per se at low dose. In the latter group, both components of locomotor activity were reduced (Table 4). According to the data in the literature, in hyperuricemic mice receiving PO per se [13] or in hypouricemic mice receiving ALL as monotherapy at higher doses (10 mg/kg and 39 mg/kg [31]), such changes were not evident. Thus, our results might indicate the dose-dependence of ALL effects or, probably, the reaction of the animals to the stressing intragastric administration. When ALL was given against the background of PO per se or together with *A. podagraria* extract, the decrease of locomotor activity was less significant (*P* =.1 and *P* = .07 respectively vs the value of M+ALL group). Locomotor activity in animals receiving the tincture did not differ significantly from the value of M+ALL group. In normouricemic mice, *A. podagraria*

extract at the same dose decreased only the number of rearings, while the tincture did not influence on this test results [12].

**Table 4. The effect of allopurinol and *A. podagraria* preparations on the behavioural responses of potassium oxonate-treated mice in the combined open field test, $Q_{50}$ ($Q_{25}$–$Q_{75}$);** M±m; n=6–8

| | | IC | M + ALL | PO + ALL | PO + ALL + EXTR | PO + ALL + TINCT |
|---|---|---|---|---|---|---|
| Locomotor activity: | Number of squares crossed | **48 (46–54)** 51±3.6 | **28 (23–33)**$^{***}$ 29±3.0 | **45 (33–48)** 41±5.7 | **36 (31–42)**$^{*}$ 40±5.8 | **31 (27–38)**$^{*}$ 34±4.6 |
| | Number of rearings | **11 (8–14)** 10±1.7 | **5 (2–6)**$^{*}$ 4±1.0 | **7 (6–9)** 7±1.6 | **4 (4–7)** 6±1.4 | **3 (2–4)**$^{**}$ 3±0.5 |
| Number of exploratory nose-pokes | | **35 (27–42)** 35±3.1 | **32 (31–32)** 31±2.6 | **35 (31–36)** 33±1.5 | **31 (25–35)** 29±3.0 | **30 (26–34)** 32±3.7 |
| Sum of the vegetative manifestations | | **0 (0–0.5)** 0.5±0.3 | **1.0 (0–1.0)** 0.6±0.2 | **0 (0–0)** 0.2±0.2 | **0.5 (0–1.8)** 0.8±0.4 | **1.0 (0.3–1.0)** 0.8±0.3 |
| Total activity sum | | **95 (87–101)** 96±5.7 | **63 (59–71)**$^{***}$ 66±6.2 | **88 (75–90)** 81±8.0 | **75 (71–77)**$^{*}$ 76±5.1 | **67 (64–78)**$^{***}$ 70±4.5 |

** – P < .05 compared to IC values; ** – P < .02 compared to IC values; *** – P < .01 compared to IC values.*

Exploratory activity was not changed in any of the groups (Table 4) indicating the safety of *A. podagraria* preparations use against the background of purine metabolism shifts. Previously it was shown that exploratory activity was decreased in hyperuricemic mice receiving PO per se [13], thus, ALL counteracted to this decrease. Uric acid content in brain did not correlate with the total activity in the combined open field. Interestingly, the decrease of locomotor activity (Table 4) was seen in the groups with the reduced GABA content in the brain (Table 3).

There were no shifts in the sum of the vegetative manifestations (all of the studied values, such as number of grooming acts, urinations, and faecal boli remained unchanged). Total activity sum was reduced in the groups in which locomotor activity decreased.

Thus, the combined use of allopurinol and *A. podagraria* preparations in mice receiving potassium oxonate does not induce any negative shifts in the open field test, the maintenance of the exploratory activity and absence of the increase in vegetative and emotional manifestations are of special importance.

### 3.3 The results of the elevated plus maze test

The studies in the elevated plus maze showed that in all of the groups receiving ALL, except for those additionally treated with the tincture, the mice spent more time in the open arms (Table 5, Fig. 1). The locomotor activity by the criterion of the total number of arm entries did not differ significantly among the groups. The latency of entry into the enclosed arm was increased in all groups, especially PO+ALL, a higher number of these animals immediately visited the open arm, they visited less enclosed arms and faster entered the open arms (this value tended to statistically significant differences with M+ALL group, $P$ =.09 and $P$ = .1 in groups PO+ALL and PO+ALL+EXTR respectively). The latter effect was maintained against the background of the extract, while the tincture eliminated it (Table 5). A clear tendency towards an increase in number of mice that immediately visited the open arm was also seen in PO+ALL+EXTR group, the time spent in the open arms only showed an increment in these animals ($P$ =.09 vs IC value).

