**Article**

**PREVENTIVE ACTIVITY OF THE EXTRACT OF THE DARKLING BEETLE *ULOMOIDES DERMOSTOIDES* IN THE DIET OF C57BL/6JSTO MICE IN A NEUROTOXIC MODEL OF PARKINSON’S DISEASE**

Vladimir M.Kovalzon¹, Aleksandr V.Ambaryan¹, Aleksandr V.Revishchin², Ekaterina Y.Rybalkina³,

Aleksandr I.Bastrakov¹, Nina A.Ushakova¹

¹Severstov Institute of Ecology and Evolution, Russian Academy of Sciences,
²Institute of Higher Nervous Activity and Neurophysiology, Russian Academy of Sciences,
³Blokhin Russian Cancer Research Center, Ministry of Health of the Russian Federation; Moscow, Russia

* Correspondence: V.M.Kovalzon, somnolog43@gmail.com Tel.: 74959541511

**Abstract:** The effect of aqueous extracts of the biomass of the adult beetle *Ulomoides dermestoides* on the delayed effects of defoliant paraquat causing parkinsonism in experimental mice was evaluated. The motor activity of the animals was analyzed in behavioral tests using a rotarod and a vertical pole. The number of tyrosine hydroxylase-immunopositive neurons in the ventral part of the *substantia nigra* of the midbrain of experimental and control mice were studied by immunohistochemistry. In the model *in vitro* system with SH-SY5Y–human neuroblastoma, the effect of the extracts on cell proliferation was examined in the absence and in the presence of the neurotoxin MPP⁺. The isolation of biologically active substances from raw biomass using cavitation effects made it possible to obtain extracts with protective properties in the model of an early stage of Parkinson’s disease used.

**Keywords:** Parkinson’s disease, neurotoxic model, early stage, biologically active substances from insects

**1. Introduction**

Parkinson’s disease (PD) is a chronic progressive degenerative disease of the central nervous system, which clinically manifests itself, in particular, as impaired coordination of movements. PD is one of the most socially significant, and the study of its biological foundations is the most important task of neurosciences. In this disease, for unknown reasons, a very slow (over tens of years), but steady degeneration of dopaminergic neurons of the *substantia nigra/pars compacta* of the midbrain (SNpc), projected onto the neurons of striatum nuclei, occurs. The process is asymptomatic as a result of the activation of compensatory mechanisms. Only at a late stage of the development of the disease, when less than half of the initial number of dopamine-containing neurons remains and the level of dopamine delivered to the striatum by these neurons drops by 4-fold, motor impairment appears, and later cognitive impairment too.
However, at that stage it is too late to start treatment. In the history of world medicine there has not yet been a single patient who could be healed. Modern medicine can only alleviate symptoms and slightly slow down the progression of the disease. Therefore, the creation of adequate experimental models and the search for early markers of the disease are now the primary tasks [1-5].

In this regard, the models of preclinical and early clinical stages of PD are of the greatest interest. These stages are characterized by a threshold level of degradation of the nigrostriatal dopaminergic system and the first minor impairments of motor function. Such models provide a unique opportunity to study the pathogenesis of a disease (the molecular mechanisms of neurodegeneration and neuroplasticity), search for peripheral biomarkers as a basis for creating preclinical diagnostics, and identify new molecular targets for pharmacotherapy and neuroprotection [6]. Obviously, neuroprotective treatment can be effective only if it is started at an early stage, not later, when most of the neurons have already died [3,7-11].

Since animals are not susceptible to PD, the creation of such models is not an easy task [12]. The most common are neurotoxic models, in which, to recreate motor and some other symptoms of a Parkinson-like state (parkinsonism) in laboratory mice, the dopaminergic systems of the brain are destroyed by central or peripheral administration of substances such as MPTP (methyl-phenyl-tetrahydropyridine), 6-hydroxodopamine (HDA) and lactacystin [11,13-20]. MPTP is a proneurotoxin that penetrates into astrocytes and there, under the influence of the MAO-B enzyme, it is converted into the neurotoxic ion MPP+(1-methyl-4-phenyl-pyridine ion). Recently, the widespread herbicides, rotenone and paraquat, have also been increasingly used. The mechanism of their destructive effect on the dopaminergic system of the brain has been widely studied and published in a number of reviews [19,21-26].

