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SUNFLOWER SEED HUSK BIOCHAR: SYNTHESIS AND TOXICITY RISK ASSESSMENT

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Aim. Searching for efficient biocompatible sorbents that possess zero neurotoxicity is an actual task. Biochars are auspicious carbon materials for the adsorption of heavy metals in the environment, wastewater, and also in human organisms.

Methods. Biochar from sunflower seed husk (SB) was synthesized by pyrolysis at 800 °C without special functionalization. Neurotoxicity risk of SB was assessed in an animal model using presynaptic nerve terminals isolated from rat cortex (synaptosomes).

Results. It was shown in radiolabelled experiments that SB did not change the synaptosomal ambient levels of the excitatory neurotransmitter L-[^{3}H] glutamate and inhibitory neurotransmitter [^{3}H] GABA within the concentration range 0.25–1.0 mg/ml. In the fluorimetric experiments using the dye JC-1, SB at a concentration of 1.0 mg/ml did not change the mitochondrial membrane potential of the nerve terminals.

Conclusions. SB demonstrated the absence of neurotoxicity signs and high biocompatibility, and therefore, SB has the potential to be used as an adsorbent in biotechnology and medicine.

Key words: agricultural waste; sunflower seed husk; biochar; non-functionalized carbon materials; neurotoxicity risk; glutamate; GABA; presynaptic terminals, brain.

Usage of agricultural waste for the synthesis of carbon materials based on “green” principles is a very promising stream in modern biotechnology as well as environmental sciences and management [1]. Among carbon materials, biochar, a black solid carbon-rich substance, has been attracting a lot of attention for various applications due to its unique specific characteristics and low-cost production. Biochar is produced by pyrolysis,

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i.e, heating organic materials in an oxygen-free or low-oxygen environment. Biochar possesses a large specific surface area, high cation exchange capacity, high porosity, has functional groups at the surface, and is stable in different media (generally in the absence of oxidizers) [2, 3]. The above properties may vary depending on the type of biomass used as a starting material, temperature, heating rate, residence time of the pyrolysis formulation, and the modification technique [4].

Many studies have reported the considerable adsorption efficiency of various biochars in the removal of multiple contaminants [5]. The adsorption mechanisms are different and depend on pollutant physical and chemical properties and biochar surface characteristics, and include binding by electrostatic interaction, ion exchange, adsorption on surface or pore filling, and chemical binding with the formation of insoluble compounds [5–7]. Literature data describe biochars as the eco-friendly, cheap, multipurpose materials with high potential for recovering soil efficiency, managing toxic metals and organic pollutants, which could be employed as a green alternative to conventional sorbents [2, 3, 7]. However, biochar can have toxic effects on living organisms as harmful substances may be present in it [8]. Biochar contaminants can be both organic and/or inorganic. They can be byproducts of pyrolysis during biochar production and/or components of the feedstock that remain more concentrated after the pyrolysis process [8].

Among different organic agricultural waste materials, sunflower seed husks recently appeared in the mainstream of biotechnological research. Different condition of synthesis of biochar from sunflower seed husks and numerous applications of this biochar were proposed in numerous studies and were reported in the literature. In particular, sunflower seed husks were pyrolyzed at 450 °C, and the produced biochar was tested for its ability to filter out NO gas. It was found that the sunflower seed husk biochar could be proposed as a low-cost and effective solution for reducing air pollution with NO in industrial and urban environment [9].

Biochar was considered as an additive for improving soil [10]. It was revealed that biochar improved soil parameters, and soil enrichment with sunflower husk biochar increased CH_4 oxidation, favouring to reduction of CH_4 exhaust [10]. In another study, biochar produced from sunflower husks had a significant effect on soil respiration, soil water flux, and soil temperature [11].

A promising approach for eliminating various contaminants from wastewater was proposed using bacterial consortium-biochar composites [4]. These composites exhibited higher remediation capacity with respect to lead and phenol than the sum of bacterial consortium and biochar *per se*, showing thus the synergistic adsorptive capability of biochar and bacterial consortium [4]. In another study, sunflower seed husk biochar was used for the purification of water contaminated with cadmium [12].