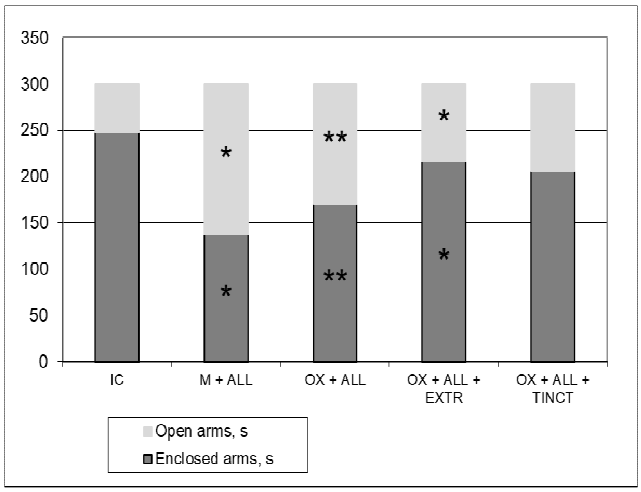


**Fig. 1. The effect of allopurinol and *A. podagraria* preparations on the time spent in open and closed arms of the elevated plus maze, seconds**

** – P < .05 compared to IC values; ** – P < .02 compared to IC values*

Thus, the effects of the extract are similar to those in the intact normouricemic mice, while the ability of the tincture to reduce anxiety signs registered in normouricemic mice [12] was not evident against the background of ALL and PO. In the group receiving the extract, a positive correlation between the latency of entry into the open arm and uricemia appeared ($\rho = +0.97$; $P < .01$), that was not present in any other group (and the previously seen correlation between the latency of entry into the enclosed arm and uricemia [13] did not reach the level of statistical significance in this study). Besides, in mice treated with the extract a negative correlation between the latency of entry into the open arm and serotonin content in brain was registered ($\rho = -0.90$; $P < .02$), in other groups it was within the range of –0.50 – +0.49 and did not reach significance. Since this correlation differed between PO+ALL and PO+ALL+EXTR groups, while there were no differences in both serotonin level and the latency of entry into the open arm, the influence of the extract on serotonin transport, pool or the interrelated neurochemical mechanisms can be supposed (besides, the use of the whole brain homogenate for analysis did not allow registering the changes in certain regions). Hydroxycinnamic acids are among the most important components of the extract [9,10], and it is notable that ferulic acid is able to exert psychotropic effects through serotonergic system [32]. Furthermore, this effect is registered at a respectively high doses 40 and 80 mg/kg, and the approximating intake of hydroxycinnamic acids is reached with the extract at a dose of 1 g/kg, but not with the tincture at a dose of 1 ml/kg [9]. The latter, on the contrary, decreased serotonin level (Table 3), while the latency of entry into the open arm was maximal just in this group (Table 5).

The changes in the preferences of the illuminated and dark compartments may be also attributed to ALL administration since this drug at the same dose reduced the number of mice that immediately visited the enclosed arm and significantly increased the time spent in the open arms [13]. Besides, it has been shown that stress can not only increase anxiety level, but also reduce it depending on the timing of the stressor [33], and the situation of the chronic stress was highly possible in animals undergoing the constant intragastric administrations.

As to the extract partial efficacy, it is unlikely that the slight increase in uricemia could influence on the anxiety level, and this effect could be attributed to its components direct effects on the CNS. In this context the data concerning the anxiolytic effect of chlorogenic acid at a dose of 20 mg/kg [34] are of special interest, as the quantity of hydroxicinnamic acids that the animal receives with the extract at a dose of 1 g/kg reaches 53 mg/kg [9].

Thus, *A. podagraria* tincture approximated the values registered in the elevated plus maze to those of the intact mice, namely eliminated the increase in the duration of stay in the open arms, caused by ALL against the background of potassium oxonate, and reduced the number of mice that immediately visit the open arm (the extract caused changes in the same direction, but they were less pronounced

and did not reach the level of statistical significance). Negative shifts in locomotion and vegetative manifestations were not registered in animals receiving *A. podagraria* preparations.