Insects have long attracted the attention of researchers as a source of biologically active substances. For example, Apis mellifera bee, larvae and adults of the large flour beetle Tenebrio molitor, carrion flies of the Calliphoridae family, wax moth Galleria mellonella, silkworm Bombyx mori contain new antimicrobial peptides, chitin-melanin complexes, flavonoids, aminoacids, organic acids [27-38]. Darkling beetles of the Tenebrionidae family, which are used in traditional medicine to treat a wide range of diseases, are of great interest. These insects synthesize protective secretions, which are a mixture of repellent and blocking chemoreceptor substances. They are found in cuticular inclusions or abdominal glands and are released when the beetles are stressed. It is believed that the secretions of these beetles are also necessary for the insect to prevent drying out and protect against pathogenic microorganisms [27, 31-33]. It has been shown that these secrets are a source of pharmacologically active compounds that are promising for the treatment of respiratory diseases [39]. There is information about the possibility of medical use of substances isolated from extracts of a representative of this family, the darkling beetle Ulomoides dermestoides, the substances with anti-inflammatory and immunomodulatory properties, as well as cytotoxicity in relation to the cells of some tumors [40-42]. We assumed that some components of the biomass of a representative of the same family, the beetle Alphitobius diaperinus, have an inhibitory activity against the delayed effects of the proneurotoxin MPTP, which causes experimental parkinsonism in C57Bl/6jstjo mice [43-45]. Our experiments using the model of the early clinical PD stage showed that the primary aqueous extract and, especially, the secondary aqueous-methanol extract of the biomass of this beetle had a powerful protective influence in response to the neurotoxic effect of MPTP, both according to behavioral tests and according to the results of morphocontrol.

The present study evaluated the effect of aqueous extracts of biomass of the adult beetle Ulomoides dermestoides in relation to the delayed effects of paraquat defoliant causing early parkinsonism in experimental mice [22,23]. The motor activity of animals was analyzed in behavioral tests using a rotarod and a vertical pole [46,47]. Changes in the
number of tyrosine hydroxylase (TH)-immunopositive neurons in the ventral part of the substantia nigra of the midbrain of mice were studied using immunohistochemistry [48]. In a model system with SH-SY5Y–human neuroblastoma, we examined the effect of the extracts on cell proliferation without and in the presence of the neurotoxin MPP+ [49].

2. Results

The rotarod test, assessing the biological effect of beetle extracts in the diet of mice under conditions of a neurotoxic model of an early stage of Parkinson’s disease, revealed (Fig. 2, Table 1) that continuous administration of “extract-cavitation” with food caused a slight insignificant increase in the mean rotation speed which the mouse could sustain before falling down compared with the control-toxin group (from 12.5±1.2 to 15±1.6 rpm; M±S.E.M.; p>0.1). The mean time of holding the mouse on the rotarod before falling increased significantly (from 701 to 825 sec, p<0.05, n=12/24, U-test).

The preparation “extract-lightning” caused a sharp increase in the ability of the animal to hold on the rotarod rotating both with constant and increasing speed. Improvement occurred on both parameters: rotarod rotation speed and mouse retention time (Fig. 2, Table 1). The indicators in this group did not differ from those of the intact control, with a significant difference from the control-toxin group (for both indicators, p <0.001).

Figure 1. Results of the behavioral test on rotarod. Groups of mice: 1 - toxin+“extract-cavitation”, N=12; 2 - toxin+“extract-lightning”, N=11; 3 - intact control; N=12; 4 – toxin-control, N=24. M±S.E.M. Differences from group 4, *p<0.05, **p<0.001, U-test. The y-scale shows the percentage of the test completion (see "Methods").

Table 1.

|               | "Extract-cavitation" | "Extract-lightning" | Intact control | Paraquat control |
|---------------|----------------------|---------------------|----------------|-----------------|
|               | Rod speed (rpm)      | Time of exposition (sec) | Rod speed (rpm) | Time of exposition (sec) | Rod speed (rpm) | Time of exposition (sec) | Rod speed (rpm) | Time of exposition (sec) |
| Mean          | 14.7                 | 825.3*              | 19.2**         | 972.9**         | 18.8**          | 960.9**          | 12.5           | 700.8                      |
| SD            | 5.6                  | 208.4               | 1.9            | 42.8            | 3.0             | 77.3             | 5.8            | 300.6                      |
Note: *revolutions per minute* - an indicator of the sustained rotarod rotation speed; †time - duration of holding the mouse on the rotarod at a rotation speed of 6 rpm.

