NATIONAL ACADEMY OF SCIENCES OF UKRAINE
Palladin Institute of Biochemistry

# BIOTECHNOLOGIA ACTA

***Vol. 15, No 5, 2022***

***BIMONTHLY***



**Editorial address:**
Palladin Institute of Biochemistry of the NAS of Ukraine, 9, Leontovich Street, Kyiv, 01054, Ukraine;
Tel.: +3 8 044–235–14–72; ***E-mail:*** biotech@biochem.kiev.ua; ***Web-site***: www.biotechnology.kiev.ua

According to the resolution of the Presidium of the National Academy of Sciences of Ukraine from 27.05.2009 №1-05 / 2 as amended on 25.04.2013 number 463 Biotechnologia Acta has been included in High Attestation Certification Commission list of Ukraine for publishing dissertations on specialties "Biology" and "Technology".

Certificate of registration of print media КВ series №19650-9450ПР on 01.30.2013

Literary editor — H. **Shevchenko**; Computer-aided makeup — **O. Melezhyk**
Authorized for printing 31.10.2022, Format — 210×297. Paper 115 g/m$^2$. Gaqrn. SchoolBookC. Print — digital. Sheets 10.8. An edition of 100 copies. Order 6.6. Make-up page is done in Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine. Print — **O. Moskalenko** FOP

NATIONAL ACADEMY OF SCIENCES OF UKRAINE
Palladin Institute of Biochemistry

# BIOTECHNOLOGIA ACTA

## Scientific journal

*Bimonthly*

*Vol. 15, No 5, 2022*

### *REVIEWS*



### *EXPERIMENTAL ARTICLES*

**НАЦІОНАЛЬНА АКАДЕМІЯ НАУК УКРАЇНИ**
**Інститут біохімії ім. О. В. Палладіна**

# BIOTECHNOLOGIA ACTA

## Науковий журнал

***Том 15, № 5, 2022***

UDC 581.6:582.37:631.5 https://doi.org/10.15407/biotech15.05.005

# WATER FERNS OF *Salviniaceae* FAMILY IN PHYTOREMEDIATION AND PHYTOINDICATION OF CONTAMINATED WATER

*KOSAKIVSKA I. V., VEDENICHEVA N. P., SHCHERBATIUK M. M., VOYTENKO L. V., VASYUK V. A.*

Kholodny Institute of Botany of the National Academy of Sciences of Ukraine, Kyiv

*E-mail: irynakosakivska@gmail.com*

Received 29.07.2022
Revised 27.09.2022
Accepted 31.10.2022

*Background*. Aquatic ecosystems are subjected to significant stress loads and depletion due to the influx of pollutants of inorganic and organic origin, that pose a serious threat to human health. The United Nations Environment Program has defined phytoremediation as an effective eco-technology for the removal, detoxification and immobilization of pollutants using plants. Water ferns of the *Salviniaceae* family belong to promising phytoremediants. They are characterized by high growth rates, resistance to adverse environmental factors, capable of adsorbing pollutants, including heavy metals. Species of the genus *Salvinia* and *Azolla* are used to assess the ecological state of water and study ecotoxicological effects of pollutants.

*Aim*. Analysis and generalization of the latest scientific results on the use of species of the *Salviniaceae* family for phytoremediation and phytoindication of contaminated water.

*Results*. In this review, we have highlighted key information on emerging phytotechnologies, including phytodegradation, phytostabilization, rhizofiltration, rhizodegradation, and phytovolatization. The growth and distribution features of species of the genus *Salvinia* and *Azolla* were described and current information on the use of water ferns for cleaning polluted water from heavy metals, inorganic and organic pollutants was presented. Data on the physiological and molecular mechanisms of the genus *Salvinia* and *Azolla* species adaptation to the toxic effect of pollutants of various origins were discussed. We focused special attention on the use of water ferns of the Salviniaceae family to control water pollution.

***Key words:*** *Salviniaceae*, aquatic ecosystems, phytoremediation, bioindication, organic and inorganic pollutants.

Anthropogenic impact, intensive industrial production and unbalanced environmental policy have led to serious environmental pollution. Waters are particularly adversely affected, as large, industrially developed regions are located on the banks of reservoirs and rivers. At the same time, within cities, even small closed reservoirs are subject to pollution, as they are sewage reservoirs for natural precipitation and absorbers of vehicle exhaust gases.

The United Nations Environment Program has defined phytoremediation as an effective eco-technology that involves the use of plants to remove, detoxify and immobilize environmental pollutants [1]. To diagnose the degree of anthropogenic pollution of water ecosystems, the method of phytoindication is used, which suggests detection of the dependence between the state of waters and the biological indicators of both individual plants and plant groups, including phytocenoses. Today, the achievements, tasks and prospects of phytoremediation and phytoindication are actively discussed by scientists of many countries of the world [2–5].

Vascular cryptogamous plants are one of the most ancient higher plants that appeared

on the planet more than 300 million years ago. The most widespread among them are representatives of the Polypodiophyta division, which grow in all climatic zones, are distinguished by a significant diversity of life forms, have a wide range of adaptive features, which allows them to exist in any environmental conditions [6]. Since these plants have a high potential in the accumulation of pollutants and water detoxification, significant stress tolerance and a high rate of biomass formation [7], they deserve attention as promising biological objects for the development of the latest biotechnologies for phytoremediation and phytoindication.

In our review, we focused on water ferns of the Salviniaceae family, analyzed and summarized the latest information on the use of these plants for cleaning and biotesting of polluted waters, discussed the mechanisms of resistance to the action of pollutants.

## Phytoremediation: eco-technology of cleaning using plants

Phytoremediation is an ecological method of purification of a contaminated environment with the participation of plants, the mechanism of which involves the absorbtion of pollutants by plants, the accumulation them in tissues, decomposition and transformations into harmless forms [1, 4, 5, 8, 9]. The use of plants for wastewater treatment began about 300 years ago [10]. Today, an effective accumulators of the inorganic and organic pollutants from reservoirs have been recognized the species of water macrophytes from families *Ranunculaceae, Lemnaceae, Cyperaceae, Salviniaceae, Haloragaceae, Hydrocharitaceae, Potamogetonaceae, Typhaceae, Najadaceae, Pontederiaceae* and *Juncaceae* [3].

Phytoremediation technology is a successful tool for reducing contamination of the aquatic environment. The initial stage is screening of plants able to store the heavy metals and other pollutants. For phytoremediation, the fast-growing species, that are easy to collect and handle, are selected [11]. The ontogenetic, physiological and biochemical traits as well as photosynthetic activity should be taken into account. The success of phytoremediation also depends on the intensity of pollution [12].

Different phytotechnologies are used to purify contaminated ecosystems, including phytodegradation, phytostabilization, rhizofiltration, rhizodegradation and phytovolatization [3]. The reduction of the content of pollutants in the soil occurs due to their uptake and binding by plant root system. In the process of immobilization, the roots accumulate, adsorb and precipitate pollutants, which is important for the removal of organic and inorganic contaminants from the soil, sludge and silt media [13-15]. During phytoextraction, pollutants are absorbed and hyperacumulated in different parts of the plant [16]. Absorption from soil, groundwater, residues and sludge with subsequent evaporation of pollutants into the atmosphere occurs during phytovolatilization [17]. Plants metabolize contaminants by means of compounds formed in their tissues [18, 19]. Rhizofiltration includes adsorption and precipitation of pollution into the substrate surrounding the root area [20]. Plants secrete various organic compounds that attract microbial communities present in the soil, which contributes to the decomposition of pollutants. This technology is called biosorption [21] and is used to remove heavy metals (HM) from wastewater [2, 22].

HM, such as cadmium (CD), lead (Pb), chromium (Cr), copper (Cu), zinc (Zn), nickel (Ni), vanadium (V) as well as metalloid selenium (SE) are the most common pollutants of aquatic ecosystems. When exceeding the threshold concentration, they become toxic to plants, induce the formation of active forms of oxygen, inhibit photosynthesis and respiration, can cause plant death [23].

## Hydrophyte ferns of the Salviniaceae family in phytoremediation of contaminated waters

Hydrophyte ferns, in particular representatives of the *Salviniaceae* family, which includes two genera *Salvinia* and *Azolla* [24], belong to promising plant species for water purification. Water ferns of the genus *Salvinia*, which includes 12 species [24], are characterized by high growth rates, adaptability and tolerance to adverse environmental factors, they are able to adsorb pollutants.

*Salvinia natans* (L.) All. is an annual hydrophyte fern widespread in Ukraine with a summer-green phenoritmotype [25]. It grows on the border of air and water environments, is characterized by a different structure of floating and submerged in the water layer photosynthetic organs — fronds. Submerged fronds are morphologically similar to roots (Fig. 1). The species occurs sporadically within its range in the temperate climate zone. It is

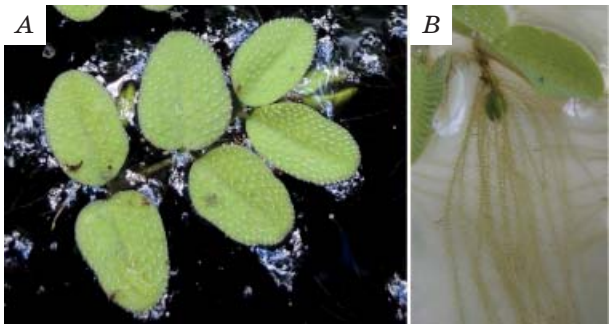


*Fig. 1. A — Salvinia natans* **in wild nature on the surface of the pond (Mizhrichynskyi Regional Landscape Park);** ***B*** **— the single submerged frond of Salvinia natans and stem apical bud with the young floating fronds**

widespread in mesoeutrophic and eutrophic freshwater closed or low-flow reservoirs and irrigation canals with a silty-sandy bottom.

In Ukraine, it occurs in the reservoirs of the Dnipro, Desna, Siversky Donets, Southern Bug, Dniester, Danube, Uzh, Latoritsa, Borzhava valleys, as well as in the ponds of the Forest Steppe and Steppe (Fig. 2) [26]. Due to the softening of the climate, the range of the fern has recently expanded. Significant populations of *S. natans* were reported in northern Europe in the Vistula River Delta [27].

The life cycle of Salvinia is represented by two independent generations: an asexual sporophyte and a sexual gametophyte. An adult Salvinia sporophyte is characterized by a clonal structure, formed during the formation of new modules that develop radially around the central (oldest) part of the plant. The complexity of the clone structure is determined by its age. The older the clone, the more complex its structure. The growth of clone modules proceeds similarly to the growth of side branches in other higher plants [28].

Modern climatic conditions have led to an extension of the growth period of the sporophyte, resulting in an increase in the number of vegetative generations from five or more, instead of two or three, which contributes to the spread of this fern and its occupation of new territories [29].

We have investigated the microstructure of the surface and the cell ultrastructure of floating and submerged fronds [30, 31], determined biometric indicators [28] and phytohormonal balance in the organs of the *S. natans* sporophyte at different stages of ontogenesis [6], analyzed the features of the photosynthetic apparatus functioning [32]. According to our observations, the species settled naturally and successfully develops on the surface of closed reservoirs on the left bank of the Dnieper within the city of Kyiv. These reservoirs are largely eutrified and have been subject to long-term lead pollution due to emissions of exhaust gases from automobile transport and waste of the now defunct Radykal enterprise — one of the most problematic in terms of environmental impact of industrial facilities within Kyiv. Significant pollution does not prevent the water fern *S. natans* from successful growth and reproduction, covering large surface areas (up to 20%, according to our estimates). Since the increase in biomass of *S. natans* is extremely fast, it is possible to periodically collect it and take it away for further burial in landfills, thus contributing to the gradual purification of these waters. In addition, *S. natans* can be easily spread into uninhabited waters by the transfer of green, free-floating sporophytes that proliferate rapidly.

It was reported previously that many species of terrestrial ferns are able to tolerate such concentrations of heavy metals that are toxic to other plants [33] and, accordingly, about the possibility of effective soil restoration using these plants. At the same time, a significant amount of pollutants adsorbed from the soil accumulated in plant tissues. Plant species considered to be weeds, including aquatic weeds, also show a high ability to hyperaccumulate some pollutants, such as herbicides, metalloids, and synthetic dyes [34, 35]. Macrophytes, particularly the free-floating ferns of the genus *Salvinia*, are well known for their physiological properties that allow them to minimize the cellular toxicity of hazardous chemicals when these pollutants hyperaccumulate [36, 37]. Along with macrophytes *Eicchornia* spp., *Pistia stratiotes*, *Lemna* spp. representatives of the genus Salvinia have the highest potential for hyperaccumulation of pollutants, and, therefore, for phytoremediation of contaminated waters [38]. *S. natans* can be used as an effective agent for phytoremediation of polluted waters.

Thus, the expediency of using *S. natans* for phytoremediation of wastewater contaminated with chromium and zinc salts was shown [39]. Also, *S. natans* was successfully used in experimental work on the purification of untreated complexly contaminated wastewater containing, in addition to other pollutants, dissolved ammonia in the form of $NH_4^+$ ions and $NO_2^-$ nitrites [40]. *S. natans* neutralizes significant concentrations of auxin herbicides, in particular 2,4-D in culture medium [41],

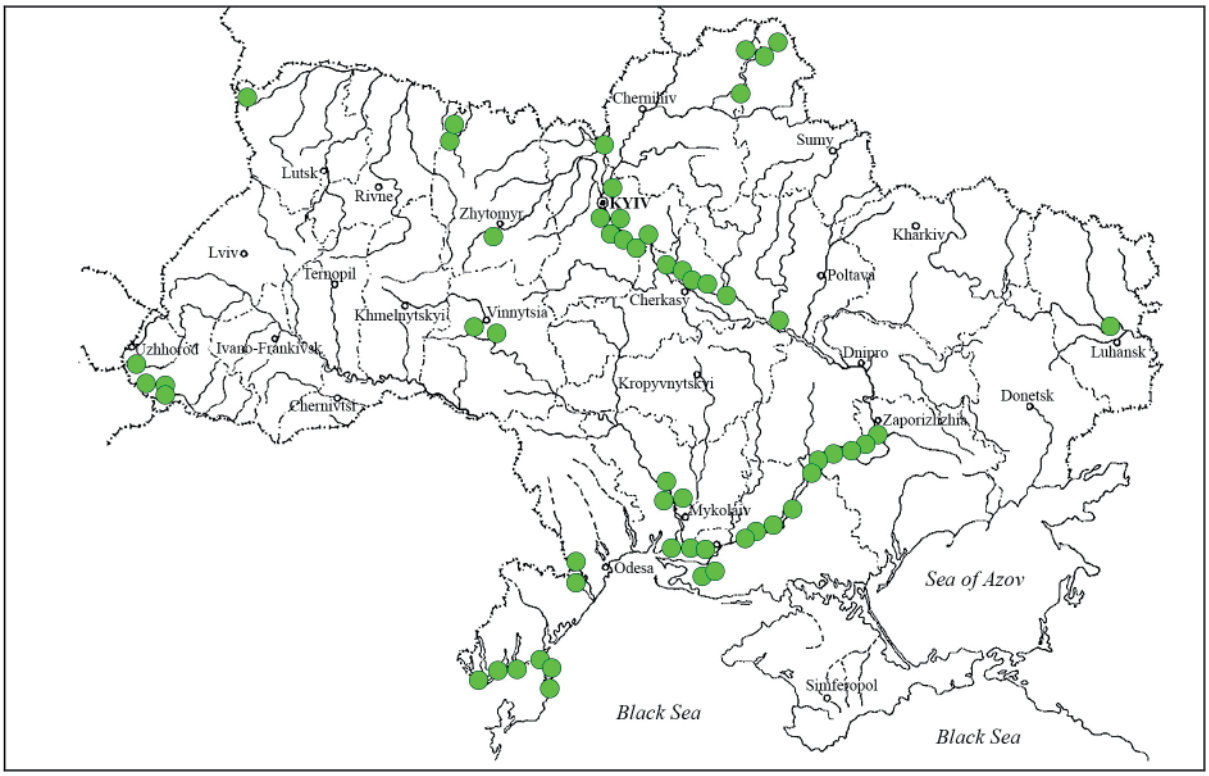


*Fig. 2.* **Map of distribution of the macrophyte *Salvinia natans* in Ukraine [26]**

actively binds complex aluminum compounds [42] and nitrogen-containing dyes dissolved in water [43]. This species of free-floating fern is characterized by high efficiency of the antioxidant system and osmotic stability of cells, which minimizes intracellular damage from herbicides [44]. In addition, it was reported that exogenous priming of *S. natans* with a solution of 500 µM 2,4-D for eight hours improves the tolerance of vegetative sporophytes to the impact of sodium (Na) and metalloid arsenic (As) [35]. In this study, primed sporophytes accumulated dissolved pollutants more actively than control ones.