**Table 5. The effect of allopurinol and *A. podagraria* preparations on the behavioural responses of potassium oxonate-treated mice in the elevated plus maze, $Q_{50}$ ($Q_{25}$–$Q_{75}$); M±m; n=5–8**

| | IC | M + ALL | PO + ALL | PO + ALL + EXTR | PO + ALL + TINCT |
|---|---|---|---|---|---|
| Latency of entry into the enclosed arm, seconds | **2.5 (1.0–18)** 14±7.6 | **18 (8–135)** 76±34 | **54 (36–84)*** 61±14 | **29 (18–48)** 48±24 | **25 (16–39)** 41±20 |
| The number of entries into the enclosed arms | **8 (6–9)** 7±1.1 | **4 (3–5)*** 4±0.7 | **3 (2–3)*** 3±0.9 | **5 (3–7)** 5±1.1 | **6 (3–6)** 5±1.0 |
| The number of entries into the open arms | **12 (8–15)** 11±1.8 | **9 (6–9)** 8±1.0 | **8 (8–9)** 8±1.3 | **9 (7–12)** 9±2.0 | **7 (4–11)** 8±2.7 |
| The number of maze center crossings | **4 (3–5)** 4±1.0 | **4 (3–6)** 5±0.8 | **6 (5–7)** 5±0.9 | **5 (3–7)** 5±1.0 | **6 (2–10)** 6±2.2 |
| Total number of arm entries | **24 (17–30)** 23±3.7 | **18 (13–19)** 17±1.7 | **16 (15–19)** 17±2.8 | **18 (14–24)** 19±3.9 | **23 (9–23)** 19±5.5 |
| Latency of entry into the open arm, seconds | **20 (7–35)** 24±7.1 | **27 (10–41)** 34±12.7 | **6 (4–12)** 11±5.7 | **8 (7–19)** 19±9.7 | **44 (21–54)**# 86±54 |
| Time spent in the open arms only, seconds | **21 (12–46)** 32±10 | **148 (50–170)**** 119±28 | **99 (92–101)**** 98±20 | **65 (35–84)** 79±27 | **50 (29–71)** 66±31 |
| Time spent in open arms and maze center excepting the latency of entry into the enclosed arm, seconds | **42 (25–59)** 45±11 | **60 (42–71)** 79±29 | **46 (11–113)** 73±34 | **58 (50–68)** 67±15 | **57 (45–90)** 62±20 |
| Sum of the vegetative manifestations | **0 (0–1.0)** 0.4±0.2 | **0** | **0 (0–0)** 0.2±0.2 | **0** | **0 (0–0)** 0.2±0.2 |
| Number of mice that immediately visited the open arm, % | **37,5** | **57,1** | **100*** | **83,3** | **40,0** |

** – P < .05 compared to IC values; ** – P < .02 compared to IC values*

### 3.4 The results of depressivity signs evaluation

The results of the tail suspension test evidenced that the extract and especially the tincture were able to moderately decrease the depressivity level in mice with purine metabolism disorders.

In mice receiving ALL at low dose (and undergoing chronic intragastric administrations) a significant increase in depressivity level was seen by criterion of the duration of immobility in the tail suspension test (Fig. 2). These changes were reduced in animals receiving PO, and in mice receiving *A. podagraria* preparations this value had no statistical differences from IC value (the effect was especially stable in mice receiving the tincture). The number of faecal boli during the test did not vary between the groups (data not shown).

It has been shown previously that ALL per se at a dose of 10 mg/kg does not change depressivity level [13], in contrast to data confirming the reduction in the immobility period in mice chronically treated with ALL at a dose of 39 mg/kg (the similar effect was exerted by febuxostat) [31]. On the other hand, the decreased locomotor activity and possibly stress-induced changes in anxiety level are in good agreement with the increase in depressivity level. Besides, the above-mentioned changes of uric acid precursors are possible, and in the context of ALL influence on the CNS special attention is given to the changes in adenosine level [35]. It is known that adenosine and inhibitors of its transport increase immobility of animals in the tests of behavioural despair [36]. The involvement of the serotoninergic system into the depressivity changes is also possible. The correlation coefficients between brain serotonin level and immobility time underwent changes: the negative interrelationship inherent in intact mice, ρ = –1.0 was eliminated in all other groups, in PO+ALL group it equalled –0.50; *P* > .05, in the other groups it was within the range of –0.21 – +0.20.