H_3PO_4-treated sunflower seed husk biochar effectively adsorbed antibiotics tetracycline, ciprofloxacin, ibuprofen, and sulfamethoxazole from the aquatic environment [3].

Taking into account the above mentioned facts, the aims of this study were:

1) to synthesize and characterize biochar from sunflower seed husks; and

2) to assess its toxicity and biocompatibility for further biotechnological and medical application as adsorbent, measuring the ambient levels of the excitatory and inhibitory neurotransmitters L-[^{3}H] glutamate and [^{3}H] GABA, respectively, and the mitochondrial membrane potential in isolated rat cortex nerve terminals (synaptosomes). The activated carbon studied herein is referred to as the sunflower seed husk biochar (SB).

Methods and Materials

Synthesis of SB

The preparation of biological material was carried out according to the established procedure: sunflower seed husk was thoroughly ground using a laboratory mill until a homogeneous powdery mass was obtained. Direct pyrolysis of sunflower waste was carried out in two stages by heat treatment in argon. At the first stage, the crushed sunflower seed husk material was subjected to thermal destruction at a temperature of 600 °C for 3 hours. At the second stage, the resulting pyrolyzed residue was mixed with sodium hydroxide and kept for 3 hours at a temperature of 800 °C. After completion of the heat treatment, the carbon material (SB) was washed with distilled water until a neutral pH value was reached.

Experiments using brain nerve terminals

Ethics

Males, Wistar rats, were kept at 22–23 °C in a quiet temperature-controlled room in the vivarium of the Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine. Rats were supplied with dry food pellets and water *ad libitum*. Experiments involving animals were carried out in accordance with the Guidelines of the European Community (2010/63/EU), Scientific Requirements and Research Protocols; Research Ethics Committees of the Declaration of Helsinki; and ARRIVE Guidelines for reporting experiments involving animals [13, 14], and Ukrainian laws and policies. The protocols of the experiments were approved by the Animal Care and Use Committee of the Palladin Institute of Biochemistry (Protocol # 1 from 10/01/2024). The overall number of animals was 12.

Isolation of nerve terminals from the rat cortex (rat cortex synaptosomes)

The synaptosomes were isolated from the rat cortex. The rat cortex was homogenized in the following solution: sucrose 0.32 M; HEPES-NaOH 5 mM, pH 7.4; EDTA 0.2 mM. All procedures were carried out at + 4 °C. The isolation was carried out as described by Cotman with minor modifications [15–18]. The standard saline solution in the synaptosome experiments was as follows: NaCl 126 mM; KCl 5 mM; $MgCl_2$ 2.0 mM; NaH_2PO_4 1.0 mM; HEPES 20 mM, pH 7.4; and D-glucose 10 mM. Protein concentrations were recorded as described by Larson [19].

The ambient level of L-[^{3}H] glutamate in synaptosomes

The synaptosome suspension with a concentration of 2 mg of protein/ml was preincubated at 37 °C for 10 min; after that, the suspension was loaded with L-[^{3}H] glutamate at 37 °C for 10 min. The synaptosome suspension was diluted with 10 volumes of the standard saline solution, and centrifuged at 10,000×g for 20 s. The pellets were suspended in the standard saline solution up to a concentration of 1 mg protein/ml. The ambient L-[^{3}H] glutamate level was monitored in 125 µl aliquots with a concentration of 0.5 mg of protein/ml The aliquots were preincubated for 8 min, and after that, SB was added, and the suspension was incubated at 37 °C for 0 and 6 min. Then the suspension was centrifuged at 10,000 × *g* for 20 s. The value of the ambient L-[^{3}H] glutamate level was recorded in the supernatant and pellets (preliminary treated with SDS, 100 µl of 10% SDS stock solution) using the liquid scintillation counting with Sigma-Fluor® High Performance LSC Cocktail and counter Hidex 600SL (Finland). The experimental data were from "n" independent experiments with different synaptosome preparations.