Although *S. natans* is currently not used for mass industrial purification of wastewater and polluted waters, a set of experimental data convincingly proves that this free-floating macrophyte can be successfully used for phytoremediation (Table 1). First of all, due to their high tolerance to general water pollution and the ability to accumulate pollutants in significant concentrations, *S. natans* sporophytes effectively reduce the level of heavy metals and metalloids in contaminated water.

*Salvinia molesta* D.S. Mitchell also known as giant Salvinia is one of the most common aquatic weeds with a natural habitat in Brazil, from where it has spread to many tropical and subtropical regions of Africa, Asia, North and South America, Oceania, Australia, India, Indonesia since the beginning of the last century [45–47]. Colonies of *S. molesta* are formed from a tangled network about 100 free-floating plants. Each plant is 2.5 to 4.0 cm long, has two floating fronds, a submerged "root-like" frond and internodes. Floating fronds are bilobed, oval in shape with short petioles 1–5 mm long, the abaxial and adaxial surfaces are covered with trichomes. Immerged fronds up to 24 cm long, with short or long petioles, 0.2–1 cm long. Three stages in the ontogeny of *S. molesta* are distinguished: the first, when the plants have small floating fronds, which lie on the surface of the water; the second, when groups of plants with shuttle-shaped fronds are formed, and the third, when plants have vertically stacked fronds and form dense mats [48, 49]. *S. molesta* is found in slow-moving waters, including lakes, ponds, ditches, streams, rivers, and marshes. Under favorable conditions, mass groups of ferns form dense carpets up to 1 m thick, which double in size in 2–3 days [50]. Spores of *S. molesta* are sterile and non-viable. This fern is pentaploid, the number of chromosomes is

*Table 1.* **Summarized information on the phytoremediation of contaminated reservoirs by the aquatic ferns of the genus *Salvinia***

| ***Salvinia* spp.** | **Indicator of contamination** | **Indicator of phytoremediation** | **Source** |
|---|---|---|---|
| *Salvinia natans* | 15 mg/L | Zn — 84.8%; Cu — 73.8%; Ni — 56.8%; Cr — 41.4% | [91] |
| *Salvinia natans* | Cd — 80 mg/L; Pb — 50 mg/L; Ni — 20 mg/L | Cd — 23550 μg/g DW Pb — 9570 μg/g DW Ni — 42363 μg/g DW | [92] |
| *Salvinia minima* | Zn –1.00 mg/L Ni — 0.40 mg/L Cd — 0.03 mg/L Pb — 1.00 mg/L | Zn — 0.4046 mg/m$^2$ Ni — 0.0595 mg/m$^2$ Cd — 0.0045 mg/m$^2$ Pb — 0.1423 mg/m$^2$ | [71] |
| *Salvinia minima* | 0, 20, 40, 80, 160 M $NiCl_2$ | 16.3 mg/g | [76] |
| *Salvinia minima* | Pb (II) — 20–40 μM $AsO_4^{3-}$ — 200 μM | Pb — 34 mg/g As — 0.5 mg/g DW | [78] |
| *Salvinia minima* | Cr (VI) in the form $K_2Cr_2O_7$ 1 and 2 mg/L | 302.61 mg/g DW 451.39 mg/g DW | [79] |
| *Salvinia minima* | Cd (II) — 4 mg/L Pb (II) — 3 mg/L Cr (VI) — 4 mg/L | Cd (II) — 82.59%, Pb (II) — 97.44%, Cr (VI) — 80.31% | [73] |
| *Salvinia minima* | Wastewater | $PO_4$ — 59% $NO_3$ — 67.4% | [80] |
| *Salvinia minima* | $CuSO_4$ and $ZnSO_4$ — 80 μM/L | Cu — 6.96 mg/g DW Zn 19.6 mg/g DW | [93] |
| *Salvinia minima* | Wastewater $Pb^{2+}$, $Zn^{2+}$, $Ni^{2+}$ 10 mg/L; Dyes methylene blue (MB), crystal violet (CV), Bismarck brown (BB) — 10 mg/L | $Pb^{2+}$, $Zn^{2+}$ and $Ni^{2+}$ 98.56, 95.69 and 92.99%; CV, MB and BB 99.4, 99.1 and 96.5 5 | [77] |
| *Salvinia molesta* | Waste of coal mines | Pb — 96.96%; Ni — 97.01%; Cu — 96.77%; Zn — 96.38%; Mn — 96.22%; Fe — 94.12%; Cr — 92.85%; Cd — 80.99% | [94] |
| *Salvinia molesta* | Cu — 1.092 mg/L; Cr– 2.201 mg/L; Pb — 2.974 mg/L; Cd — 0.251 mg/L | Cu — 2.035 mg/L Cr — 1.05 mg/L Pb — 1.924 mg/L Cd — 0.018 mg/L | [95] |
| *Salvinia molesta* | Cu — 0,01 ppm Fe — 0,775 ppm Ni — 0,009 ppm Zn — 0,135 ppm | Cu –20% Fe — 4,5% Ni — 50% Zn — 10,3% | [56] |
| *Salvinia molesta* | Aqueous solutions of mercury chloride and lead chloride — 25, 50, 75, 100 mg/L | Pb — 85% Hg — 74% | [57] |
| *Salvinia molesta* | Wastewater $Ni^{2+}$, $Cr^{3+}$, $Cd^{2+}$, $Pb^{2+}$,10 μg/L | Ni, Cr, Cd, Pb — 85–90% | [58] |
| *Salvinia molesta* | Industrial wastewater | Na — 30% | [59] |
| *Salvinia molesta* | Textile wastewater | BOD and COD — 99%, | [60] |
| *Salvinia molesta* | Household wastewate | Turbidity — 97.7 % phosphates — 97.7% ammonia nitrogen — 99 % nitrates — 90.6% | [61] |
| *Salvinia biloba* | Cu 5 μg/mL | 11861 μg/L | [65] |

*Table 1. End*

| *Salvinia* spp. | Indicator of contamination | Indicator of phytoremediation | Source |
|---|---|---|---|
| *Salvinia biloba* | River water<br>Pb — 30,57 mg/L | Pb — 86.7% | [37] |
| *Salvinia biloba* | $Pb^{2+}$<br>4.8 mg/L<br>9.1 mg/L<br>19.6 mg/L | $Pb^{2+}$<br>97.7%<br>96.6%<br>91.6% | [66] |
| *Salvinia biloba* | Contaminated water 100 μM Cd | Floating fronds — 23 mg/g DW<br>Submerged fronds — 12 mg/g DW | [96] |
| *Salvinia biloba* | Contaminated water Cd, Cu, Pb and Zn<br>50 and 100 μM | Cu and Pb ≥96%,<br>Cd — 79% and 56% under 50 and 100 μM,<br>Zn — 77 and 70% under 50 and 100 μM | [69] |
| *Salvinia biloba* | $Pb^{2+}$<br>5.9, 8.2 and 22 ppm | 5–10% | [67] |
| *Salvinia biloba* | Hg<br>0.05, 0.1 and 0.2 μg/mL | 277.9 μg/g | [68] |
| *Salvinia cucullata* | $NH_4^+$ — 0.5, 1, 5, 10 and 15 mM | The content of nitrogen increased, potassium absorption decreased | [81] |
| *Salvinia cucullata* | Wastewater | BOD — 43.02%, COD — 31.04%, nitrates — 20.00%, ammonium — 5.26%, total phosphorus — 81.25% | [82] |
| *Salvinia rotundifolia* | Industrial wastewater Pb(II) 0.651 ppm | 50 g of fresh biomass removed 85-95% Pb(II) from 1.5 l of wastewater | [89] |
| *Salvinia auriculata* | River water<br>Ti, Fe, Mn, Cu, Zn, Sr | Ti — 3303, Fe — 4344,<br>Mn — 2882, Cu — 1366,<br>Zn — 34, Sr — 66 μL/μg | [88] |
| *Salvinia auriculata* | Artificial reservoirs Hg — 0.2 ng/L | Floating fronds — 85–246 ng/g DW<br>Submerged fronds — 88–265 ng/g DW | [87] |
| *Salvinia herzogii* | Water solutions<br>Cr, Cd, Pb | Submerged fronds –<br>Pb > Cd > Cr,<br>Floating fronds —<br>Cd > Pb > Cr | [84] |
| *Salvinia herzogii* | River water<br>Cr < 1 g/L;<br>Ni < 3 g/L;<br>Zn < 25 g/L | Zn — 35–42%,<br>Ni — 47–52%,<br>Cr — 99–100 | [85] |

45, which makes it genetically incapable of sexual reproduction and the completion of meiosis. Asexual vegetative reproduction at a fast pace is ensured by rhizome fragmentation and bud growth [49, 51]. Morphological and molecular studies have shown that *S. molesta* often mutates, which allows this species to quickly adapt to new environments [52]. The success of the growth of *S. molesta* depends on the temperature, illumination, pH, electrical conductivity, salinity and availability of nutrients in the aquatic environment [53]. *S. molesta* biomass is used for ethanol production. Monosaccharides from fern hydrolyzate were obtained by thermal acid hydrolysis, ultrasonic treatment, and enzymatic saccharification [54]. It has been established that *S. molesta* can absorb a significant amount of nutrients, in particular up to 8 mg of nitrogen per g of dry biomass per day [55].

Floating and submerged fronds of *S. molesta* accumulate and remove pollutants, including lead, copper, mercury, arsenic, zinc, and cadmium, from industrial and municipal wastewater [56, 57]. Fern removes up to 85% of lead and 74% of mercury within ten days [57]. *S. molesta* effectively purifies wastewater from phenolic compounds and dyes [58], sodium compounds [59] and [60]. In domestic wastewater, *S. molesta* plants reduced turbidity, phosphate, ammonia nitrogen,

and nitrate content by 97.7, 99.7, 99%, and 90.6%, respectively. Plants with greater biomass were more effective in removing excess nutrients [61]. *S. molesta* counteracts the harmful effects of HM due to the high activity of antioxidant defense enzymes: superoxide dismutase, catalase, peroxidase and ascorbate peroxidase. In floating fronds, enzyme activity was higher and less arsenic accumulated than in submerged ones [62]. In general, the fern *S. molesta* has proven itself as an effective phytoremediant of harmful substances of various nature from polluted waters and wastewater (Table 1).

*Salvinia biloba* Raddi is an autochthonous free-floating macrophyte originating from South America. This species is common in Argentina, Bolivia, Brazil, Chile, Paraguay and Uruguay. As a decorative culture, ferns can be found in various regions of the globe. The floating bilobed fronds of *S. biloba* have short petioles 1–9 mm long, a heart-shaped base with a system of hairs, long papillae, and heterogeneous areolar veins. Submerged fronds up to 45 cm long, with short or long petioles up to 2 cm long [63]. Floating and submerged fronds under favorable conditions form a huge vegetative mass on the surface of the water [64].

The use of *S. biloba* to treat water contaminated with cadmium, chromium, zinc, nickel, copper, and lead has been reported [37, 65, 66]. *S. biloba* proved to be tolerant to long-term (for 30 days) exposure to high concentrations of lead, which made it possible to use the fern to purify waters polluted by this heavy metal [67]. *S. biloba* is tolerant to mercury, which allows the fern to be used as a phytoremediant in waters contaminated with this metal [68]. Floating *S. biloba* fronds adsorbed HM ions from artificially polluted water in different ways. Removal of copper and lead ions (≥96%) was more active, cadmium (79 ± 4%) and zinc (77 ± 5%) ions were less active [69]. Pollutants are removed from contaminated waters by their adsorption and subsequent accumulation in *S. biloba* cells [70].

Therefore, the fast-growing and capable of hyperaccumulation of pollutants water fern *S. biloba* is suitable for phytoremediation of contaminated waters (Table 1).

*Salvinia minima* (Willd) is a floating fern, which, due to its high productivity and tolerance to a wide range of temperatures, is classified as a weed in tropical and subtropical regions. It is widespread in North, Central and South America. The floating fronds of *S. minima* are rounded in shape, contain hairs and heterogeneous areolar veins; have short petioles, 1–2 mm long. Underwater submerged fronds up to 4.5 cm long with short 1–2 mm petioles. The upper side of the floating fronds folds to the axis of the stem and is morphologically abaxial [63].

*S. minima* grows normally at low concentrations of cadmium (0.03 mg/l), nickel (0.40 mg/l), lead (1.00 mg/l), zinc (1.00 mg/l) and is able to adsorb HM at higher concentrations in the culture medium [71]. Hyperaccumulation of lead after exposure of ferns to a solution of 40 μM $Pb(NO_3)_2$ depended on the chelation and biosequestration of metals mediated by phytochelatins. Submerged fronds accumulated significantly more lead (II) than floating fronds, which was correlated with increased phytochelatin synthase (PCS) activity. Lead (II) accumulation occurred in the floating fronds due to a marked increase in the expression of the *SmPCS* gene [36]. Lead accumulates in ferns in the form of quasi-spherical and elongated nanoparticles (PbNP), which are localized on the cell membranes of floating and submerged fronds. Cellulose, lignin and pectin act as lead ion reducers [72]. The removal of lead from waters occurs by bioadsorption and subsequent accumulation in the cells of fronds. The distribution of lead between different compartments of the fern depends on the availability of nutrients, chelating agents and environmental conditions [73, 74]. In lead-contaminated waters, the rate of photosynthesis in *S. minima* was reduced by 44%, membrane damage was observed in the cells of submerged fronds, stomata were closed in floating fronds, and as a result, $CO_2$ intake decreased [75]. A concentration of nackel above 80 μM changed the integrity of cell membranes, affected photosynthesis, the efficiency of photosystem II, and reduced the content of photosynthetic pigments [76]. The fern is a hyperaccumulator of nickel, which is stored mainly in submerged fronds (16.3 mg/g of dry biomass). Quick absorption of nickel occurs in the first 6-12 hours of contact with the metal, and slows down over time [76].