*A. podagraria* extract and tincture did not exert an antidepressive effect in normouricemic male mice (while in female animals it was present after administration of the extract at a tenfold lower dose) [12]. It is important that this effect was evident in our study – in animals with the increased depressivity signs. In accordance with the previous results [13], a positive correlation between the duration of immobility and uricemia (ρ = +0.71, *P* = .07) was present in IC group. It was eliminated in M+ALL group (ρ = –0.20, *P* > .05), while in the group receiving ALL and PO the direction of a relationship changed (ρ = –0.80, *P* = .1), that was further enhanced by the extract (ρ = –0.99, p<0.001) but not by the tincture (ρ = –0.50, P > .05).

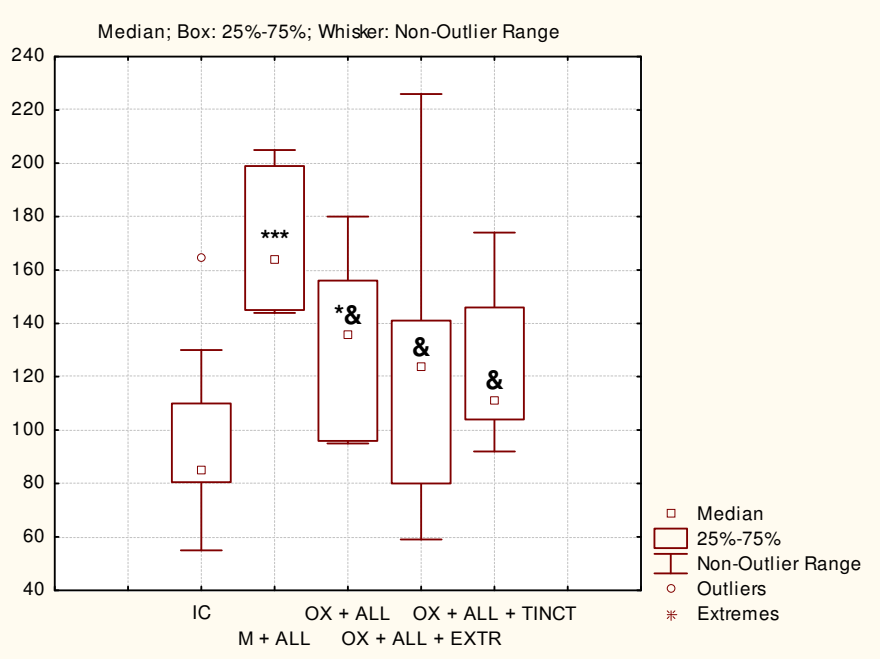


**Fig. 2. The effect of allopurinol and *A. podagraria* preparations on the duration of immobility of potassium oxonate-treated mice in the tail suspension test, seconds**
** – P < .05 compared to IC values; *** – P < .01 compared to IC values;*
*& – P < .05 compared to M +ALL values*

Together with the changes in purine metabolism, the direct influence of *A. podagraria* components on the CNS is possible, and hydroxycinnamic acids are of special interest in this context. Much evidence exists about the beneficial activity of chlorogenic acid (which takes part in the development of the favourable central effects of coffee) including antidepressive as well as anxiolytic properties in different experimental models. Some of the effects of the hydroxycinnamic acids are dose-dependent [37,38] as well as the effects of the *A. podagraria* preparations. Further research is expected to clarify whether the modulatory effect on the purinergic mechanisms is inherent in these compounds.

## 3.5 The results of the physical endurance evaluation

The physical endurance by criterion of duration of swimming in the weight-loading forced swimming test was reduced in all of the groups receiving ALL (Fig. 3). This decrease was especially significant in M+ALL group, while the tincture approximated this value to the level of the IC.