The ambient level of [^{3}H] GABA in synaptosomes

The synaptosome suspension with a concentration of 2 mg of protein/ml was preincubated at 37 °C for 10 min; after that, the suspension was loaded with [^{3}H]GABA in the standard saline solution at 37 °C for 10 min. Throughout all experiments, 100 µM aminooxyacetic acid was added to the incubation media. Then, the synaptosomes were diluted with 10 volumes of the standard saline solution, centrifuged, and the pellets were suspended in the solution up to a concentration of 1 mg of protein/ml. The aliquots were preincubated for 8 min, and after that, SB was added and further incubated at 37 °C for 0 and 5 min; after that centrifuged at 10,000 × *g* for 20 s [20]. The value of the ambient [^{3}H] GABA level was monitored in the supernatant aliquots using the liquid scintillation counting with Sigma-Fluor® High Performance LSC Cocktail and the counter Hidex 600SL (Finland). The experimental data were obtained from "n" independent experiments with different synaptosome preparations.

The mitochondrial membrane potential in synaptosomes

The cationic fluorescent membrane-permeable dye JC-1, a mitochondrial membrane potential assay kit, was applied to measure the mitochondrial membrane potential in synaptosomes in the control and in the presence of SB. Synaptosome suspension with the concentration of 0.15 mg of protein /ml was incubated in a stirring cuvette; after that, JC-1 at a concentration of 5 µM was added and preincubated at 37 °C for 10 minutes; and then the fluorescence spectra were measured at a 485 nm excitation wavelength and from 510 to 610 nm emission wavelengths. The ratio of the fluorescence intensity at 590 versus 530 nm was used in the calculations. The fluorescence measurements were carried out using a spectrofluorimeter, Hitachi 650-10S, and Shimadzu RF-6000.

Statistical analysis

The experimental results were expressed as the mean ± SEM of *n* independent experiments. One-way ANOVA was used with the accepted significance $P < 0.05$.

Materials

HEPES, EGTA, EDTA, salts of the analytical grade, High Performance LSC Cocktail were obtained from Sigma, USA; L-[^{3}H] glutamate and [^{3}H] GABA were from Revvity, Waltham, MA, USA.

Scanning electronic microscopy and energy-dispersive X-ray spectroscopy

Scanning electron microscopy (SEM) images were obtained using the FEI Inspect Instrument at 20 kV. Samples were placed on a carbon film without any special treatment. Analysis of the surface of the biochar was performed using energy-dispersive X-ray spectroscopy (EDX) using an Apollo XL SDD EDAX instrument.

Results and Discussion

SB characterization

SEM studied the morphology of the biochar prepared from sunflower seed husk. The material contained particles of irregular shape (Fig. 1) with a wide size distribution (10–100 μm). A large number of voids were observed, which could be due to the partial preservation of the cellular microstructure of the material of biological origin. Such heterogeneous particle morphology and the presence of voids of different sizes can influence the sorption properties of the resulting material, as well as the kinetics of the processes occurring on its surface.

The element composition of the surface of the SB sample was analyzed using energy-dispersive X-ray spectroscopy. The carbon content (Fig. 2) was expectedly the highest and was equal to 89.5%, and the oxygen content was 8.3%. Other typical biogenic elements were also present in small amounts: magnesium (1.0%), silica (0.8%), and calcium (0.4%). These elements apparently originate from the starting material used for pyrolysis.

Toxicity assessment of SB using rat brain nerve terminals: measurements of the ambient levels of *L*-[^{3}H] glutamate and [^{3}H]GABA as well, as the mitochondrial membrane potential

Neurotoxicity risk assessment of SB was carried out in the nerve terminal preparations according to its influence on the ambient levels of the key excitatory neurotransmitter L-[^{3}H] glutamate and the inhibitory neurotransmitter [^{3}H] GABA. The changes in the ambient level of the neurotransmitters reflect the plasma membrane disintegration and changes in the transportation of neurotransmitters [21, 22]. It was proven that SB within the concentration range 0.25–1.0 mg/ml did not change the ambient levels of L-[^{3}H] glutamate and [^{3}H] GABA in the nerve terminal preparations (Fig. 3). Therefore, it can be concluded that SB was biocompatible and did not demonstrate any neurotoxic signs at these concentrations.