*S. minima* is an effective biosorbent of methylene blue (MB), crystal violet (CV) and Bismarck brown (BB) dyes and HM ions lead (II), zinc (II) and nickel (II). The removal efficiency of dyes and metal ions exceeded 90% (99.4, 99.1 and 96.5% for CV, MB, BB and 98.56, 95.69 and 92.99% for lead, zinc and nickel respectively) under an initial dye concentration of 10 mg/l and a high concentration of metal ions. The maximum

adsorption capacity for CV, MB, BB dyes was 94.13, 150.98 and 228.81 mg/g, and for lead, zinc and nickel ions — 174.32, 232.43, 171.40 mg/g. It was established that the accumulation of dyes and HM ions occurred mainly due to chemisorption. In general, the fern was found to be an effective ecological means of purification of wastewater containing dyes and heavy metal ions [77]. *S. minima* accumulates in large quantities and removes from aqueous solutions HM cadmium, lead, chromium and metalloid arsenic [78, 79]. It was reported that the intensity of pollutant accumulation is affected by the intensity of lighting and the pH of aqueous solution [73]. *S. minima* also adsorbs numerous nutrients from eutrophied waters. This fern grows rapidly in municipal wastewater and can effectively remove excess $NO_3$ and $PO_4$, as well as reduce biological and chemical oxygen consumption (BOD and COD) by 67.4% and 72.4% and conductivity by 89%, 59% and 59% respectively during 28 days [80]. So, *S. minima* was an effective purifier of inorganic and organic pollutants (Table 1).

*Salvinia cucullata* Roxb. ex Bory comes from India. Its floating fronds have short petioles 0.5–1 mm long; the plates are rounded at the top, truncated at the base, 0.5–1 × 1,1–1.5 cm. Hairs are only on the abaxial surface, the papillae are short or absent, the areolar veins are heterogeneous. Submerged fronds are 3.5 cm long, with short 0.2–1 mm petioles [63].

Fern grows well in a nitrate-contaminated environment (0.5–1 mM). With an increase in pollutant concentrations up to 5 mM, growth is suppressed, potassium absorption was inhibited, but the amount of nitrogen in fronds increased, which became the basis for the use of *S. cucullata* as fertilizers, animal feed and in waters purification [81]. A significant decrease in biological and chemical oxygen consumption, nitrates and phosphates content (Table 1) was observed in wastewater after cultivation of ferns during 45 days [82].

*Salvinia herzogii* de la Sota is common in Argentina and Brazil. Floating two-bladed fronds with a high-cut top of up to 1/3 of the length of the plate and a heart-shaped base 1.5–2.5 × 2.7–3.8 cm are attached to short petioles 0.4–1.0 mm. The frond surface is covered with hairs that are divided into four segments at the apex and connected to the tips. It has long papillae and heterogeneous areolar veins. Submerged fronds are up to 10 cm long, with long 0.5–1 cm petioles [63]. *S. herzogii* is capable to change the morphology of fronds depending on the population density, which allows to compete for resources [83]. Submerged fronds of *S. herzogii* accumulate more chromium, cadmium, lead, zinc and nickel than floating fronds, and the removal of chromium from wastewater occurred faster than zinc and nickel [84, 85]. The absorption of chromium and cadmium by floating fronds of *S. herzogii* occurred due to the bioadsorption, helating and ion exchange [86].

*Salvinia auriculata* together with other macrophytes *Elodea densa, Sagittaria montevidensis, Pistia stratiotes* and *Eichhornia crassipes* in two artificial reservoirs actively accumulated mercury. The concentration of HM in the organs of plants ranged from 46–246 ng/g to 37–314 ng/g of fresh weigh. Negative correlation between mercury content and plant biomass has emphasized the importance of juvenile plants using to absorb mercury [87]. *S. auriculata* accumulated potassium, calcium, titanium, iron, manganese, chromium, cuprum, zinc and strontium, the content of which increased over time (Table 1). Coefficients of concentrations for all metals except strontium reached the highest value in 46 days of cultivation of ferns in contaminated river water [88].

It was reported that *S. rotundifolia* removed 85–95% of lead from contaminated industrial wastewater [89]. Ferns *S. auriculata, S. biloba, S. herzogii, S. minima, S. molesta, S. natans* and *S. rotundifolia* accumulated HM gold, cadmium, chromium, cesium, cooper, iron, manganese, nickel, lead, strontium and zinc up to 6000–18000 mg/kg of dry biomass, which allows to use them for effective purification of industrial and wastewater (Table 1) [90].

## Hydrophyte ferns of the *Azolla* genus in phytoremediation of polluted waters

The genus *Azolla* unites aquatic ferns, which are characterized by small fronds and bright colors from green to burgundy. Due to the significant water repellency of the scaly fronds, the fern floats on the surface of stagnant water in tropical, subtropical, and temperate regions around the world [97, 98]. Reproduction occurs mainly through rapid vegetative segmentation, the biomass of the fern doubles in two to four days [99]. The degree of sporulation is quite low and requires certain conditions for instance, it can

be induced by far-red light [100]. A unique feature of ferns of this genus is the formation of symbiosis with the nitrogen-fixing cyanobacterium *Anabaena azollae* [101]. Due to the significant rate of nitrogen assimilation and extremely high productivity, ferns of the genus Azolla are used in agricultural production as biofertilizers [102, 103] and feed bioadditive for animals in aquaculture [104]. Recently, *Azolla* ferns have attracted attention as potential phytoremediants. The ability of *Azolla* ferns to absorb heavy metals attracts special attention of researchers (Table 2).

*Azolla filiculoides* Lam. is an invasive plant that is widespread in tropical and temperate regions throughout the world. An adult plant is 1–2 cm in size, colored at the edges in pink, orange or red. It differs from other Azolla species by the presence of single-cell trichomes on the surface, which provide water-repellent properties of plant. The ability to successful absorb nitrogen and phosphorus compounds from wastewater was found in *A. filiculoides* [105]. Growth stimulation of *A. filiculoides* under the addition of nitrogen and phosphorus had a limiting concentration of 50 μM/L, at higher concentrations chlorosis due to iron deficiency was observed [106]. The fern *A. filiculoides* is known as a hyperaccumulator of lead, cadmium, chromium, nickel, silver and gold [107]. When *A. filiculoides* was grown for 15 days in solutions containing 5, 10, and 25 mg/L of lead, nickel, and cadmium, the cleaning efficiency reached a maximum at day 10 at a metal concentration of 5 mg/L and decreased at a higher level of pollution [108]. Accumulation of cadmium by A. *filiculoides* was an order of magnitude lower than that of copper when these compounds were added in a complex with EDTA, while cadmium alone caused significant damage to photosystem II [109]. Water pollution with iron, chromium and aluminum did not prevent the growth of the fern *A. filiculoides*, instead, aluminum had even a small stimulating effect. Fern removed 92% of iron, 96% of aluminum and more than 80% of chromium [110]. Water pollution with iron, chromium and aluminum did not prevent the growth of the fern *A. filiculoides*, instead, aluminum had even a small stimulating effect. *A. filiculoides* effectively absorb nickel from aqueous and galvanic solutions, even at extreme pH values [111]. Ferns of the genus Azolla are also able to accumulate and remove organic compounds from the water environment. Thus, *A. filiculoides* removed up to 50% of diclofenac and 60% of levofloxacin from the water [110]. *A. filiculoides* absorbs up to 90% of the phenolic substance pyrocatechol, which is a precursor of pesticides and flavorings and one of the most famous water pollutants [112]. Phenanthrene, a tricyclic aromatic hydrocarbon, one of the most common environmental pollutants from vehicle exhaust and asphalt heating, is absorbed by *A. filiculoides* by 88, 69, and 60% at contamination levels of 1, 5, and 10 mg/L, respectively [113].

*Azolla pinnata* R. Brown, the smallest species from the genus Azolla, endemic to the coastal areas of Africa, Asia and Australia. Triangular stems 2.5 cm in length that bears many rounded or angular green, blue-green, or dark red leaves each 1-2 mm long, coated in tiny hairs, giving them a velvety appearance. The growth of this fern did not depend on the presence of nitrogen in the environment, apparently, the fern supplied itself with this macroelement due to symbiotic nitrogen fixation [114]. *A. pinnata* plants absorbed 86.97% of iron sulfate and 81.14% of zinc sulfate at an initial concentration of 100 ppm for 20 days [115]. *A. pinnata* actively accumulated the herbicide 2,4-D and converted it into less toxic compounds that were deposited in the cell walls [116].

*A. caroliniana* Willd. is native for North and South America, the Caribbean. Scaly leaves 5–10 mm long are green or red, they are coated with two-cell trichomes. Plants purified water from mercury and chromium compounds by almost 100% in 12 days of the experiment. At the same time, the content of metals in fern tissues increased from 71 to 964 mg/kg of biomass, chromium absorption was more effective [117]. This fern also accumulated up to 5 mg of lead per 1 kg of dry matter at a concentration of 20 mg/L of lead acetate, but the toxicity of the metal had a significant negative effect on the photosynthetic apparatus and plant metabolism [118].

*A. microphylla* Kaulf. occurs in North America. Floating fronds 0.6–2 mm long are green or red, submerged fronds reach a length of 5 cm. Cultivation of *A. microphylla* significantly improved the quality of wastewater from fish breeding ponds (temperature, turbidity, pH, ammonium content) [119]. The accumulation of aluminum in the body of *A. microphylla* occurred in proportion to the increase in the concentration of $AlCl_3$ in the water, while significant activation of the antioxidant system contributed to detoxification and maintenance of metabolic and growth processes in floating fern fronds [120].

*Table 2.* **Summarized information on the phytoaccumulation of heavy metals by aquatic ferns of the genus *Azolla* from polluted waters (adapted from [107])**

| *Azolla* spp. | Heavy metal (HM) | Initial concentration of HM | Duration of the experiment (d) | Accumulation of HM (DW) or absorption efficiency (%) | Source |
|---|---|---|---|---|---|
| *Azolla pinnata* | Hg | 3.0 mg/L | 13 | 667 µg/g | [125] |
| | Hg | 10.0 µg/L | 21 | 450 µg/g | [126] |
| | Hg | 3.0 mg/L | 6 | 940 µg/g | [127] |
| | Cd | 3.0 mg/L | 13 | 740 µg/g | [125] |
| | Cd | 10.0 mg/L | 7 | 2759 µg/g | [128] |
| | Cr(III) | 3.0 mg/L | 13 | 1095 µg/g | [129] |
| | Cr(VI) | 20.0 µg/L | 14 | 9125 µg/g | [130] |
| | Ni | 500 mg/L | 7 | 16252 µg/g | [128] |
| | Fe | 100 ppm | 20 | 87 % | [118] |
| | Zn | 100 ppm | 20 | 81 % | [118] |
| *Azolla caroliniana* | As | 80.0 µg/L | 7 | >120 µg/g | [131] |
| | Pb | 1.0 mg/L | 12 | 416 µg/g | [132] |
| | Cd | 1.0 mg/L | 12 | 259 µg/g | [132] |
| | Cr(VI) | 1.0 mg/L | 12 | 356 µg/g | [110] |
| | Cr(III) | 1.0 mg/L | 12 | 964 µg/g | [110] |
| | Hg | 1.0 mg/L | 12 | 578 µg/g | [110] |
| | Pb | 20 mg/L | 10 | 5 mg/g | [111] |
| *Azolla filiculoides* | As | 80.0 µg/L | 7 | >60 µg/g | [131] |
| | Cr(VI) | 20.0 µg/L | 14 | 12 383 µg/g | [130] |
| | Cr(III) | 9.0 mg/L (ppm) | 4 | 1904 ppm | [133] |
| | Cd | 9.0 mg/L (ppm) | 4 | 10441 ppm | [133] |
| | Cd | 10.0 mg/L | 7 | 2608 µg/g | [128] |
| | Ni | 9.0 mg/L (ppm) | 4 | 8814 ppm | [133] |
| | Ni | 500 mg/L | 7 | 28 443 µg/g | [128] |
| | Cu | 9.0 mg/L (ppm) | 4 | 9224 ppm | [133] |
| | Zn | 9.0 mg/L (ppm) | 4 | 6408 ppm | [133] |
| | Fe | 5.0 mg/L | 8 | 92% | [112] |
| | Al | 5.0 mg/L | 8 | 96% | [112] |
| | Cr | 5.0 mg/L | 8 | 10% | [112] |
| | Pb | 5 mg/L<br>10 mg/L<br>25 mg/L | 15 | 95%<br>97%<br>79% | [115] |
| | Ni | 5 mg/L<br>10 mg/L<br>25 mg/L | 15 | 71%<br>69%<br>77% | [115] |
| | Cd | 5 mg/L<br>10 mg/L<br>25 mg/L | 15 | 93%<br>89%<br>66% | [115] |
| | Cd | 1 mg/L<br>2.5 mg/L<br>2.7 mg/L | | 188.7 mg/kg<br>673.5 mg/kg<br>93.11 mg/kg | [117] |
| | Cu | 2.6 mg/L | | 1169.45 mg/kg | [117] |
| *A. microphylla* | Cr(VI) | 20.0 µg/L | 14 | 14 931 µg/g | [130] |
| | Ni | 500 mg/L | 7 | 21 785 µg/g | [128] |
| | Cd | 10.0 mg/L | 7 | 1805 µg/g | [128] |
| | Al | 100, 250, 500 and 750 µM | 6 | 195.8 µg/g FW | [119] |
| *A. imbricata* | Cd | 0.5 µg/L | 9 | 183 µg/g | [134] |

It was shown also, that in *A. imbricata*, excess cadmium induced the expression of genes encoding anthocyanin biosynthesis [121]. Cultivation of *A. japonica* reduced the nitrogen content in the medium by half in less than a week [122]. It was determined that purification of environment from antibiotics by ferns Azolla spp. occurred in three stages: absorption of the substance by the fern with the formation of reactive oxygen species, which were partially neutralized, conjugation of the substance with the participation of glutathione transferase and glutathione, and deposition of the assimilated substance in the apoplast, vacuoles, and cell wall [123].

The ability to adsorb an excess of macronutrients has been established for all species of ferns of this genus, although they differ in their tolerance to pollutants. Thus, *A. microphylla* showed greater tolerance to supraoptimal nitrogen concentrations than *A. caroliniana*, *A. imbricata*, and *A. mexicana*. In addition, this species had the highest nitrogen absorption efficiency [124].

## Hydrophyte ferns of the *Salviniaceae* family in phytoindication of water pollution

To determine the pollution of waters, bioindication methods are used. Special signs that allow us to assess changes in mineralization and purity of the environment are distinguished in indicator plants. These include physiological (level of transpiration, pigmentation, salt content), morphological (size, branching), phenological (anomalies of the development rhythm, growing season) indicator signs. The most sensitive according to these indicators are aquatic macrophytes, the species composition and productivity of which reflect the nature of water pollution with organic substances, heavy metals, pesticides, etc. Due to a closer connection with the aquatic environment, aquatic macrophytes are the most convenient object for phytoindication of waters [135].

Thus, it was shown that the growth rate of the fern *S. natans* increased by 20% when the water was polluted with nickel at a concentration of 0.25 mg/l, while it was significantly inhibited at metal concentrations of 0.5, 0.75, 1 and 2 mg/L [136]. Phenotypic changes of the floating fronds of *S. biloba* were detected on the fifth day of cultivation in artificially contaminated water with cadmium (100 μM), which were manifested in the form of chlorosis and necrosis. In the floating fronds, the metal content on the third day was 3 mg/g DW, and on the tenth — 23 mg/g DW, while in the submerged fronds, it was 3 and 12 mg/g DW, respectively [96]. Prolonged exposure to lead and cadmium (for 10 days) induced changes in the content of photosynthetic pigments (carotenoids, chlorophylls *a* and *b*), secondary metabolites (anthocyanins and flavonoids), soluble carbohydrates, changed the stability of cell membranes of floating and submerged fronds. Such adverde effects were correlated with qualitative changes in the fern phenotype. The plants showed typical signs of toxicity, such as chlorosis and necrosis of floating fronds, the appearance of a brownish-red color on the surface of plants, and a decrease in total plant biomass [37, 96]. *S. biloba* is a bioindicator of cuprum contamination in aquatic ecosystems. At the high concentrations of metal, symptoms of plant intoxication and death were observed [65]. When studying the phytoextraction capacity of *S. natans*, it was shown that the fern actively accumulated lead and copper (>3.328±0.032 and >2.641±0.014 mg/kg DW, respectively). High concentrations of HM negatively affected the growth and habit of the fern, which allows the use of *S. natans* for biotesting [137].