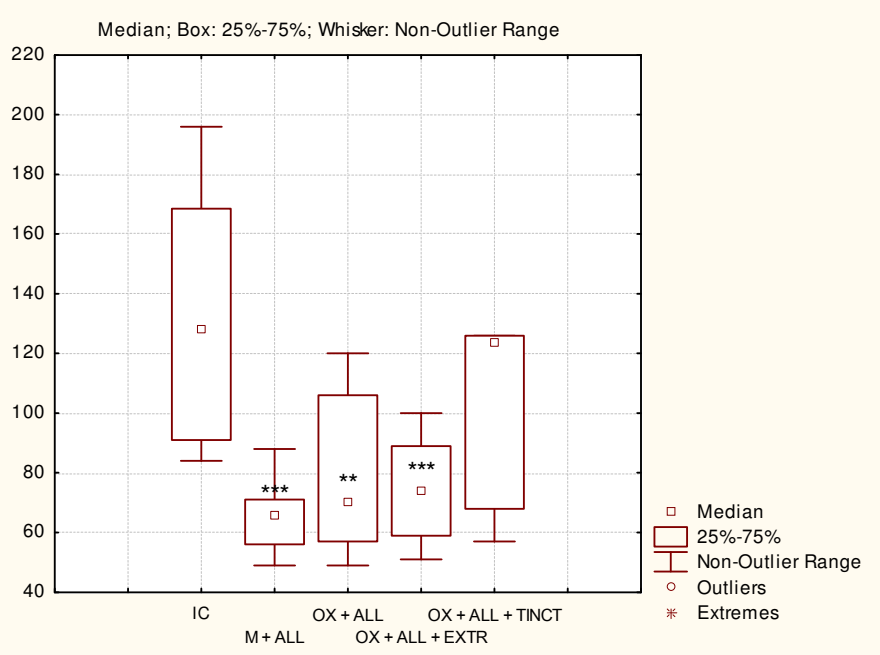


**Fig. 3. The effect of allopurinol and *A. podagraria* preparations on the physical endurance of potassium oxonate-treated mice (duration of swimming in the weight-loading forced swimming test), seconds**

*** – P < .02 compared to IC values; *** – P < .01 compared to IC values.*

Body weight of all mice was not changed (only in animals receiving the extract there was a slight but significant decrease compared to the IC value), thus the loading did not differ significantly among the groups. In our previous experiments, ALL per se at a dose of 10 mg/kg did not reduce the duration of swimming, on the contrary, there was a tendency to the increment of this value [13], while the other authors have demonstrated decreased swimming duration in rats treated with xanthine oxidase inhibitor [39]. The open space swimming test results may be influenced by the state of peripheral metabolism, and ALL is known to support anabolic processes through the increased availability of hypoxanthine for the synthesis of nucleotides [40]. Nevertheless, this effect was not evident and the decrease in swimming duration might be caused by the chronic stress which is known to reduce physical activity in most cases [41].

The role of uricemia reduction is also possible, since the correlation between the duration of swimming and uricemia changed in a similar direction as did correlation between the duration of immobility and uricemia discussed above. Namely, a positive correlation inherent in IC group was eliminated in M+ALL group, while in the group receiving ALL and PO the direction of a relationship changed to negative, and at both groups receiving *A. podagraria* preparations this correlation was not significant. The tincture also normalized glutamic acid – aspartic acid correlation (described above) that also may be associated with depressivity changes. In previous experiments the tincture did not change the physical endurance, while the extract increased it at a significantly lower dose [42]. It can be supposed that the direct action of *A. podagraria* components is realized at respectively low doses, thus the tincture with the lower content of hydroxycinnamic acids (compared with the extract) is more effective. It seems to be favourable that the effect is present just in the model with the decreased physical performance.

### 3.6 The results of the extrapolation escape task

There were no differences in all the measures taken in the extrapolation escape task, namely the latency of escape and number of animals in each group capable of solving the task (data not shown). Thus, the cognitive functions remained unaltered demonstrating the well-known brain plasticity. These aspects are favourably combined with the absence of the decrease in the exploratory behaviour in the combined open field test (discussed above). Ambiguous results of the extrapolation escape task were seen previously in rats receiving *A. podagraria* preparations [42], and the absence of worsening of this test results in hypouricemic mice treated with these preparations can be regarded positively.

## 4. CONCLUSIONS

The results substantiate the combined use of allopurinol and *A. podagraria* preparations, which do not counteract the main effect of allopurinol and do not cause the negative changes of the CNS functional state (which could be expected in hypouricemia). Thus, the possibilities of hyperuricemia correction may be increased by herbal preparations combined use with allopurinol (in this case the decrease of allopurinol dose and development of favourable concomitant effects are possible). *A. podagraria* aerial part extract and tincture are promising for such combinations development.

## ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws (“Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes”).
All experiments have been examined and approved by the bioethics committee of the National University of Pharmacy.

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