In the following sets of experiments, the possible effect of SB on the mitochondrial

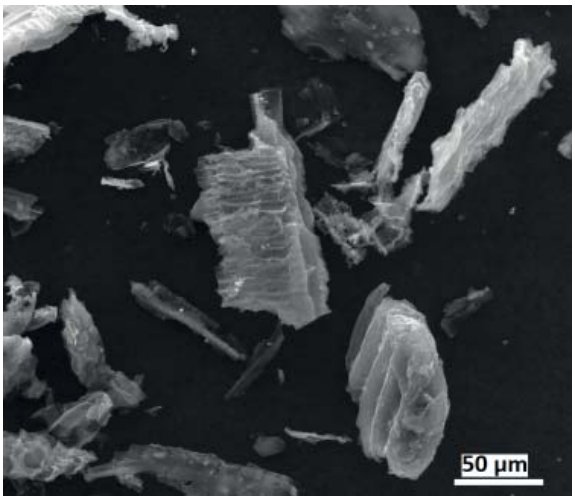


Fig. 1. **SEM microphotograph of SB**

membrane potential was assessed in the nerve terminals. The measurement was performed using the cationic carbocyanine fluorescent dye JC-1. It was revealed that SB at a concentration of 1 mg/ml was inert regarding modulation of the synaptosomal mitochondrial membrane (Fig. 4). Therefore, SB (1 mg/ml) did not depolarize the mitochondria membrane in nerve terminals. These fluorimetric data entirely corresponded to the above results obtained using L-[^{3}H] glutamate and [^{3}H]GABA (Fig. 3).

It was found that SB did not change the ambient levels of neurotransmitters and the mitochondria membrane potential, thus it can be concluded that SB did not have