A number of investigations are devoted to the elucidation of the impact of HM pollution on the physiological state of aquatic macrophytes in natural and experimental conditions. It has been reported that *S. natans* is able to accumulate high levels of HM. Thus, the accumulation of chromium, iron, nickel, copper, lead, and cadmium ranged from 6 to 9 mg/g DW, while the accumulation of cobalt, zinc, and manganese was ~4 mg/g DW. The accumulation of HM affected the photosynthetic activity of fern, in particular, the efficiency of carbon assimilation, photochemical activity, and photophosphorylation [91]. Significant growth rate, simplicity of cultivation, distribution and sensitivity to various harmful substances as well as the ability to hyperaccumulate pollutants contribute to the successful use of *S. natans* for biotesting and purification of contaminated waters [138].

Chlorosis was detected in the fern *S. cucullata*, which was grown in a medium containing 0.5, 1, 2, and 4 mg/L cadmium and 5, 10, and 40 mg/L lead. With an increase in the duration of the negative impact and a raise in the concentration of HM, the growth of plants slowed down, the accumulation of biomass decreased, and the content of chlorophyll reduced. Submerged fronds accumulated more

cadmium and lead than floating fronds [139]. The fern grew well in a medium containing 0.5–1 mM $NH_4^+$. On the other hand, the concentration of the pollutant above 5 mM inhibited the growth rate, the number and length of the submerged branches decreased, signs of chlorosis appeared [81]. It was reported that with increasing concentrations of cadmium, copper, chromium, mercury, lead, nickel, and zinc in the aquatic environment, growth and raw biomass accumulation by macrophytes *S. natans*, *S. molesta*, and *S. auriculata* were inhibited [140].

In *A. microphylla* plants under high concentrations of aluminum in water (up to 750 μM), the size of the submerged fronds and the content of phenols and flavonoids significantly decreased, but the level of chlorophylls, sucrose, starch, photosynthesis efficiency and nitrogen-fixing capacity remained almost normal [119]. The growth of *A. filiculoides* was inhibited by 42% under the contamination with phenathrene at a concentration of 10 mg/L, simultaneously the content of photosynthetic pigments was significantly reduced [123]. The presence of 1 mM phenol in the environment also negatively affected the morpho-biochemical parameters of this fern: numerous necrosis was observed, the malondialdehyde content increased significantly [141].

Therefore, aquatic ferns of the Salviniacea family can be used as a valuable tool for biotesting water contaminated with pollutants of various nature.

## Conclusion

Aquatic macrophytes of the Salviniaceae family play an important role in the phytoremediation of contaminated waters, improve water quality, promote the circulation of nutrients, stabilize and optimize the habitat of other species of flora and fauna. They are characterized by rapid growth, accumulation of significant biomass, are able to absorb heavy metals and other hazardous waste, possess physiological and molecular mechanisms of adaptation to the toxic effects of pollutants. Aquatic macrophytes of the Salviniaceae family remove pollutants from waters by surface adsorption and incorporate them into their own system or store them in bound form. Species of the genera Salvinia and Azolla are successfully used to assess the ecological state of waters, the ecotoxicological effects of pollutants are studied on them, and biotechnological approaches for biotesting are developed. The properties and characteristics summarized in the review reveal the enormous potential of water ferns for the creation of ecologically acceptable and economically profitable modern biotechnologies for the purification of large volumes of polluted waters from substances harmful to the environment.

This review was prepared as a part of research work 0121U114018 “Exogenous phytohormones in the regulation of growth and development of aquatic macrophytes of the genus *Salvinia* — perspective for phytoindication and phytoremediation of contaminated water” supported by the National Academy of Sciences of Ukraine.

## REFERENCES

1. *UNEP*. Phytoremediation: An Environmentally Sound Technology for Pollution Prevention, Control and Remediation. 2019. http://www.unep.or.jp/Ietc/
2. *Ali S., Abbas Z., Rizwa M., Zaheer I.E., Yavas I., Ünay A., Abdel-Daim M.M., Bin-Jumah M., Hasanuzzaman M., Kalderis D.* (2020) Application of Floating Aquatic Plants in Phytoremediation of Heavy Metals Polluted Water: A Review. *Sustainability*. 2020, 12, 1927. https://doi.org/10.3390/su12051927
3. *Ansari A.A., Naeem M., Gill S.S., AlZuaibr F.M.* Phytoremediation of contaminated waters: An eco-friendly technology based on aquatic macrophytes application. *Egyptian Journal of Aquatic Research*. 2020, 46, 371–376. https://doi.org/10.1016/j.ejar.2020.03.002
4. *Favas P.J.C., Pratas J., Rodrigues N., D'Souza R., Varun M., Paul M.S.* Metal(loid) accumulation in aquatic plants of a mining area: Potential for water quality biomonitoring and biogeochemical prospecting. *Chemosphere*. 2017, 194, 158–170. https://doi.org/10.1016/j.chemosphere.2017.11.139
5. *Yadav K.K., Gupta N., Kumar A., Reecec L.M., Singh N., Rezania S., Khan S.A.* Mechanistic understanding and holistic approach of phytoremediation: A review on application and future prospects. *Ecol. Engineer*. 2018. 120, 274–298. doi: 10.1016/j.ecoleng.2018.05.039
6. Phytohormonal system and structural-physiological features of pteridophytes (Polypodiaphyta). Ed. I.V. Kosakivska. Kyiv: Nash Format. 2019. 250 p.

7. *Dhir B.* Role of Ferns in Environmental Cleanup. In: Fernández, H. (eds) *Current Advances in Fern Research*. Springer, Cham. 2018. https://doi.org/10.1007/978-3-319-75103-0_25
8. *Mustafa H.M., Hayder G.* Recent studies on applications of aquatic weed plants in phytoremediation of wastewater: A review article. *Ain Shams Engineering Journal*. 2021, 12(1), 355–365. https://doi.org/10.1016/j.asej.2020.05.009
9. *Vidal C.F., Oliveira J.A., da Silva A.A., Ribeiro C., Farnese F.D.S.* Phytoremediation of arsenite-contaminated environments: Is *Pistia stratiotes* L. a useful tool? *Ecol. Indicat*. 2019, 104, 794–801
10. *Carolin C.F., Kumar P.S., Saravanan A., Joshiba G.J., Naushad M.* Efficient techniques for the removal of toxic heavy metals from aquatic environment: A review. *J. Environ. Chem. Eng*. 2017, 5, 2782–2799.
11. *Stefani G.D., Tocchetto D., Salvato M., Borin M*. Performance of a floating treatment wetland for in-stream water amelioration in NE Italy. *Hydrobiol*. 2011, 674, 157–167.
12. *Jamuna S., Noorjahan C.M*. Treatment of sewage waste water using water hyacinth — Eichhornia sp. and its reuse for fish culture. *Toxicology International*. 2009, 16 (2), 103–106.
13. *Baker A.J.M., McGrath S.P., Sidoli C.M.D., Reeves R.D.* The possibility of *in situ* heavy metal decontamination of polluted soils using crops of metalaccumulating plants. *Resources, Conservation and Recycling*. 1994, 11, 41–49.
14. *Brooks R.R.* Plants that Hyperaccumulate Heavy Metals. In: Plants and chemical elements: Biochemistry, uptake, tolerance and toxicity. Ed. Margaret E. Farago. VCH Verlagsgesellschaft, Weinheim, Germany, 1994, 88–105.
15. *Cunningham S.D., Ow D.W*. Promises and prospects of phytoremediation. *Plant Physiol*. 1996, 110, 715–719.
16. *Rulkens W.H., Tichy R., Grotenhuis J.T.C.* Remediation of polluted soil and sediment: Perspectives and failures. *Water Science and Technology*. 1998, 37, 27–35.
17. *Girdhar M., Sharma N.R., Rehman H., Kumar A., Mohan A.* Comparative assessment for hyper-accumulatory and phytoremediation capability of three wild weeds. *Biotech*. 2014, 4, 579–589.
18. *Black H*. Absorbing possibilities: Phyto-remediation. *Environmental Health Perspectives*. 1995, 103, 1106–1108.
19. *Chaudhry T.M., Hayes W.J., Khan A.G., Khoo C.S.* Phytoremediation — focusing on accumulator plants that remediate metal contaminated soils. *Austral. J. Ecotoxicol*. 1998, 4, 37–51.
20. *Dushenkov V., Kumar P.B.A.N., Motto H., Raskin I*. Rhizofiltration: The use of plants to remove heavy metals from aqueous streams. *Environ. Sci. Technol*. 1995, 29, 1239–1245.
21. *Verma V.K., Tewari S., Rai J.P.N*. Ion exchange during heavy metal biosorption from aqueous solution by dried biomass of macrophytes. *Bioresource Technol*. 2008, 99, 1932–1938.
22. *Fard R.F., Azimi A.A., Nabi Bidhendi* G.R. Batch kinetics and isotherms for biosorption of cadmium onto biosolids. *Desalination and Water Treatment*. 2011, 28, 69–74.
23. *Zhang T., Lu Q., Su C., Yang Y., Hu D., Xu Q*. Mercury induced oxidative stress, DNA damage, and activation of antioxidative system and Hsp70 induction in duckweed (*Lemna minor*). *Ecotoxicol. Environ. Safety*. 2017, 143, 46–56.
24. *Smith A.R., Pryer K.M., Schuettpelz E., Korall P., Schneider H., Wolf P.G.* A classification for extant ferns. *Taxon*. 2006, 55, 705–731.
25. *Dubyna D.V., Shelyag-Sosnko Yu.R., Zhmud O.I., Zhmud M.E., Dvoretsky T.V., Dzyuba T.P., Tymoshenko P.A.* Danube Biosphere Reserve. The plant world. Kyiv: Phytosociotsentr. 2003, 448 p. [In Ukrainian]
26. Green Book of Ukraine. Гол. Ed. Ya.P. Didukh. Kyiv: Alterpress. 2009, 448 p. [In Ukrainian]
27. *Galka A., Szmeja J*. Phenology of the aquatic fern *Salvinia natans* (L.) All. in the Vistula Delta in the context of climate warming. *Limnologica*. 2013, 43, 100–105.
28. *Babenko L., Vasheka O., Shcherbatiuk M., Romanenko P., Voytenko L., Kosakivska I.* Biometric characteristics and surface microstructure of vegetative and reproductive organs of heterosporous water fern *Salvinia natans*. *Flora*. 2019, 252, 44–50. https://doi.org/10.1016/j.flora.2019.02.006
29. *Seastedt T*. Ecology: Traits of plant invaders. *Nature*. 2009, 459, 783–784. https://doi.org/10.1038/459783a
30. *Shcherbatiuk M.M., Babenko L.M., Sheyko O.A., Kosakivska I.V*. Microstructural features of water fern *Salvinia natans* (L.) All. Modern Phytomorphol. 2015, 7, 129–133. [In Ukrainian]
31. *Shcherbatiuk M.M., Babenko L.M., Kosakivska I.V*. The ultrastructure of chloroplasts and photosynthetic pigments in floating and submerged leaves of water fern *Salvinia natans* (L.) All during ontogeny. Modern Phytomorphol. 2016, 9, 85–95. [In Ukrainian]
32. *Kosakivska I.V., Shcherbatiuk M.M., Babenko L.M., Polishchuk O.V*. Characteristics of photosynthetic apparatus of aquatic fern *Salvinia natans* floating and submerged fronds. *Adv. Biol. Earth Sci*. 2018, 3(1), 13–26. http://jomardpublishing.com/UploadFiles/Files/journals/ABES/V3N1/KasakivskaI.pdf

33. *Kachenko A.G., Singh B., Bhatia N.P.* Heavy metal tolerance in common fern species. *Austral. J. Botan*. 2007, 55 (1), 63–73. https://doi.org/10.1071/BT06063
34. *Mohebi Z., Nazari M*. Phytoremediation of wastewater using aquatic plants. A review. *J. Appl. Res. Water Wastewater*. 2021, 8 (1), 50–58.
35. *Dolui D., Hasanuzzaman M., Saha I., Ghosh A., Adak M.K*. Amelioration of sodium and arsenic toxicity in *Salvinia natans* L. with 2,4-D priming through physiological responses. *Environ. Sci. Pollut. Res*. 2022, 29, 9232–9247. https://doi.org/10.1007/s11356-021-16246-7
36. *Estrella-Gómez N., Mendoza-Cózatl D., Moreno-Sánchez R., González-Mendoza D., Zapata-Pérez O., Martínez-Hernández A., Santamaría J.M*. Pb-hyperaccumulator aquatic fern *Salvinia minima* Baker, responds to $Pb^{2+}$ by increasing phytochelatins via changes in SmPCS expression and in phytochelatin synthase activity. *Aquat. Toxicol*. 2009, 91 (4), 320–328. https://doi.org/10.1016/j.aquatox.2008.11.002
37. *Loría K.C., Emiliani J., Bergara C.D., Herrero M.S., Salvatierra L.M., Pérez L.M*. Effect of daily exposure to Pb-contaminated water on *Salvinia biloba* physiology and phytoremediation performance. *Aquat. Toxicol*. 2019, 210, 158–166. https://doi.org/10.1016/j.aquatox.2019.02.019
38. *Rezania S., Taib S.M., Din M.F.M., Dahalan F.A., Kamyab H*. Comprehensive review on phytotechnology: heavy metals removal by diverse aquatic plants species from wastewater. *J. Hazardous Mater*. 2016, 318, 587–599. https://doi.org/10.1016/j.jhazmat.2016.07.053
39. *Dhir B., Sharmila P., Saradhi P.P*. Photosynthetic performance of *Salvinia natans* exposed to chromium and zinc rich wastewater. *Braz. J. Plant Physiol*. 2008, 20, 61–70.
40. *Laabassi A., Boudehane A*. Wastewater treatment by floating macrophytes (*Salvinia natans*) under Algerian semi-arid climate. *Europ. J. Engineer. Nat. Sci*. 2019, 3, 103–110.
41. *Dolui D., Saha I., Adak M.K*. 2, 4-D removal efficiency of *Salvinia natans* L. and its tolerance to oxidative stresses through glutathione metabolism under induction of light and darkness. *Ecotoxicol. Environ. Saf*. 2021, 208, 111708. https://doi.org/10.1016/j.ecoenv.2020.111708
42. *Mandal C., Ghosh N., Maiti S., Das K., Sudha Gupta, Dey N., Adak M.K*. Antioxidative responses of Salvinia (*Salvinia natans* Linn.) to aluminium stress and it's modulation by polyamine. *Physiol. Mol. Biol. Plants*. 2013, 19, 91–103. https://doi.org/10.1007/s12298-012-0144-4
43. *Ohtani S., Gon M., Tanaka K., Chujo Y*. The design strategy for an aggregation-and crystallization-induced emission-active molecule based on the introduction of skeletal distortion by boron complexation with a tridentate ligand. *Crystals*. 2020, 10 (7), 615. https://doi.org/10.3390/cryst10070615
44. *Pandey P.K., Singh S., Singh A.K., Samanta R., Yadav R.N., Singh M.C*. Inside the plant: bacterial endophytes and abiotic stress alleviation. *J. Appl. Nat. Sci*. 2016, 18 (4), 1899–1904. https://doi.org/10.31018/jans.v8i4.1059
45. *Julien M.H., Hill M.P., Tipping P.W. Salvinia molesta* D.S. Mitchell (Salviniaceae). In: Muniappan R., Reddy G.V., Raman A. (eds.). *Biological control of tropical weeds using arthropods*. Cambridge University Press, Cambridge, UK, 2009, 378–407.
46. *Koncki N.G., Aronson M.F.J*. Invasion risk in a warmer world: Modeling range expansion and habitat preferences of three nonnative aquatic invasive plants. *Invas. Plant Sci. Manag*. 2015, 8(4), 436–449.
47. *Madani I., Sinada F., Mohammed T. Salvinia molesta* (Salviniaceae) a new record for the flora of Sudan. *Int. J. Sci. Environ. Technol*. 2019, 8 (1), 132–140.
48. *McFarland D.G., Nelson L.S., Grodowitz M.J., Smart R.M., Owens C. Salvinia molesta* D. S. Mitchell (Giant Salvinia) in the United States: A Review of Species Ecology and Approaches to Management. *Aquatic Plant Control Research Program*, 2004, 1–41. http://acwc.sdp.sirsi.net/client/search/asset/1002855
49. *Nelson L.* Giant and Common Salvinia. In: Gettys L.A., Haller W.T. and Petty D.G. eds., *Biology and Control of Aquatic Plants: A Best Management Practices Handbook*, 3rd Edition, Aquatic Ecosystem Restoration Foundation, Marietta, GA, 2014, 157–164. http://www.aquatics.org/bmp%203rd%20edition.pdf
50. *Andama M., Ongom R., Lukubye B.* Proliferation of *Salvinia molesta* at Lake Kyoga Landing Sites as a Result of Anthropogenic Influences. *J. Geosci. Envir. Protec*. 2017, 5 (11), 160–173. https://doi.org/10.4236/gep.2017.511012
51. *Jacono C.C. Salvinia molesta* (Salviniaceae), new to Texas and Louisiana. *Sida*. 1999, 18, 927–928.
52. *Galam D., Silva J., Sanders D., Oard J.H*. Morphological and genetic survey of giant Salvinia populations in Louisiana and Texas. *Aquatic Botany*. 2015, 127, 20–25. https://doi.org/10.1016/j.aquabot.2015.07.005
53. *Lal A. Salvinia molesta*: An Assessment of the Effects and Methods of Eradication. *Master's Projects and Capstones*. 2016, 572. https://repository.usfca.edu/capstone/572