*p<0.05, **p<0.001, difference from the paraquat-control group.

The results of intergroup comparisons of the maximum, minimum and mean duration of exposition on the vertical pole are presented in Table 2.

**Table 2.** Intergroup comparisons of the duration of the pole exposition (in three attempts).

| Timing parameters | Groups in comparison       | Mean duration of the pole exposition (sec) |
|-------------------|-----------------------------|--------------------------------------------|
| Maximal time      | Control intact              | 28.9±7.2                                   |
|                   | Control-toxin               | 18.1±2.1                                   |
|                   | "Extract-cavitation"       | 32.4±5.2                                   |
|                   | "Extract-lightning"        | 41.9±10.1                                  |
| Minimal time      | Control intact              | 7.5±0.6                                    |
|                   | Control-toxin               | 9.9±0.9                                    |
|                   | "Extract-cavitation"       | 9.0±0.7                                    |
|                   | "Extract-lightning"        | 12.1±0.8                                   |
| Mean time         | Control intact              | 15.4±2.5                                   |
|                   | Control-toxin               | 13.6±1.4                                   |
|                   | "Extract-cavitation"       | 17.8±2.0                                   |
|                   | "Extract-lightning"        | 24.0±3.9                                   |

Comparison of the maximum, minimum and mean times (out of three attempts) spent by mice on the pole using the Kruskal-Wallis test showed that there were significant differences between the groups in maximal and mean times only (maximum time: H3=10.19, P=0.017; mean time: H3=11.10, P=0.011), therefore, further, in statistical analysis, the minimal time is excluded from the account.

Pairwise *a posteriori* comparisons using the Steel-Dvaz-Critchloo-Flyiner test showed that the maximal time spent on the pole was significantly higher in the "extract-cavitation" group than in the control-toxin group. And the mean time spent on the pole was significantly higher in the "extract-lightning" group than in the control-toxin group (Table 3).

**Table 3.**

| Timing parameters | Groups in comparison                   | Criterion statistics Meanings (Hodges-Lehmann location shift) | Significance of intergroup differences (P) |
|-------------------|----------------------------------------|-------------------------------------------------------------|-------------------------------------------|
| Maximal time      | "Extract-cavitation"/control toxin     | 10.0                                                        | 0.02*                                      |
|                   | "Extract-lightning"/control toxin      | 9.2                                                         | 0.12                                       |
|                   | "Extract-cavitation"/control intact    | 7.6                                                         | 0.24                                       |
The ratio of the maximum in three attempts to the maximum possible time spent on the pole (Table 4) in the "extract-lightning" group was significantly higher than in the other groups. The same relation was significantly higher in the control group without toxin, as well as in the "extract-cavitation" group, compared to the control-toxin group. There were no significant differences in this parameter between the "extract-cavitation" and toxin-free control intact groups. The relation of the mean exposure time on the pole to the maximal possible in three attempts in the "extract-lightning" group was also significantly higher than in the other groups, and in the "extract-cavitation" group - compared with the control-toxin group. There were no significant differences in this parameter between the other groups.

Table 4. Pairwise intergroup comparisons of the maximum and mean proportion of exposition time on the pole versus maximum possible duration in three attempts (180 seconds) (Marasquilo’s procedure at a significance level of α=0.05).