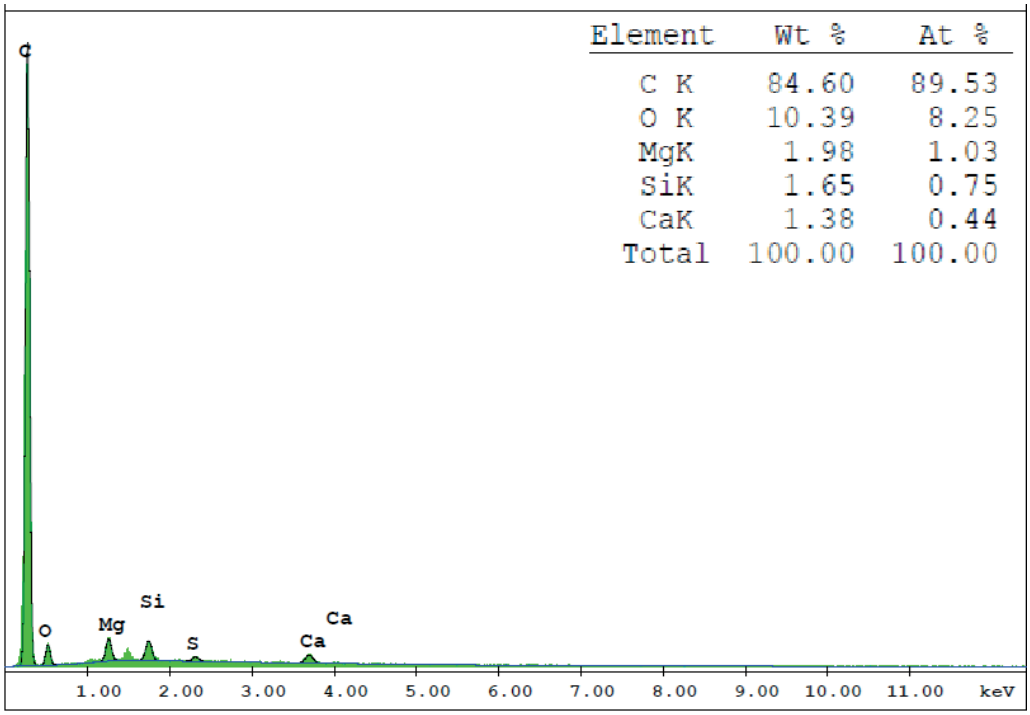
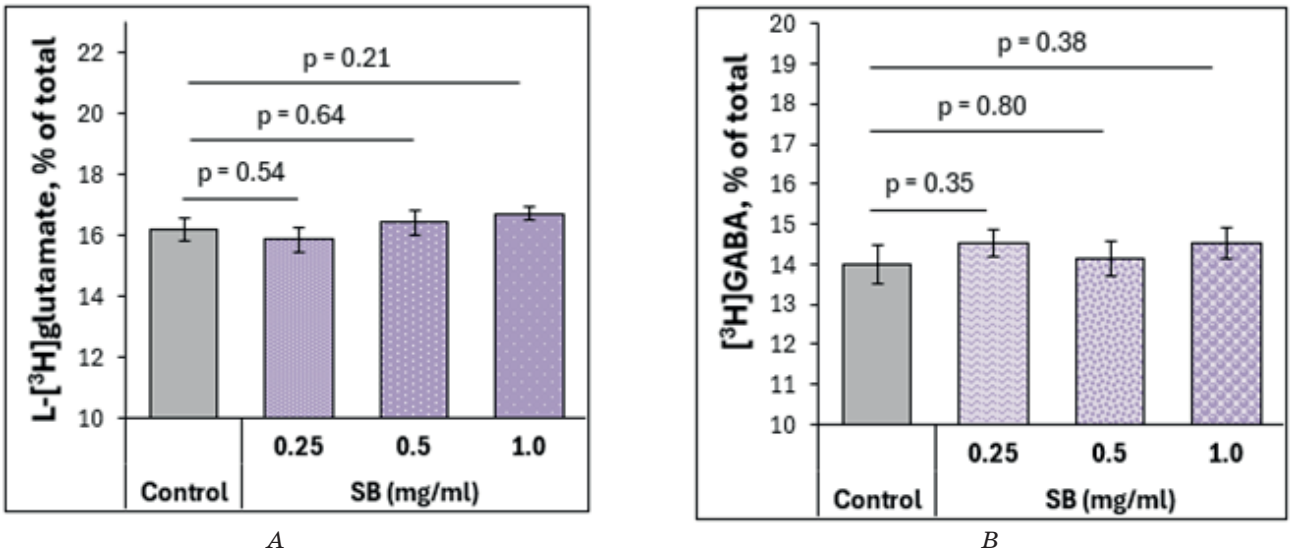


Fig. 2. **Energy-Dispersive X-ray Spectroscopy (EDX)** and element composition of the surface of SB



A *B*

Fig. 3. **The ambient levels of neurotransmitters *L*-[^{3}H] glutamate (*A*) and [^{3}H]GABA (*B*) in the nerve terminals in the presence of SB (0.25–1.0 mg/ml)**

Note. Data are the mean ± SEM. $n = 12$.