54. *Kityo M.K., Sunwoo I., Kim S.H., Park Y.R., Jeong G.T., Kim S.K.* Enhanced Bioethanol Fermentation by Sonication Using Three Yeasts Species and Kariba Weed (*Salvinia molesta*) as Biomass Collected from Lake Victoria, Uganda. *Appl. Biochem. Biotechnol.* 2020, 192 (1), 180–195. https://doi.org/10.1007/s12010-020-03305-x
55. *Room P.M., Thomas P.A.* Nitrogen, phosphorus and potassium in *Salvinia molesta* Mitchell in the field: effects of weather, insect damage, fertilizers and age. *Aquat. Bot.* 1986, 24 (3), 213–232. https://doi.org/10.1016/0304-3770(86)90058-6
56. *George G.T., Gabriel J.J.* Phytoremediation of heavy metals from municipal wastewater by *Salvinia molesta* Mitchell Haya: *The Saudi J. Life Sci.* 2017, 2 (3), 108–115.
57. *Kumari S., Kumar B., Sheel R.* Biological control of Heavy metal pollutants in water by *Salvinia molesta*. *Int. J. Cur. Microbiol. Appl. Sci.* 2017, 6 (4), 2838–2843. https://doi.org/10.20546/ijcmas.2017.604.325
58. *Ugya A.Y., Hua X., Ma J.* Phytoremediation as a Tool for the Remediation of Wastewater Resulting from Dyeing Activities. *Appl. Ecol. Environ. Res.* 2019, 17, 3723–3735. https://doi.org/10.15666/aeer/1702_37233735
59. *Pavithra M., Kousar H.* Potential of *Salvinia molesta* for Removal of Sodium in Textile Wastewater. *J. Bioremed. Biodegrad.* 2016, 7, 364. https://doi.org/10.4172/2155-6199.1000364
60. *Tangahuand B.V., Putri A.P.* The Degradation of BOD and COD of Batik Industry Wastewater Using *Egeria densa* and *Salvinia molesta*. *J. Sains dan Teknolog.* 2017, 9 (2), 82–91. https://doi.org/10.20885/jstl.vol9.iss2.art2
61. *Mustafa H.M., Hayder G.* Cultivation of *S. molesta* plants for phytoremediation of secondary treated domestic wastewater. *Ain Shams Engineering Journal*. 2021, 12(3), 1–8. https://doi.org/10.1016/j.asej.2020.11.028
62. *da Silva A.A., De Oliveira J.A., De Campos F.V., Ribeiro C., Farnese F.D.S., Costa A.C.* Phytoremediation potential of *Salvinia molesta* for arsenite contaminated water: role of antioxidant enzymes. *Theor. Exp. Plant Physiol.* 2018, 30 (4), 275–286. https://doi.org/10.1007/s40626-018-0121-6
63. *Miranda C.V., Schwartsburd P.B. Salvinia* (Salviniaceae) in southern and southeastern Brazil—including new taxa, new distribution records, and new morphological characters. *Braz. J. Bot.* 2019, 42, 171–188. https://doi.org/10.1007/s40415-019-00522-5
64. *Gardenal P., Morbelli M.A., Guidice G.* Spore Morphology and Ultrastructure in Species of Salvinia from Southern South America. *Palynol.* 2008, 32 (1), 143–156. https://doi.org/10.2113/gspalynol.32.1.143
65. *Freitas F., Lunardi S., Souza L.B., von der Osten J.S.C., Arruda R., Andrade R.L.T., Battirola L.D.* Accumulation of copper by the aquatic macrophyte *Salvinia biloba* Raddi (Salviniaceae). *Braz. J. Biol.* 2018, 78, 133–139. https://doi.org/10.1590/1519-6984.166377
66. *Zevallos W.T., Salvatierra L.M., Loureiro D.B., Morató J., Pérez L.M.* Evaluation of the autochthonous free-floating macrophyte *Salvinia biloba* Raddi for use in the phytoremediation of water contaminated. *Desalin. Water Treat.* 2018, 103, 282–289. https://doi.org/10.5004/dwt.2018.21709
67. *Reeves M.C., Yujnovsky F.A., Bergara C.D., Litardo V.G., Landínez G., Fideleff S., Salvatierra L.M., Pérez L.M.* Fitorremediacion de aguas contaminadas con plomo ($Pb^{+2}$), empleandoespecies nativas de flotacion libre. *Congreso Nacional de Conservación de la Biodiversidad. Las Grutas*, 2017, 1. https://doi.org/10.13140/RG.2.2.22058.52165
68. *Casagrande G.C.R., dos Reis C., Arruda R., de Andrade R.L.T., Battirola L.D.* Bioaccumulation and Biosorption of Mercury by *Salvinia biloba* Raddi (Salviniaceae). *Water Air Soil Pollut.* 2018, 229, 166. https://doi.org/10.1007/s11270-018-3819-9
69. *Emiliani J., Wendi G.L.O., Bergara C.D., Salvatierra L.M., Novo L.A.B., Leonardo M., Pérez L.M.* Variations in the Phytoremediation Efficiency of Metal-polluted Water with *Salvinia biloba*: Prospects and Toxicological Impacts. *Water.* 2020, 12(6), 1737. https://doi.org/10.3390/w12061737
70. *Zevallos W.T., Salvatierra L.M., Pérez L.M.* Evaluacion de los mecanismos de eliminacion de $Pb^{2+}$ en sistemas de fitorremediacion en lotesoperados con *Salvinia biloba* raddi (acordeon de agua). *Energeia*. 2015, 13 (13), 10–17.
71. *Iha D.S., Bianchini I.* Phytoremediation of Cd, Ni, Pb and Zn by *Salvinia minima*. *Int. J. Phytoremediation*. 2015, 17(10), 929–935. https://doi.org/10.1080/15226514.2014.1003793
72. *Castro-Longoria E., Trejo-Guillén K., Vilchis-Nestor A.R., Avalos-Borja M., Andrade-Canto S.B., Leal-Alvarado D.A., Santamaría J.M.* Biosynthesis of lead nanoparticles by the aquatic water fern, *Salvinia minima* Baker, when exposed to high lead concentration. *Colloids and Surfaces B: Biointerfaces*. 2014, 114, 277–283. https://doi.org/10.1016/j.colsurfb.2013.09.050
73. *Olguín E., Hernández E., Ramos I.* The effect of both different light conditions and the pH value on the capacity of *Salvinia minima* Baker for removing cadmium, lead and chromium. Acta Biotechnol. 2002, 22 (1-2),

121–131. https://doi.org/10.1002/1521-3846(200205)22:1/2%3C121::AID-ABIO121%3E3.0.CO;2-F
74. *Sánchez-Galván, G., Monroy, O., Gómez, J., Olguin E.J.* Assessment of the hyperaccumulating lead capacity of *Salvinia minima* using bioadsorption and intracellular accumulation factors. *Water Air Soil Pollut*. 2008, 194, 77–90. https://doi.org/10.1007/s11270-008-9700-5
75. *Leal-Alvarado D., Espadas-Gil F., Saenz-Carbonell L., Talavera-May C., Sanramaria J. M.* Lead accumulation reduces photosynthesis in the lead hyper-accumulator *Salvinia minima* Baker by affecting the cell membrane and inducing stomata closure. *Aquat. Toxicol*. 2016, 171, 37–47. https://doi.org/10.1016/j.aquatox.2015.12.008
76. *Fuentes I.I., Espadas-Gil F., Talavera-May C., Fuentes G., Santamaría J.M*. Capacity of the aquatic fern (*Salvinia minima* Baker) to accumulate high concentrations of nickel in its tissues, and its effect on plant physiological processes. *Aquat. Toxicol*. 2014, 155, 142–150. https://doi.org/10.1016/j.aquatox.2014.06.016
77. *Sachan D., Ghosh A., Das G*. Valorization of aquatic weed *Salvinia minima* to value-added eco-friendly biosorbent: preferential removal of dye and heavy metal. *Int. J. Environ. Sci. Technol*. 2022, https://doi.org/10.1007/s13762-022-04126-7
78. *Hoffmann T., Kutter C., Santamaria J*. Capacity of *Salvinia minima* Baker to tolerate and accumulate As and Pb. *Eng. Life Sci*. 2004, 4(1), 61–65. https://doi.org/10.1002/elsc.200400008
79. *Nichols P.B., Couch J.D., Al-Hamdani S.H*. Selected physiological responses of *Salvinia minima* to different chromium concentrations. *Aquat. Bot*. 2000, 68, 313–319. https://doi.org/10.1016/S0304-3770(00)00128-5
80. *Mir B.A., Sahoo S*. Growth and nutrient removal potential of two native hydrophytes (*Pistia* sp and *Salvinia* sp) from municipal sewage water of Sambalpur Town, Odisha, India. *Eco. Env. Cons*. 2017, 23 (4), 2247–2253.
81. *Jampeetong A., Brix H., Kantawanichkul S.* Response of *Salvinia cucullata* to high $NH_4^{(+)}$ concentrations at laboratory scales. *Ecotoxicol. Environ. Saf*. 2012, 79, 69–74. https://doi.org/10.1016/j.ecoenv.2011.12.003
82. *Rashidul Alam A.K.M., Hoque S*. Phytoremediation of industrial wastewater by culturing aquatic macrophytes, *Trapa natans* L. and *Salvinia cucullata* Roxb. *Jahangirnagar University. J. Biol. Sci*. 2017, 6 (2), 19–27.
83. *Boschilia S.M., Thomaz S.M., Piana P.A*. Plasticidade morfologica de *Salvinia herzogii* (de La Sota) emresposta à densidade populacional. *Acta Scientiarum Biological Sciences*. 2006, 28 (1), 35–39.
84. *Paris C., Hadad H.R., Maine M.A., Suñe N*. Efficiency of two free floating macrophytes in the adsorption of heavy metals. *Limnetica*. 2005, 24 (3), 237–244.
85. *Hadad H.R., Maine M.A., Natale G.S., Bonetto C*. The effect of nutrient addition on metaltolerance in *Salvinia herzogii*. *Ecological Engineering*. 2007, 31 (2), 122–131. https://doi.org/10.1016/j.ecoleng.2007.06.012
86. *Suñe N., Sanchez G., Caffaratti S., Maine A.* Cadmium and chromium removal kinetics from solution by two aquatic macrophytes. *Environ. Pollut*. 2007, 145 (2), 467–473. https://doi.org/10.1016/j.envpol.2006.04.016
87. *Molisani M.M., Rocha R., Machado W., Barreto R.C., Lacerda L.D*. Mercury contents in aquatic macrophytes from two reservoirs in the Paraíba do Sul: Guandú river system, SE Brazil. *Braz. J. Biol*. 2006, 66 (1A), 101–107. https://doi.org/10.1590/s1519-69842006000100013
88. *Espinoza-Quiñones F.R., Zacarkim C.E., Palacio S.M., Obregón C.L., Zenatti D.C., Galante R.M., Rossi N., Rossi F.L., Pereira I.R.A., Welter R.A., Rizzutto M.A*. Removal of heavy metal from polluted river water using aquatic macrophytes Salvinia sp. *Appl. Phys. Instrm*. 2005, 35 (3B), 744–746. https://doi.org/10.1016/j.cej.2009.01.004
89. *Banerjee G., Sarker S*. The role of *Salvinia rotundifolia* in scavenging aquatic Pb (II) pollution: a case study. *Bioprocess Engin*. 1997, 17, 295–300. https://doi.org/10.1007/PL00008966
90. *Fasani E., Li M., Varotto C., Furini A., Dal Corso G*. Metal Detoxification in Land Plants: From Bryophytes to Vascular Plants. *Plants*. 2022, 11, 237. https://doi.org/10.3390/plants11030237
91. *Dhir B., Sharmila P., Saradhi P.P., Sharma S., Kumar R., Mehta D*. Heavy metal induced physiological alterations in *Salvinia natans*. *Ecotoxicol. Environ. Saf*. 2011, 74 (6), 1678–1684 https://doi.org/10.1016/j.ecoenv.2011.05.009
92. *Leblebici Z., Kar M., Yalçin V*. Comparative study of Cd, Pb, and Ni removal potential by *Salvinia natans* (L.) All. and *Lemna minor* L.: Interactions with Growth Parameters. *Romanian Biotechnological Letters*. 2018, 23(1), 13235–13248.
93. *Carrillo-Niquete G., Andrade J.L., Hernández-Terrones L. Cobos-Gasca V., Fuentes G., Santamaría J.M*. Copper accumulation in the aquatic fern *Salvinia minima* causes