| Timing parameters | Statistics and significance in χ2 | Groups in comparison | Meaning of criterion statistics | Critical statistical value (r) | Significance of intergroup differences |
|-------------------|----------------------------------|-----------------------|---------------------------------|-------------------------------|----------------------------------------|
| Maximal proportion | χ²=207.4, P<0.000 | "Extract-cavitation"/intact control | 0.020 | 0.029 | No |
| | | "Extract-cavitation"/toxin control | 0.080 | 0.027 | Yes |
| | | "Extract-cavitation"/"extract lightning" | 0.053 | 0.035 | Yes |
| | | Intact control/toxin control | 0.060 | 0.021 | Yes |
| | | "Extract-lightning"/intact control | 0.072 | 0.030 | Yes |
| | | "Extract-lightning"/toxin control | 0.132 | 0.028 | Yes |
| Mean proportion | χ²=61.1, P<0.000 | "Extract-cavitation"/intact control | 0.013 | 0.023 | No |
| | | "Extract-cavitation"/toxin control | 0.024 | 0.022 | Yes |
| | | "Extract-cavitation"/extra | 0.034 | 0.028 | Yes |
In the middle brain samples of 4 animals from the intact control, toxin control and two “extract” groups. The number of TH-immunopositive cells was counted. The number of TH-immunopositive cells in animals that received an injection of paraquat was significantly lower than in intact control animals, as well as in animals in the "extract-cavitation" and the "extract-lightning" groups (H3=12.794, P=0.005, Kruskal-Wallis test). At the same time, the number of TH-immunopositive cells in animals from the "extract-cavitation" group was significantly lower than in control intact animals and animals from the "extract-lightning" group (Table 5 and 6).

Table 5. The mean number of TH-immunopositive cells on 9 slices of substantia nigra in all studied animals.

| Nos of mice | Intact control | Toxin control | "Extract-lightning" | "Extract-cavitation" |
|-------------|----------------|---------------|---------------------|----------------------|
| 1           | 3992           | 2416          | 4256                | 3728                 |
| 2           | 4512           | 3408          | 5576                | 3448                 |
| 3           | 4656           | 3184          | 4016                | 3536                 |
| 4           | 4720           | 3232          | 4776                | 3712                 |
| Mean        | 4470           | 3060          | 4656                | 3606                 |
| S.E.M.      | 165            | 220           | 345                 | 68                   |

Table 6. Significance of differences in the average number of TH-immunopositive cells on 9 slices of substantia nigra between all studied groups of animals using Conover-Iman test.

| Compared groups          | Statistics criterion value (q) | Statistics criterion critical value | Significance of intergroup difference (P) |
|-------------------------|--------------------------------|------------------------------------|------------------------------------------|
| Intact control/toxin control | 6.58                          | 2.18                               | <0.000*                                  |
| Extract lightning/intact control | 0.69                          | 2.18                               | 0.502                                    |
| Extract cavitation/intact control | 3.81                          | 2.18                               | 0.003*                                   |
Representative slices of the substantia nigra of the midbrain of the intact control animal, the toxin-control one, and the one from the "extract-lightning" group, stained for TH, are shown in Fig. 3. The number of TH-immunopositive neurons in the substantia nigra and the ventral area of the midbrain tegmentum of the toxin-control animal (Fig. 3B) were significantly less compared to the intact control (Fig. 3A) and the animal from the "extract-lightning" group (Fig. 3C). In the latter sample, the number of TH-immunopositive neurons in the substantia nigra and the ventral tegmental area of the midbrain is practically the same as in the intact control animal.

Figure 2. Representative slices of the substantia nigra of the midbrain of the animals: A - intact control; B - toxin-control; C - the extract-lightning group. Stained for TH.

Evaluation of the protective effect of the extract-cavitation of the beetle Ulomoides dermestoides against 0.5 mM neurotoxin MPP⁺ in a model experiment using SH-SY5Y neuroblastoma showed the dependence of the effect on the dose of the administered extract (Fig. 4). The best result was obtained using a 5% addition of the extract: the protection was 23%. Dilution of the extract did not cause reliable protection.
Figure 3. Protective effect of *Ulomoides dermestoides* "cavitation" extract.

For the "lightning extract" *Ulomoides dermestoides*, the protection of 26.5% was detected with the administration of 1% extract (Fig. 5).

Figure 4. Protective effect of *Ulomoides dermestoides* "lightning extract" on SH-SY5Y cells in the presence of MPP⁺ neurotoxin (0.5 mM).