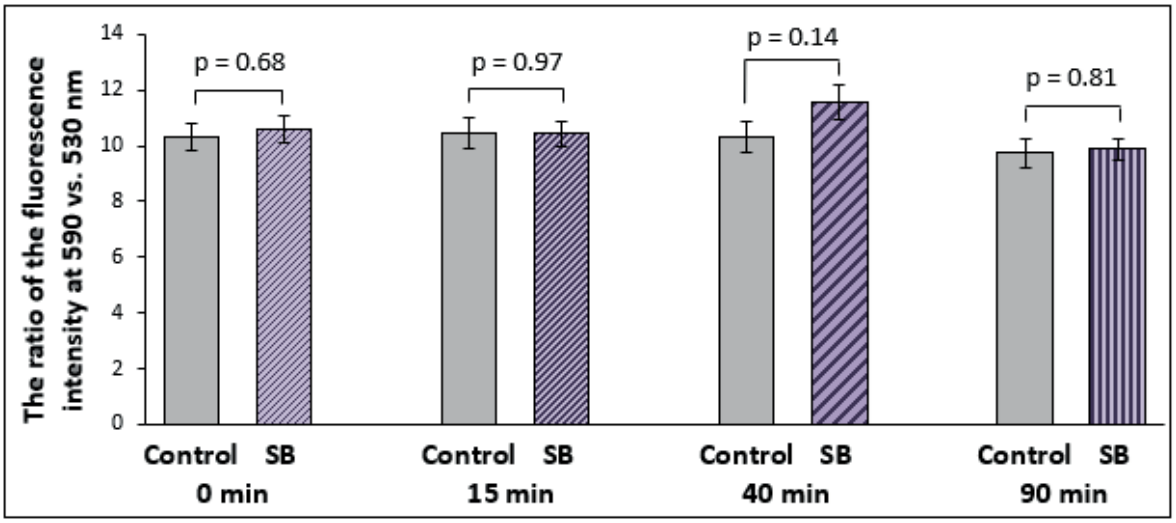


Fig. 4. **The mitochondrial membrane potential of the nerve terminals in the presence of SB (1.0 mg/ml) was measured using the cationic membrane-permeable dye JC-1 and the fluorescence intensity ratio at 590 vs. 530 nm**

Note. JC-1 at a concentration of 5 μM was added to the synaptosome incubation media (0.15 mg of protein/ml); incubated in the dark at a temperature of 37 °C for 10 min; after that SB aliquots were added to the cuvette; and the JC-1 fluorescence was recorded at 0, 15, 40, 90 min time point at an excitation wavelength of 485 nm and emission wavelength from 510 to 610 nm. Data are the mean values ± SEM. $n = 12$.

neurotoxicity signs and was biocompatible. Due to these features, we believe that SB has high potential for its application as an adsorbent in biotechnology and medicine.

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Competing interests

The authors declare no financial and non-financial competing interests exist.

Author Contributions

A. V. Terebilenko, M. O. Ivanytsya, D. O. Mazur, Ya. I. Kurys — Chemical methodology, Investigation, Data curation, Formal analysis; N. V. Krisanova, N. G. Pozdnyakova — Conceptualization, Biochemical methodology, Investigation, Data curation, Formal analysis, Writing — review & editing, Writing — original draft, Project administration; M. V. Dudarenko, A. O. Pastukhov, R. V. Sivko, L. M. Kalynovska, M. M. Driuk — Methodology, Investigation; T. A. Borisova, S. V. Kolotilov — Conceptualization, Data curation, Formal analysis, Writing — review & editing, Writing — original draft.

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БІОВУГІЛЛЯ З ЛУШПИННЯ НАСІННЯ СОНЯШНИКУ: СИНТЕЗ ТА ОЦІНКА РИЗИКУ ТОКСИЧНОСТІ

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Мета. Пошук ефективних біосумісних сорбентів, які мають нульову нейротоксичність, є актуальним завданням. Біовугілля є дуже перспективним вуглецевим матеріалом для адсорбції важких металів у навколишньому середовищі, стічних водах, а також в організмі людини.

Методи. Біовугілля з лушпиння насіння соняшнику (БЛС) було синтезовано шляхом піролізу при 800 °С без спеціальної функціоналізації. Ризик нейротоксичності БЛС оцінювали на тваринній моделі з використанням ізольованих пресинаптичних нервових закінчень кори головного мозку щурів (синаптосом).

Результати. В експериментах з використанням радіоактивно мічених нейромедіаторів було показано, що БЛС не змінює позаклітинні рівні збуджувального нейромедіатора L-[^{3}H] глутамату та гальмівного нейромедіатора [^{3}H] ГАМК у препаратах синаптосом в діапазоні концентрацій 0,25–1,0 мг/мл. У флуориметричних експериментах з використанням барвника JC-1, БЛС у концентрації 1,0 мг/мл не змінював мітохондріальний мембранний потенціал нервових закінчень.

Висновки. БЛС продемонстрував відсутність ознак нейротоксичності та високу біосумісність, тому БЛС має перспективи для використання як адсорбенту в біотехнології та медицині.

Ключові слова: сільськогосподарські відходи, лушпиння насіння соняшнику, біовугілля, нефункціоналізовані вуглецеві матеріали, ризик нейротоксичності, глутамат, ГАМК, пресинаптичні закінчення, мозок.