more severe physiological stress than zinc. *Biometals*. 2022. https://doi.org/10.1007/s10534-022-00423-3
94. *Lakra K.C., Lal B., Banerjee T.* Decontamination of coal mine effluent generated at the Rajrappa coal mine using phytoremediation technology. *Int. J. Phytoremediation*. 2017, 19(6), 530–536. https://doi.org/10.1080/15226514.2016.1267698
95. *Ranjitha J., Raj A., Kashyap R., Vijayalakshmi S., Donatus M.* Removal of heavy metals from Industrial Effluent using *Salvinia molesta*. *Int. J. ChemTech Res*. 2016, 9 (5), 608–613.
96. *Emiliani J., Oyarce W.G.L., Salvatierra L.M., Novo L.A.B., Pérez L.M.* Evaluation of Cadmium Bioaccumulation-Related Physiological Effects in *Salvinia biloba*: An Insight towards Its Use as Pollutant Bioindicator in Water Reservoirs. *Plants (Basel)*. 2021, 10(12), 2679. https://doi.org/10.3390/plants10122679
97. *Kumar U., Rout S., Kaviraj M. Swain P., Nayak A.K.* Uncovering morphological and physiological markers to distinguish *Azolla* strains. *Braz. J. Bot*. 2021, 44, 697–713. https://doi.org/10.1007/s40415-021-00725-9
98. *Raja W.* Azolla: Amazing Aquatic Fern. Lambert Academic Publishing, Saarbrucken. 2014.
99. *Carlozzi P., Padovani G.* The aquatic fern Azolla as a natural plant-factory for ammonia removal from fish-breeding fresh wastewater. *Environ. Sci. Pollut. Res.* 2016, 23, 8749–8755. doi:10.1007/s11356-016-6120-8
100. *Gevrek M.N., Samanci B., Yagmur B., Arabaci O., zkaynak E.* Studies on the adaptation of *Azolla mexicana* in the aegean and the mediterranean regions. *Plant Prod. Sci*. 2004, 7, 50–54.
101. *Eily A.N., Pryer K.M., Li F.W.* A first glimpse at genes important to the *Azolla–Nostoc* symbiosis. *Symbiosis*. 2019, 78, 149–162. https://doi.org/10.1007/s13199-019-00599-2
102. *Kumar U., Kaviraj M., Rout S., Chakraborty K., Swain P., Nayak P.K., Nayak A.K.* Combined application of ascorbic acid and endophytic N-fixing *Azotobacter chroococcum* Avi2 modulates photosynthetic efficacy, antioxidants and growth-promotion in rice under moisture deficit stress. *Microbiol. Res*. 2021, 10, 126808. https://doi.org/10.1016/j.micres.2021.126808
103. *Razavipour T., Moghaddam S.S., Doaei S., Noorhosseini S.A., Damalas C.A.* Azolla (*Azolla filiculoides*) compost improves grain yield of rice (*Oryza sativa* L.) under different irrigation regimes. *Agricult. Water Manag*. 2018, 209, 1–10. https://doi.org/10.1016/j.agwat.2018.05.020
104. *Rahmah S., Nasrah U., Lim L.-S., Ishak S.D., Rozaini M.Z.H., Liew H.J.* Aquaculture wastewater-raised Azolla as partial alternative dietary protein for *Pangasius catfish*. *Environ. Res*. 2022, 208, 112718. https://doi.org/10.1016/j.envres.2022.112718.
105. *Zheng X., Lin Z., Lu J., Ye R., Qu M., Wang J., Xu G., Ying Z., Chen S. De novo* transcriptome analysis reveals the molecular regulatory mechanism underlying the response to excess nitrogen in Azolla spp. *Aquatic Toxicol*. 2022, 248, 106202. https://doi.org/10.1016/j.aquatox.2022.106202.
106. *Costa M.L., Santos M.C., Carrapiço F., Pereira A.L.* Azolla-Anabaena's behaviour in urban wastewater and artificial media--influence of combined nitrogen. *Water Res*. 2009, 43 (15), 3743–3750. https://doi.org/10.1016/j.watres.2009.05.038
107. *Park H., Song U.* Microcosm investigation of growth and phytoremediation potential of *Azolla japonica* along nitrogen gradients. *Int. J. Phytoremed*. 2017, 19 (10), 863–869. https://doi.org/10.1080/15226514.2017.1290582
108. *Temmink R.J.M., Harpenslager S.F., Smolders A.J.P. Azolla* along a phosphorus gradient: biphasic growth response linked to diazotroph traits and phosphorus-induced iron chlorosis. Sci. Rep. 2018, 8, 4451. https://doi.org/10.1038/s41598-018-22760-5
109. *Da Silva M.E.J., Mathe L.O.J., Van Rooyen I.L., Brink H.G., Nicol W.* Optimal Growth Conditions for *Azolla pinnata* R. Brown: Impacts of Light Intensity, Nitrogen Addition, pH Control, and Humidity. *Plants*, 2022, 11(8), 1048. https://doi.org/10.3390/plants11081048
110. *Bennicelli R., Stezpniewska Z., Banach A., Szajnocha K., Ostrowski J.* The ability of *Azolla caroliniana* to removeheavy metals (Hg (II), Cr (III), Cr (VI)) from municipal waste water. *Chemosphere*. 2004, 55(1), 141–146. https://doi.org/10.1016/j.chemosphere.2003.11.015
111. *Roberts A.E., Boylen C.W., Nierzwicki-Bauer S.A.* Effects of lead accumulation on the Azolla caroliniana–Anabaena association. *Ecotoxicology and Environmental Safety*. 2014, 102, 100–104. https://doi.org/10.1016/j.ecoenv.2014.01.019
112. *Bianchi E., Biancalani A., Berardi C., Antal A., Fibbi D., Coppi A., Lastrucci L., Bussotti N., Colzi I., Renai L., Scordo C., Del Bubba M., Gonnelli C.* Improving the efficiency of

wastewater treatment plants: Bio-removal of heavy-metals and pharmaceuticals by *Azolla filiculoides* and *Lemna minuta*. *Sci. Total Environ*. 2020, 746, 141219. https://doi.org/10.1016/j.scitotenv.2020.141219

113. *Zhao M., Duncan J.R.* Removal and recovery of nickel from aqueous solution and electroplating rinse effluent using *Azolla filiculoides*. *Proc. Biochem*. 1998, 33 (3), 249–255. https://doi.org/10.1016/S0032-9592(97)00062-9
114. *Sood A., Uniyal P.L., Prasanna R., Ahluwalia A.S*. Phytoremediation potential of aquatic macrophyte, Azolla. *Ambio*. 2012, 41 (2), 122–137. https://doi.org/10.1007/s13280-011-0159-z
115. *Naghipour D., Ashrafi S.D., Gholamzadeh M., Taghavi K., Naimi-Joubani M*. Phytoremediation of heavy metals (Ni, Cd, Pb) by *Azolla filiculoides* from aqueous solution: A dataset. *Data Brief*. 2018, 21, 1409–1414. https://doi.org/10.1016/j.dib.2018.10.111
116. *Dai L.P., Dong X.J., Ma H.H*. Molecular mechanism for cadmium-induced anthocyanin accumulation in *Azolla imbricata*. *Chemosphere*. 2012, 87, 319–325.
117. *Valderrama A., Carvajal D.E., Penailillo P., Tapia J*. Accumulation capacity of cadmium and copper and their effects on photosynthetic performance in *Azolla filiculoides* lam. Under induced rhizofiltration. *Gayana Bot*. 2016, 73 (2), 283–291. http://dx.doi.org/10.4067/S0717-66432016000200283
118. *Mohamed S., Mahrous A., Elshahat R., Kassem M*. Accumulation of iron, zinc and lead by *Azolla pinnata* and *Lemna minor* and activity in contaminated water. *Egyptian J. Chem*. 2021, 64 (9), 5017–5030. https://doi.org/10.21608/ejchem.2021.50016.3036
119. *Chakraborty S., Mishra A., Verma E., Tiwari B., Mishra A.K., Singh S.S*. Physiological mechanisms of aluminum (Al) toxicity tolerance in nitrogen-fixing aquatic macrophyte *Azolla microphylla* Kaulf: phytoremediation, metabolic rearrangements, and antioxidative enzyme responses. *Environ. Sci. Pollut. Res.* 2019, 26, 9041–9054. https://doi.org/10.1007/s11356-019-04408-7
120. *Zazouli M.A., Balarak D., Mahdavi Y*. Pyrocatechol Removal From Aqueous Solutions by Using *Azolla Filiculoides*. *Health Scope*. 2013, 2 (1), 25–30. https://doi.org/10.17795/jhealthscope-9630
121. *Maldonado I., Terrazas E.G.M., Vilca F.Z*. Application of duckweed (Lemna sp.) and water fern (Azolla sp.) in the removal of pharmaceutical residues in water: State of art focus on antibiotics. *Sci. Total Environ*. 2022, 838 (4), 156565. https://doi.org/10.1016/j.scitotenv.2022.156565
122. *De A.K., Sarkar B., Adak M.K*. Physiological explanation of herbicide tolerance in *Azolla pinnata* R.Br. *Ann. Agrarian Sci*. 2017, 15 (3), 402–409. https://doi.org/10.1016/j.aasci.2017.05.021
123. *Kösesakal T., Seyhan M*. Phenanthrene stress response and phytoremediation potential of free-floating fern *Azolla filiculoides* Lam. *Intern. J. Phytoremediation*. 2022. https://doi.org/10.1080/15226514.2022.2069224
124. *Triono S.T., Amin M., Putra A.M., Haryanto A*. The utilization of wastewater from catfish pond to culture *Azolla microphylla*. *Intern. J. Adv. Sciences, Engineering and Information*. 2022, 12 (1), 423–430. https://doi.org/10.18517/ijaseit.12.1.12695
125. *Rai P.K*. Phytoremediation of Hg and Cd from industrial effluents using an aquatic free floating macrophyte *Azolla pinnata*. *Int. J. Phytoremediation*. 2008, 10(5), 430-439. https://doi.org/10.1080/15226510802100606
126. *Mishra V.K., Tripathi B.D., Kim K.H*. Removal andaccumulation of mercury by aquatic macrophytes from an opencast coal mine effluent. *J. Hazard Mater*. 2009, 172(2-3), 749–754. https://doi.org/10.1016/j.jhazmat.2009.07.059
127. *Rai P.K., Tripathi B.D*. Comparative assessment of *Azolla pinnata* and Vallisneria spiralis in Hg removal from G.B. Pant Sagar of Singrauli Industrial region, India. *Environ. Monit. Assess*. 2009, 148(1-4), 75–84. https://doi.org/10.1007/s10661-007-0140-2
128. *Arora A., Sood A., Singh P.K*. Hyperaccumulation of cadmium and nickel by Azolla species. *Indian J. Plant Physiol*. 2004, 9(3), 302– 304.
129. *Rai P.K*. Microcosom investigation of phytoremediation of Cr using *Azolla pinnata*. *Int. J. Phytoremediation*. 2010, 12(1), 96–104. https://doi.org/10.1080/15226510902767155
130. *Arora A., Saxena S., Sharma D.K*. Tolerance and phytoaccumulation of Chromium by three *Azolla* species. *World J. Microbiol. Biotechnol*. 2006, 22, 97–100. https://doi.org/10.1007/s11274-005-9000-9
131. *Zhang X., Lin A.J., Zhao F.J., Xu G.Z., Duan G.L., Zhu Y.G*. Arsenic accumulation by aquatic fern Azolla: comparisonof arsenate uptake, speciation and efflux by *A. caroliniana* and *A. flliculoides. Environ. Pollut*. 2008, 156(3), 1149–1155. https://doi.org/10.1016/j.envpol.2008.04.002
132. *Stepniewska Z., Bennicelli R.P., Balakhnina T.I., Szajnocha K., Banach A., Woliñska A.* Potential of *Azolla caroliniana* for the removal of Pb and Cd from wastewaters.

*Intern. Agrophysics*. 2005, 19(3), 251–255.
133. *Sela M., Garty J., Tel-Or E.* The accumulation and effect of heavy metal on the water fern *Azolla flliculoides*. *New Phytologist*. 1989, 112(1), 7–12.
134. *Dai L.P., Xiong Z.T., Huang Y., Li M.J.* Cadmium-induced changes in pigments, total phenolics, and phenylalanine ammonia-lyase activity in fronds of *Azolla imbricate*. *Environ. Toxicol*. 2006, 21(5), 505–512. https://doi.org/10.1002/tox.20212
135. *Dhir B., Sharmila P., Saradhi, P.P.* Potential of Aquatic Macrophytes for Removing Contaminants from the Environment. *Crit. Rev. Environ. Sci. Technol.* 2009, 39 (9), 754-781. https://doi.org/10.1080/10643380801977776
136. *Rongxue Cui R., Nam S.-H., An Y.-J.* *Salvinia natans*: A potential test species for ecotoxicity testing. *Environ. Pol*. 2020, 267, 115650. https://doi.org/10.1016/j.envpol.2020.115650
137. *Mânzatu C., Nagy B. A., Iannelli R., Giannarelli S., Majdik C.* Laboratory tests for the phytoextraction of heavy metals from polluted harbor sediments using aquatic plants. *Marine Pollut. Bull*. 2015, 101(2), 605–611. https://doi.org/10.1016/j.marpolbul.20Marin15.10.045
138. *Dhir B., Srivastava S.* Heavy metal tolerance in metal hyperaccumulator plant, *Salvinia natans*. *Bull. Environ. Contam. Toxicol.* 2013, 90 (6), 720–724. https://doi.org/10.1007/s00128-013-0988-5
139. *Phetsombat S., Kruatrachue M., Pokethitiyook P., Upatham S.* Toxicity and bioaccumulation of cadmium and lead in *Salvinia cucullata*. *J. Environ. Biol*. 2006, 27 (4), 645–652.
140. *Kumari S., Kumar B., Sheel R.* Bioremediation of Heavy Metals by Serious Aquatic Weed, *Salvinia*. *Int. J. Curr. Microbiol. Appl. Sci*. 2016, 5 (9), 355–368. http://dx.doi.org/10.20546/ijcmas.2016.509.039
141. *Bittu P., Ashis S., Swarnendu R.* Growth and physio-biochemical responses of *Azolla filiculoides* subjected to short span of phenol exposure. *Research Square*. 2021. https://doi.org/10.21203/rs.3.rs-416354/v1

## ВОДНІ ПАПОРОТІ РОДИНИ *Salviniaceae* У ФІТОРЕМЕДІАЦІЇ ТА ФІТОІНДИКАЦІЇ ЗАБРУДНЕНИХ ВОДОЙМ

*Косаківська І.В., Веденичова Н.П., Щербатюк М.М., Войтенко Л.В., Васюк В.А.*

Інститут ботаніки імені М. Г. Холодного
НАН України, Київ

*Проблематика.* Водні екосистеми зазнають значних стресових навантажень та виснаження через надходження забруднюючих речовин неорганічного та органічного походження, що створює серйозну загрозу для здоров'я людей. Програма ООН з навколишнього середовища визначила фіторемедіацію як ефективну екотехнологію видалення, детоксикації та іммобілізації полютантів за допомогою рослин. Папороті гідрофіти родини *Salviniaceae* належать до перспективних фіторемедіантів. Вони характеризуються високими темпами росту, стійкістю до несприятливих екологічних чинників, здатні адсорбувати полютанти, серед яких важкі метали. Види родів *Salvinia* та *Azolla* використовують для оцінки екологічно стану водойм та дослідження екотоксикологічних ефектів забруднюючих речовин

*Мета.* Аналіз та узагальнення новітніх наукових результатів з використання видів родини *Salviniaceae* для фіторемедіації та фітоіндикації забруднених водойм.