3. Discussion

The use of methods for the extraction of biologically active substances from the raw biomass of the darkling beetle *Ulomoides dermestoides* using cavitation effects, both directly and by electroexplosive method, made it possible to obtain extracts with protective properties in the used model of an early stage of Parkinson’s disease. Presented experiments show that the administration of the “extract-cavitation” drug had some positive effect on the motor skills...
of animals after intoxication with paraquat compared with the control group, which was intoxicated with paraquat, but did not receive the antidote. Intake of the preparation “extract-lightning” with food almost completely eliminated the toxic effect of paraquat in the rotarod test. In the vertical pole test, the mice from both “extract” groups could hold on to an upright pole longer without sliding or jumping off. That is why the proportion of the maximum and the mean (in three attempts) times spent on the pole (from the maximal possible) in mice of these groups were higher than in mice from the toxin-control group. This is probably due to a higher muscle tone and better coordination of movements, both during locomotion and maintaining a stationary position on the supporting surface of the vertical pole. The mice from the "extract-lightning" group demonstrated the best results.

The behavioral tests results were consistent with the results of histochemical examination of mouse brain slices. The significant decrease in the degree of damage to dopaminergic neurons of the compact part of the substantia nigra of the brain with paraquat toxin in the neurotoxic model of an early stage of Parkinson's disease was shown when preparations from Ulomoides dermestoides extracts were added to the food. The best results were found in animals that received the "extract-lightning".

The results of the neurotoxic model of Parkinson’s disease in mice were confirmed in vitro using the SH-SY5Y neuroblastoma model in the presence of the MPP⁺ neurotoxin (0.5 mM). Both tested beetle extracts were able to maintain cell viability by 23-26%. However, this value was achieved with the introduction of 5% extract-cavitation, and 1% extract-lightning which correlated with the in vivo effects of the extracts in mice.

The revealed activity of extracts of the darkling beetle Ulomoides dermestoides may be associated with the presence of antioxidants and quinones [41]. Previously, it was shown that the closely related darkling beetle Alphitobius diaperinus contains the antioxidant melanin [52]. According to our latest yet unpublished studies, 1 mg of the dry matter per ml of lightning extract of the beetle Ulomoides dermestoides is equivalent in antioxidant activity to 0.25 mM trolox, a water-soluble analogue of vitamin E. Beetle extract has been shown to cause a 25-30% increase in the average lifespan of nematodes C. elegans under normal conditions, a 12-17% increase under oxidative stress (with paraquat), and significantly inhibit the serum albumin fructosylation reaction. The antioxidant proteins contain superoxide dismutase (SOD) and catalase - main enzymes of the body’s antioxidant system. An important role in the biological activity of the extract under oxidative stress is probably also played by heat shock proteins with molecular weights of 60, 70, and 83 kDa, which protect biological systems from damaging effects under stress, including oxidative stress. The aqueous extract also includes non-protein substances with anti-infectious and anti-inflammatory properties: various phenolic compounds, and ethyl-p-hydroquinone. We assume that the prophylactic activity of the extract of the darkling beetle Ulomoides dermestoides in the diet of C57Bl/6jsto mice in the neurotoxic model of an early stage of Parkinson’s disease is associated not with just one, but with a whole group of antioxidant and antitoxic substances contained in it.

4. Materials and Methods

The laboratory population of Ulomoides dermestoides beetles was cultivated at the Severstov Institute Ecology/Evolution on a nutrient mixture of wheat bran (70%), milk powder (5%), corn flour (20%) and sunflower meal (5%) in a climatic chamber at 28°C and 60-70% humidity, conditions similar to cultivation of Alphitobius diaperinus [50]. Adult sexually mature beetles of both sexes separated from the substrate were immobilized by cooling at -18°C and the resulting biomass was divided into two parts. One part was crushed in distilled water using cavitation on a rotary-pulsation unit as part of RPA 0.8-55A-5.5/2 “Stalpischeprom, Ltd.”, Russia, followed by separation of the
sediment by centrifugation in an Ohaus FRONTER 5706 for 15 min at 5000 g. Received extract we named as “extract-cavitation”. Beetle substances were extracted from the second part of the biomass by the method of electro-pulse plasma-dynamic extraction (EPPDE) in distilled water at 23°C. EPPDE extraction was carried out on a “KorolevFarm LLC” setup, Russia [36]. Extraction parameters: the power of the transmitted electric discharge is 38,000 Volts, the pulse frequency is one pulse per second, the distance between the electrodes is 5 mm, the extraction time is 7 min. After exposure, the extract was separated from the solid fraction by centrifugation at 5000 g for 15 min. Received extract we named as “extract-lightning”. Antibacterial processing of extracts was carried out by radiation decontamination using a beam of accelerated electrons using a compact radiation sterilization unit with local biosecurity (CRSU) of the Moscow Radiotechnical Institute, Russian Academy of Sciences, at 15 K Gy. The energy of the accelerated electrons is 5 MeV, the power of the electron beam is 1.5 kW.