*Результати.* У цьому огляді ми навели ключову інформацію про новітні фітотехнології, серед яких фітодеградація, фітостабілізація, ризофільтрація, ризодеградація та фітоволатизація. Охарактеризували особливості росту і розповсюдження видів родів *Salvinia* та *Azolla* та представили актуальну інформацію щодо використання водних папоротей для очистки забруднених водойм від важких металів, неорганічних та органічних забруднювачів. Обговорили відомості щодо фізіологічних та молекулярних механізмів адаптації видів родів *Salvinia* та *Azolla* до токсичної дії полютантів різного походження. Окрему увагу ми зосередили на використанні водних папоротей родини *Salviniaceae* для контролю забруднення водойм.

***Ключові слова:*** *Salviniaceae*, водні екосистеми, фіторемедіація, біоіндикація, органічні та неорганічні забруднювачі.

UDC 615.014:615.456.3 https://doi.org/10.15407/biotech15.05.024

# INFLUENCE OF THE LIPID COMPOSITION ON THE PROPERTIES, TECHNOLOGY AND QUALITY INDICATORS OF LIPOSOMAL DRUGS

*D. M. PYLYPENKO*[1], *G. S. GRIGORYEVA*[2], *N. F. KONAKHOVYCH*[2], *Yu. M. KRASNOPOLSKY*[3]

[1]State Biotechnological University, Ukraine, Kharkiv
[2]State Institution "Institute of Pharmacology and Toxicology of the National Academy of Medical Sciences of Ukraine", Kharkiv
[3]National Technical University "Kharkiv Polytechnic Institute", Ukraine

*E-mail: pdmforwork@gmail.com*

Received 10.08.2022
Revised 23.09.2022
Accepted 31.10.2022

Liposomal drug delivery system is an example of the use of nanodrugs in medical practice. Encapsulation of active pharmaceutical ingredients in liposomal nanoparticles allows increasing their bioavailability and efficacy.

*Aim.* The article is devoted to the analysis of the lipid composition of liposomal drugs developed in Ukraine, its influence on the choice of technology and control parameters.

*Results.* The lipid compositions of liposomal drugs developed in Ukraine in recent years were reviewed. The advantages and disadvantages of natural phosphatidylcholine as the main membrane-forming lipid were analyzed. Data on the influence of anionic phospholipids and cholesterol in the liposomal membrane composition on the stability of liposomal nanoparticles and the level of active pharmaceutical ingredient encapsulation were given. The main technological stages of obtaining liposomes with hydrophilic and hydrophobic active pharmaceutical ingredients were considered. The main groups of quality indicators of liposomal dosage forms have been determined.

*Conclusions.* The lipid composition determines the structure and physicochemical properties of the lipid membrane, the mechanism and level of active pharmaceutical ingredient encapsulation, which significantly influences the pharmacological efficacy of liposomal drug delivery systems.

***Key words:*** nanobiotechnology; drug delivery system; liposomal drug; phospholipids; anionic phospholipids; phosphatidylcholine; cholesterol.

Using drug delivery systems, the pharmacokinetics of active pharmaceutical ingredients (APIs) can be changed, and its bioavailability and effectiveness can be increased [1]. The development of liposomal drugs (LS-drugs) is one of the promising areas of modern nanopharmacology due to a number of advantages of LSs [2–5]: they prolong the action of encapsulated APIs in the body; change the pharmacokinetics of drugs, that significantly increases their pharmacological efficacy; protect APIs from degradation; protect healthy cells and pathological organs from the toxic effects of drugs; increase the bioavailability of lipophilic APIs.

LS-drugs are the only real example of the use of nanopreparations in medicine, for example, in Ukraine LS-drugs are licensed for use in cardiology, ophthalmology, oncology, etc. [2, 6–9]. In addition, LSs show high efficiency as adjuvants in vaccines.

LSs are colloidal spherical nanorange particles formed by a phospholipid bilayer [10]. There are single-layer (unilamellar) vesicles (ULVs), multilayer vesicles, which are divided into oligolamellar (OLVs) and multilamellar

(MLVs) vesicles, and multivesicular LSs (MVLs). OLV and MLV contain 2–5 or more than 5 concentric lipid bilayers. Unlike MLVs, MVLs consist of hundreds of concentric water compartments bounded by a single lipid bilayer membrane. Depending on the particle size, ULVs are divided into small unilamellar vesicles (SUVs) with a size of 30–100 nm, large unilamellar vesicles (LUVs) with a size of more than 100 nm, and giant unilamellar vesicles (GUVs) with a size of more than 1000 nm (Fig. 1).

The structure and efficacy of LSs depend heavily on the lipid composition of the LS membrane. The lipid composition determines the particle size and stability, the level of API encapsulation, and the methods of LS preparation [11].

**The aim of the work** was to analyze the lipid composition of LS-drugs developed in Ukraine, to characterize the main functions of lipids in the composition of LS-drugs, to highlight the main control points in the preparation of LSs.

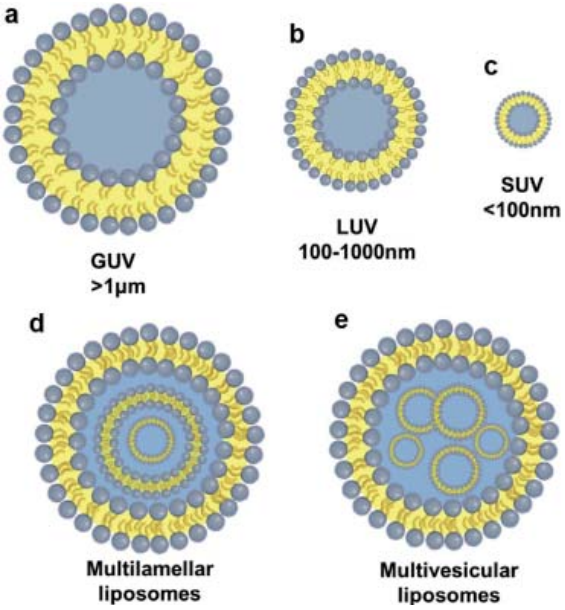


*Fig. 1.* **Classification of liposomal nanoparticles by size and number of lipid bilayer [10]**

## Lipids in LS-drug

Most of commercial LS-drugs, which are available on the market, are ULVs capable of passive targeting and long-term circulation in the body. In this article we focused on LS-drugs, which were developed by us in Ukraine in 1989–2021 (Table 1).

Analyzing the lipid composition of this LS-drugs, the following components can be identified: EPC, DPhG, DPPG, PhG, PhI, and Chol (Fig. 2).

*Table 1.* **LS-drugs, developed in Ukraine**

| Product name | Application area | API composition | LS size, nm | Lipid composition | Development stage | Ref. |
|---|---|---|---|---|---|---|
| Lipin® | Pulmonology, nephrology, cardiology | EPC | 80–140 | EPC | Licensed in 1991 | 2, 32 |
| Lipodox® | Oncology | Doxorubicin hydrochloride | 80–120 | EPC, Chol | Licensed in 1998 | 2, 9, 22 |
| Lioliv® | Hepatoprotector | Antral | 90–130 | EPC | Licensed in 2003 | 2, 27 |
| Lipoflavon® | Cardiology, ophthalmology | Quercetin | 130–160 | EPC | Licensed in 2006/2007 | 2, 26 |
| LS irinotecan | Oncology | Irinotecan hydrochloride | 80–120 | EPC, Chol | Preclinical trials | 24, 25 |
| LS cytochrome C | Cardiology | Cytochrome C | 120–170 | EPC, DPPG | Preclinical trials | 12 |
| Lipotax | Oncology | Docetaxel | 120–150 | DPhG, EPC | Laboratory studies | 2, 19, 25 |
| Lipoplat | Oncology | Cisplatin | 140–180 | EPC, Chol, PhG, PhI | Clinical trials | 2, 6, 21 |
| LS curcumin | Antioxidant, cardioprotector | Curcumin | 150–200 | DPPG, EPC | Laboratory studies | 13, 14 |
| LS coenzyme Q10 | Antioxidant, cardioprotector | Coenzyme Q10 | 140–180 | DPPG, EPC | Laboratory studies | 2 |

*Legend.* EPC — egg phosphatidylcholine, DPhG — diphosphatidylglycerol, DPPG — dipalmitoylphosphatidylglycerol, Chol — cholesterol, PhG — phosphatidylglycerol, PhI — phosphatidylinositol.

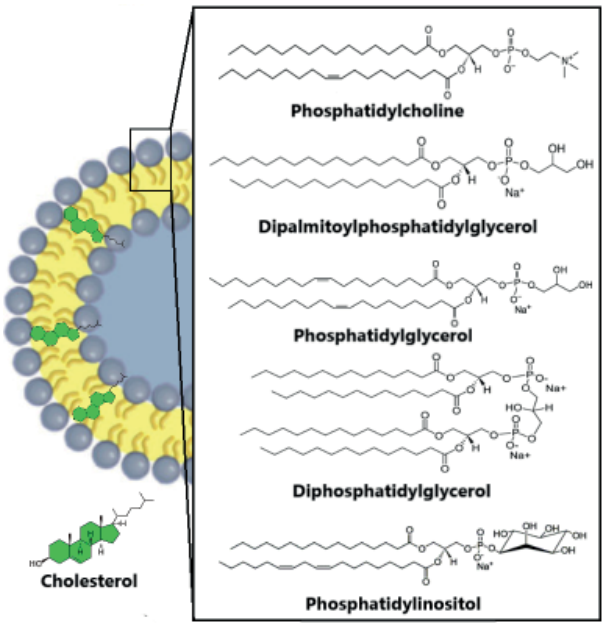


*Fig. 2.* **Structure of phospholipids**

We used EPC as the main membrane-forming lipid in LS-drug developing. The advantage of natural EPC is the low cost of production compared with semi-synthetic and synthetic PCs, such as DSPC, DPPC, DOPC, etc. EPC is composed of a family of fatty acids, and a wide range of fatty acid composition causes a low standardization of the EPC. LysoPC (1.0%) and sphingomyelin (3.0%) are detected in the natural EPC, obtained according to the previously developed technology [12], and the purification of them leads to reducing the cost of the product due to a significant decrease of the product yield. Presumably, sphingomyelin of the EPC can influence the stability of the LS structure [13], especially in acidic medium with pH 2.0–3.0 used in the preparation of LSs by transmembrane pH gradient method of hydrophilic APIs encapsulation, such as doxorubicin hydrochloride or irinotecan hydrochloride.

We used semi-synthetic DPPG in the preparation of a number of LS-drugs, for example, in the preparation of a complex with cytochrome C. DPPG can interact with a positively charged API, such as cytochrome C, to form a stable complex. In addition, DPPG has a negative charge and can prevent the aggregation of LSs [14]. DPPG was also used in the preparation of LS-drug with bilayer-encapsulated lipophilic APIs, curcumin and coenzyme Q10 (Table 1). The introduction of anionic DPPG allowed not only to increase the API incorporation (by 10–15 %) into the bilayer of the nanoparticle, but also to stabilize LSs [15–17]. Using curcumin-containing LSs, the pharmacological activity of the LS-drug was confirmed, namely, cardioprotective and antioxidant properties were shown. Based on the high pharmacological activity of curcumin [18–20], the development of its hydrophilic form in LS gives hope for using the product in various pharmacological models. The use of natural DPhG stabilized the structure of a docetaxel-containing LSs [21, 22].

We used Chol as a component of a lipid bilayer in preparation of LSs with hydrophilic APIs, doxorubicin [23–25] and irinotecan [26, 27]. The encapsulation of Chol into the LS bilayer facilitates the packing of fatty acid chains and thereby forms and stabilizes the lipid bilayer. Furthermore, Chol in nanoparticles largely determines the rigidity of the LS membrane, which can influence the API encapsulation into the inner water space of LSs and the subsequent release in the organism. The Chol encapsulation into LS-drugs containing irinotecan or doxorubicin can stabilize APIs in acidic medium. In addition, Chol was not used in LS-drugs with lipophilic APIs, which are incorporated in the lipid bilayer, for example, quercetin in Lipoflavon® [2, 28] (used in cardiology, ophthalmology, oncology), and antral in Lioliv® (hepatoprotector) [2, 29, 30]. In our opinion, lipophilic substances incorporated in the LS bilayer can independently stabilize the LS membrane and influence the membrane

rigidity. A number of authors have also reported the high pharmacological activity of quercetin-containing LS-drugs [2, 31–33]. The influence of the lipid composition on the LS structure and the pharmacokinetics of anthracycline antibiotics and 5-fluorouracil encapsulated LS-drugs was studied [6]. The Chol increase in LS composition leads to an increase in the size of LSs and consequently to decrease in the API encapsulation and complicate the sterilizing filtration. The cumulative and prolonging effect of aforementioned LS-drugs have been established, and the distribution of anticancer APIs in organs was shown.

## Technological methods for LS-drugs preparation

The lipid and API composition of LS-drugs largely determines the technology for producing products. The production of developed LS-drugs has been discussed in detail in our previous studies [2, 7, 27]. To prepare LS-drugs with lipophilic APIs, we used the lipid film method followed by high-pressure homogenization, sterilizing filtration, and lyophilization. According to this scheme, Lipin® [34, 35], Lipoflavon® [28], Lyoliv® [29], Lipotax [22], and Latanoprost [36] were prepared. LS-drugs with hydrophilic APIs were prepared by lipid film method, emulsion rehydration, high pressure homogenization or sonication, transmembrane pH gradient method, sterilizing filtration and lyophilization. LS forms of doxorubicin hydrochloride (Lipodox®) [24] and irinotecan hydrochloride [26] were prepared in this way. Non-encapsulated APIs were removed using sterilizing filtration through a cascade of membrane filters, centrifugation, ultrafiltration or gel filtration. Sterilizing filtration was applied in following stages: preparation of sterile solutions of lipid components in organic solvents, cryoprotectant solutions and buffer solutions. In addition, a number of stages were carried out under aseptic conditions [2]. The lyophilization was carried out using cryoprotectant solutions, lactose or trehalose.

It has been established that physicochemical characteristics of LS-drugs depend on a number of factors in their obtaining: pressure of homogenization, intensity of sonication, process temperature, lipid concentration, number of cycles, etc. Even minor deviations from the established regulatory standards result in changes in properties of LS samples. At the same time, it is well known that the size of LSs, their charge, and fatty acids oxidation determine pharmacological properties of LS-drugs, which is primarily associated with altered pharmacokinetics in the organism. The influence of lyophilization modes on the stability of the physicochemical parameters of LS-drug was shown. The properties of the product are also affected by the structure and properties of the API [2, 27].

## Control of LS-drugs

The standardization and control of LS-drugs containing different compositions of lipids and APIs need to be considered. LS-drugs were controlled in accordance with international [38] and national [39, 40] requirements. We proceeded from the definition of three groups of indicators [37]: I — indicators characterizing the identification and quantity of individual biologically active components of the drug: API, lipids (EPC, Chol, anionic phospholipids), cryoprotectant; II — quality indicators characterizing the dosage form of the drug (sterility, pH value, abnormal toxicity, pyrogenicity (endotoxins)); III — indicators characterizing the properties of LSs (encapsulation of API in LSs, size and charge of LSs, etc.). Tests should control those properties of the product, which are subject to changes during the storage and may affect the quality of the finished product, and the methods of quantitative determination should characterize the stability. The profile of new products of degradation of the drug components also need to be taken into account. In this case, new products of degradation must be identified. Thus, the limit concentrations of impurities should be identified and indicated, such as limit concentrations of lysoproducts or free fatty acids for EPC, and limit concentrations of impurities and degradation products for API. When developing LS-drugs, the identity of the qualitative and quantitative compositions of the APIs and lipid composition after freeze-drying and subsequent rehydration were proved.