“Extract-lightning” contained 20 mg/ml, and “extract-cavitation” - 12 mg/ml of dry matter. The obtained extracts were immobilized on sterile food wheat bran; the final moisture content of the mass was 8%. The preparations were stored in a refrigerator and fed to experimental mice, for which dry preparations were added to the main food mixture for mice by fractional and thorough mixing (at the rate of 4 g of the “preparation-lightning” and 8 g of the “preparation-cavitation” per 1 kg of the food mixture). The main food mixture consisted of porridge, including boiled oats and peas, with the addition of vegetable oil.

To study the biological activity of the extracts, we used a neurotoxic model of an early stage of Parkinson’s disease in male C57Bl/6jsto mice administrated with paraquat toxin [19, 21-26]. The animals were divided into 4 groups. Group 1 (n=12) - toxin+“extract-cavitation”; the animals were injected i.p. twice (with an interval of 1 week) with 10 mg/kg of paraquat, dissolved in 0.3 ml of saline; an extract obtained by cavitation was added to the food as an antidote as described above; the supplement began one week before the 1st injection of the toxin and continued throughout the entire experiment. Group 2 (n=11) - toxin+“extract-lightning”; the animals were injected with paraquat and received the beetle extract with the food – everything according to the same regimen as Group 1. Group 3 (n=12) - intact control; the animals were not injected with paraquat and did not receive anything additional to the food. Group 4 (n=24) – toxin-control; the animals were injected with paraquat as groups 1 and 2, but they did not receive any antidote. Four days after the 2nd injection of the toxin, the motor activity of all the animals was tested using a rotarod and then a vertical pole.

The mice were placed on a rod rotating at a constant speed of 6 rpm for a period of 600 sec; then the rotation speed was increased automatically by 1 rpm every 30 sec up to 20 rpm. The entire duration of the test was 990 sec. It was recorded how long each mouse can hold out on the rod without falling at a constant speed, and at what maximum speed of rotation. If the mouse was able to stay on the rotarod for 990 seconds, and do not fall at 20 rpm within 30 sec, then the test was considered 100% complete. Statistical analysis was performed using the nonparametric U test (Mann-Whitney).

The test for locomotor activity on a vertical pole was used in accordance with the previously described method [12, 18, 46, 47]. Individual testing was performed in a familiar environment for the animals. At the beginning of testing, a vertical pole with an unpolished rough surface 50 cm high and 1 cm in diameter was placed in the cuvette. A mouse was placed on it close to the top of the pole so that its head was oriented upward. Once on the pole, the mouse reoriented its body position with its head down and began descent from the pole to the bottom of the cage. Even if the animal, after reorientation, did not go down the pole completely and jumped off the pole, the time it took to reach the bottom of the cage was recorded. In our variant of the method, 3 tests were carried out with each mouse. The
minimum time interval between tests was 30 seconds. If the mouse did not reorient its body and start descending within the first 3 minutes from the start of testing in two or all three trials, data were not included in the statistical analysis. All experiments were recorded using Panasonic HC-VX1 video camera and then processed by the BORIS 7.9.8 program for the analysis and presentation of behavioral data. In the statistical analysis, only the duration of the descent of the mouse from the pole was taken into account. The groups were compared in terms of maximum and mean time of descent from the pole from three attempts. When analyzing the data, the nonparametric Kruskal-Wallis test was used. Pairwise post hoc comparisons were made using the Steel-Dvash-Critchlow-Flyiner test. In addition, intergroup comparisons were made for maximal and mean pole exposition relatively to maximal possible duration in three attempts (180 sec). The relations were compared using the χ² test. For pairwise comparisons of groups, the Marasquilo procedure was used. Statistical analysis was performed using XLSTAT 2019 2.2 and Analyze-it 5.66 software.

After completion of the behavioral tests, the animals were perfused under deep general anesthesia through the heart with phosphate buffered saline (PBS) and then 4% formaldehyde in PBS. The brain was removed and, after additional fixation in a perfusion solution for 12 hours at 4°C, it was soaked in a 30% sucrose solution in PBS for 24 hours. Coronal sections of the midbrain 40 and 20 µm thick were obtained on a freezing microtome and collected in PBS. There were 3 series of 40 µm slices and 2 series of 20 µm of each brain. Sections were placed in antifreeze and stored at -20°C until staining. Serial sections with a thickness of 20 µm with a spacing between the sections of 160 µm containing substantia nigra of the midbrain, were immunohistochemically stained for TH using monoclonal antibodies against TH (T2928 Sigma), diluted 1:300 in a PBS solution with the addition of 2% normal horse serum, 0.5% Triton X100 detergent (Sigma, USA) and 0.01% sodium azide (Sigma, USA). The free floating sections were kept in the primary antibody solution with stirring at 4°C for 48 hours. Then, after the reaction in a solution of biotinylated horse antibodies against mouse immunoglobulin (Vector Laboratories, USA), and then in a solution of the ABC complex (Vector Laboratories, USA), a standard procedure for staining for peroxidase was carried out using 0.03% diaminobenzidine solution (Sigma, USA) in PBS with the addition of 0.01% hydrogen peroxide. Stained sections were placed on glass slides, covered with 50% glycerol and a cover slip.

The quantitative analysis of TH-immunopositive (TH⁺) cells was performed using an Olympus IX81 microscope equipped with a Märzhäuser motorized stage (FRG) computer controlled stage and an Olympus DP72 digital camera. Cells were counted on a computer monitor using the Cell* software (Olympus Soft Imaging Solution GmbH, Germany). At low magnification (10x objective), an overview image of the section with the substantia nigra and the ventral tegmental area (VTA) was obtained. Then, at high magnification (objective 40x), the number of TH⁺ cells was counted using the method of an optical fractionator. The position of the test square measuring 50x50 µm was changed with a step of 200 µm along the X axis and 100 µm along the Y axis within the ventral part of the midbrain. An uninformed operator counted the number of TH-immunopositive cells in the test square, using unstained nuclei of TH-immunopositive cells as the object of counting. A cell stained for TH was included in the count if its nucleus lay inside the test square or touched two of its adjacent sides (Fig. 1) and did not touch the other two sides marked in this figure with a thicker outline. Since the calculated fraction was 1/8 of the area of the investigated section, to determine the total number of TH-positive cells in the section the counted number was multiplied by 8. Subsequently, the number of cells in 9 consecutive sections containing substantia nigra of the midbrain was determined. Intergroup comparisons of the number of TH-immunopositive cells were performed using the Kruskal-Wallis test. Since the compared samples were small, the Conover-Iman test was used for pairwise a posteriori comparisons.
The effect of darkling beetle extract on SH-SY5Y human neuroblastoma cells was evaluated using the MTT test. This test is based on the ability of mitochondrial dehydrogenases of living cells to convert “yellow” MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide) into “blue” formazan, insoluble in aqueous solutions. For analysis, cells are placed in 96 well plates at 5000 cells per well in 100 μl of standard culture medium. After 24 hours of incubation, 10 μl (1/10 of the medium volume) of the extract at various concentrations was added to the cells. The cells were incubated for another 48 or 72 hours (the incubation time depended on the rate of cell proliferation). After that, 20 μl of MTT preparation (diluted in saline solution 5 μg/ml) was added to each well for 3 hours. Then the solution was removed from the wells and 60 μl of dimethyl sulfoxide (DMSO) was added to each well. Shake thoroughly until the formazan crystals are completely dissolved. The quantitative determination of formazan was carried out on a brand multichannel photometer with a 530 nm filter. Cell viability was assessed by the ratio of optical density in the control wells without the tested extract and in the wells with the extract [51].

6. Patent

Zagorinsky, A.A.; Bastrakov, A.I.; Ushakova, N.A.; Mislavsky, B.V.; Iliev, R.L. A method of growing the darkling beetle *Alphitobius diaperinus* on vegetable raw materials. RF patent No. 2737117 dated 06/11/2020. Published: 24.11.2020 Bul. No. 33.
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