## Conclusions

The main task of the LS drug delivery system is to increase the bioavailability and effectiveness of the API. The lipid composition determines the structure and physicochemical properties of the lipid membrane, the mechanism and level of API encapsulation into the LS nanoparticle, that fundamentally impacts the pharmacological

efficiency of this drug delivery system. The LS-drug composition (both lipids and APIs) has the greatest influence on the choice of technological methods for obtaining LSs and the main control indicators of the finished product.

*Funding source*. The work was performed within the research program "Comprehensive research and optimization of industrial and pharmaceutical biotechnologies" (State Registration No.0118U002336, 2018–2021). There is no conflict of interest.

## REFERENCES

1. *Rommasi F, Esfandiari N*. Liposomal nanomedicine: applications for drug delivery in cancer therapy. *Nanoscale Res. Lett*. 2021, 16(1), 95. https://doi:10.1186/s11671-021-03553-8
2. *Shvets V.I., Krasnopolskiy Yu.M., Sorokoumova G.M*. Liposomal forms of drugs: technological features of production and use in the clinic. *M.: Remedium*, 2016. 200 p. (In Russian).
3. *Bulbake U., Doppalapudi S., Kommineni N., Khan W*. Liposomal formulations in clinical use: an updated review. *Pharmaceutics*. 2017, 9(2), 12. https://doi.org/10.3390/pharmaceutics9020012
4. *Allen T. M., Cullis P. R.* Liposomal drug delivery systems: from concept to clinical application. *Adv. Drug. Deliv. Rev*. 2013, 65(1), 36–48. https://doi.org/10.1016/j.addr.2012.09.037
5. *Khromov A. S*. Liposomal drugs — implementation of nanotechnology in medicine. *Pharmacol. Drug Toxicol*. 2016, 2(48), 14–23. (In Russian).
6. *Dudnitchenko A. S., Krasnopolsky Yu. M*. Preparation and pharmacokinetics in vivo of liposome-associated anthracyclines and 5-fluorouracil. *Exp. Oncol*. 1996, 18(4), 392–396.
7. *Krasnopolskii Y.M., Grigor'eva A.S., Katsai A.G., Konakhovich N. F., Prokhorov V. V., Stadnichenko A. V. Balaban'yan V. Yu., Lyutik A. I., Shvets V. I*. Technologies and perspectives of liposomal drug application in clinical practice. *Nanotechnol. Russ*. 2017, 12 (7-8), 461–470. http://doi.org/10.1134/s1995078017040139
8. *Anchordoquy, T. J., Barenholz, Y., Boraschi, D., Chorny, M., Decuzzi, P., Dobrovolskaia, M. A., Farhangrazi, Z. S., Farrell, D., Gabizon, A., Ghandehari, H., Godin, B., La-Beck, N. M., Ljubimova, J., Moghimi, S. M., Pagliaro, L., Park, J.H., Peer, D., Ruoslahti, E., Serkova, N.J., Simberg, D*. Mechanisms and barriers in cancer nanomedicine: addressing challenges, looking for solutions. *ACS Nano*. 2017, 11(1), 12–18. https://doi.org/10.1021/acsnano.6b08244
9. *Pivnuk V.M., Tymovska Yu.O., Ponomareva O.V., Kulyk G.I., Olyinichenko G.P., Anikusko M.F., Krasnopolsky Yu.M., Chekhun V.F*. Applying of liposomal form of doxorubicin in patients with doxorubicin-resistant breast cancer. *Oncol*. 2007, 9(2), 120–124. (In Ukrainian).
10. *Magar K. T., Boafo G. F., Li X., Chen Z., He W*. Liposome-based delivery of biological drugs. *Chinese Chem. Lett*. 2022, 33(2), 587–596. https://doi.org/10.1016/j.cclet.2021.08.020
11. *Krasnopolsky Yu. M., Pylypenko D. M*. "Quality By Design" in liposomal drugs creation. *Biotechnologia ACTA*. 2020, 13(6), 5–12. https://doi.org/10.15407/biotech13.06.005
12. *Shvets V. I., Sennikov G. A., Golbec I. I. Krasnopolsky Y. M*. Production of purified lecithin. *Farmac. J*. 1977, 4, 79–81. (In Ukrainian).
13. *Webb M.S., Bally M.B., Mayer L.D., Miller J.J., Tardi P. G*. Sphingosomes for enhanced drug delivery. *US Patent. 5741516*. April 21, 1998.
14. *Hryhorieva H. S., Katsai O. H., Krasnopolskyi Yu. M., Prokhorov V. V., Khromov O. S., Pasiechnikova N. V., Dobrelia N. V*. The method of obtaining a pharmacologically active liposomal composition containing cytochrome C, and a liposomal composition obtained by this method. *Ukrainian patent № 118583*. February 11, 2019.
15. *Pylypenko D. M., Krasnopolsky Yu. M*. A method of preparing a complex liposomal composition containing quercetin and curcumin. *Ukrainian patent № 147767*. June 09, 2021.
16. *Pylypenko D. M., Gorbach T. V., Katsai O. G., Grigoryeva A. S., Krasnopolsky Y. M*. A Stu dy of Oxidative Stress Markers when Using the Liposomal Antioxidant Complex. *Pharmakeftiki*. 2019, 31(1), 40–47.
17. *Pylypenko D., Gorbach T. Krasnopolsky Yu*. Study of antioxidant activity of liposomal forms of quercetin and curcumin ischemic heart disease. *BioTechnologia*. 2020, 101(4), 273–282. https://doi.org/10.5114/bta.2020.100420
18. *Feng T., Wei Y., Lee R. J., Zhao L*. Liposomal curcumin and its application in cancer. *Int. J. Nanomedicine*. 2017, 12, 6027–6044. http://doi:10.2147/IJN.S13243417
19. *Vetha B. S. S., Kim E. M., Oh P. S., Kim S. H, Lim S. T., Sohn M. H., Jeong H. J*. Curcumin encapsulated micellar nanoplatform for blue light emitting diode induced apoptosis as a new class of cancer therapy. *Macromol. Res*. 2019. 27, 1179–1184. https://doi.org/10.1007/s13233-019-7168-3

**20.** *Subramani P. A., Panati K., Narala V. R.* Curcumin nanotechnologies and its anticancer activity. *Nutr Cancer*. 2017, 69(3), 381–393. http://doi:10.1080/01635581.2017.1285405

21. *Krasnopolsky Y. M., Dudnichenko A. S.* Experimental study of liposomal docetaxel incorporation and stability. *Exp. Oncol*. 2017, 39(2), 121–123.

22. *Shobolov D.L., Krasnopol'skij Ju.M., Ul'janov A.M. Natykan A. A., Tarasov V. V., Balaban'jan V. Ju., Shvec V. I., Prohorov V. V.* Method for producing liposomal form of docetaxel. *Eurasian patent № 022182*. November 30, 2015.

23. *Dudnychenko O. S., Temirov Yu. P., Butenko K. A., Krasnopolskyi Yu. M.* The method of obtaining the liposomal form of an anticancer drug. *Ukrainian patent № 14629*. January 20, 1997.

24. *Dudnychenko A. S., Temirov Yu. P., Shvets V. I., Krasnopolskyi Yu. M., Sennikova I. H.* A method of obtaining a liposomal form of an antitumor antibiotic. *Ukrainian patent № 64591*. January 16, 2006.

25. *Pobedimsky D. D., Stepanov A. E., Kaplun A. P., Krasnopolsky Yu. M., Dudnichenko A. S., Pobedimsky D. G., Shvets V. I. Development of technology for the production of Lipodox — a liposomal form of doxorubicin*. *Chemistry and Market*. 2004, 3, 13–22.

26. *Shobolov D. L., Krasnopol'skij Ju. M., Ul'janov A. M. Natykan A. A., Tarasov V. V., Balaban'jan V. Ju., Shvec V. I.* Method of obtaining liposomal form of irinotecan. *Eurasian patent № 023079*. April 29, 2016.

27. *Stadnichenko A. V., Dudnichenko A. S., Krasnopolskiy Yu. M.* Liposomal anticancer drugs. *Kharkov : «Madrid»*, 2018. 256 p. (In Russian).

28. *Hryhorieva G. S., Krasnopolsky Yu. M., Konakhovich N. F., Pasechnikova N. V.* The method of obtaining a pharmacologically active liposomal agent containing quercetin. *Ukrainian patent № 111762*. June 18, 2016.

29. *Hryhorieva A. S., Konakhovych N. F., Stefanov O. V., Krasnopolskyi Yu. M., Temirov Yu. P., Riabushev M. B.* A method of obtaining a liposomal hepatoprotective agent. *Ukrainian patent № 46528*. December 15, 2003.

30. *Ryabushev M.B., Grigor'eva G.S., Konakhovich N.F.* Membranoprotective action of liposomal hepatoprotector "Lioliv". *Medicine*. 2001, 5–6, 74–79. (In Ukrainian).

31. *Vafadar A., Shabaninejad Z., Movahedpour A., Fallahi F., Taghavipour M., Ghasemi Y., Akbari M., Shafiee A., Hajighadimi S., Moradizarmehri S., Razi E., Savardashtaki A., Mirzaei H.* Quercetin and cancer: new insights into its therapeutic effects on ovarian cancer cells. Cell Biosci. 2020, 10, 32. https://doi.org/10.1186/s13578-020-00397-0

32. *Hashemzaei M., Delarami Far A., Yari A., Heravi R. E., Tabrizian K., Taghdisi S. M., Sadegh S. E., Tsarouhas K., Kouretas D., Tzanakakis G., Nikitovic D., Anisimov N. Y., Spandidos D. A., Tsatsakis A. M., Rezaee R.* Anticancer and apoptosis-inducing effects of quercetin in vitro and in vivo. *Oncol. Rep*. 2017, 38(2), 819–828. https://doi.org/10.3892/or.2017.5766

33. *Liu Y., Gong W., Yang Z. Y., Zhou X. S., Gong C., Zhang T. R., Wei X., Ma D., Ye F., Gao Q. L.* Quercetin induces protective autophagy and apoptosis through ER stress via the p-STAT3/Bcl-2 axis in ovarian cancer. *Apoptosis*. 2017, 22(4), 544–557. https://doi.org/10.1007/s10495-016-1334-2

34. *Stefanov A. V., Bryginsky S. A., Lishko V. K., Krasnopolsky Yu. M.* Method for obtaining an antihypoxic agent in liposomal form. *Author's certificate of the USSR No. 1699343 A3*. December 15, 1991.

35. *Grigorieva G. S., Krasnopolsky Yu. M.* Liposomes per se pharmacotherapeutic status. *Pharmacol. Drug Toxicol*. 2020, 14(4), 264–271. https://doi.org/10.33250/14.04.264

36. *Pylypenko O. Ya., Grigoryeva G. S., Krasnopolskyi Yu. M., Konakhovich N. F., Myheitseva I. M., Pasyechnikova N. V., Prokhorov V. V.* The method of obtaining a liposomal composition containing latanoprost, and a pharmacologically active liposomal composition for ophthalmology obtained by this method. *Ukrainian patent № 124724*. November 02, 21.

37. *Krasnopolsky Yu.M., Stepanov A.E., Shvets V.I.* Analysis of Risk Factors Under Production of Preparation Based on Biotechnology. *Third Russian Symposium with International Participation BIOPHARMA-2011: from science to industry*. Tel Aviv, 2011. P. 12–13.

38. Liposome Drug Products: Chemistry, Manufacturing, and Controls; Human Pharmacokinetics and Bioavailability; and Labeling Documentation. Guidance for industry. *FDA, Center for Drug Evaluation and Research (CDER),* 2018, 15 p.

39. *Borschevsky G.I., Tovmasyan E.K., Krasnopolsky Yu.M., Gryzodub O.I.* Standardization of liposome drug products. *Farmakom*. 2013, 2, 5–11. (In Russian)

40. State Pharmacopoeia of Ukraine 2.0. Vol. 1. 2015, 1036–1038. (In Ukrainian)

# ВПЛИВ ЛІПІДНОЇ КОМПОЗИЦІЇ НА ВЛАСТИВОСТІ, ТЕХНОЛОГІЮ ТА ПОКАЗНИКИ ЯКОСТІ ЛІПОСОМАЛЬНИХ ПРЕПАРАТІВ

*Д. М. Пилипенко*[1], *Г. С. Григор'єва*[2], *Н. Ф. Конахович*[2], *Ю. М. Краснопольський*[3]

[1]Державний біотехнологічний університет, Україна, Харків
[2]Державна установа «Інститут фармакології та токсикології Національної академії медичних наук України», Харків
[3]Національний технічний університет «Харківський політехнічний інститут», Україна

*E-mail: pdmforwork@gmail.com*

Ліпосомальні системи доставки ліків є прикладом застосування нанопрепаратів у медичній практиці. Інкапсуляція активних фармацевтичних інгредієнтів у ліпосомальні наночастинки дозволяє збільшити їх біодоступність та ефективність.

*Мета*. Стаття присвячена аналізу ліпідного складу розроблених в Україні ліпосомальних препаратів, його впливу на вибір технології одержання та контрольних параметрів.

*Результати*. Розглянуто ліпідний склад ліпосомальних препаратів, розроблених в Україна за останні роки. Проаналізовано переваги та недоліки природного фосфатидилхоліну як основного мембраноутворюючого ліпіду. Наведені дані щодо впливу аніонних фосфоліпідів та холестерину у складі ліпосомальної мембрани на стабільність ліпосомальних наночастинок та ступінь інкапсуляції активного фармацевтичного інгредієнту. Розглянуто основні технологічні етапи одержання ліпосом з гідрофільними та гідрофобними активними фармацевтичними інгредієнтами. Визначено основні групи показників якості ліпосомальних лікарських форм.

*Висновки*. Ліпідна композиція визначає структуру та фізико-хімічні властивості ліпідної мембрани, механізм та ступінь інкапсуляції активного фармацевтичного інгредієнту, що суттєво впливає на фармакологічну ефективність ліпосомальних систем доставки ліків.

***Ключові слова:*** нанобіотехнологія, система доставки ліків, ліпосомальний препарат, фосфоліпіди, аніонні фосфоліпіди, фосфатидилхолін, холестерин.

UDC 577.27:606.61:617.7 https://doi.org/10.15407/biotech15.05.031

# PRODUCTION OF ANTI-LACTOFERRIN ANTIBODIES AND THEIR APPLICATION IN ANALYSIS OF THE TEAR FLUID IN THE HEALTHY EYE AND CORNEAL INJURIES

*A. Tykhomyrov*[1]
*O. Yusova*[1]
*L. Kapustianenko*[1]
*V. Bilous*[1]
*T. Drobotko*[1]
*I. Gavryliak*[2]
*N. Greben*[2]
*C. A. Ağca*[3]

[1]Palladin Institute of Biochemistry of NAS of Ukraine, Kyiv

[2]Bogomolets National Medical University, Kyiv, Ukraine

[3]Bingol University, Bingol, Turkiye

*E-mail: artem_tykhomyrov@ukr.net*

Received 08.08.2022
Revised 28.08.2022
Accepted 31.10.2022

Lactoferrin is a ubiquitous and multifunctional protein, which has antimicrobial and immunomodulatory activities. Lactoferrin plays an important role in the maintenance of ocular health.

*The aim* of the study was to produce polyclonal antibodies against human lactoferrin in order to apply them in evaluation of lactoferrin levels in the tear fluid collected from healthy eye and after corneal injury.

*Materials and methods.* Affine chromatography on Protein A-sepharose was applied in order to isolate immunoglobulin G (IgG) fraction from the blood serum of lactoferrin-immunized rabbits. Each step of protein purification was monitored by denaturing gel electrophoresis (SDS-PAGE). Target antigen recognition by produced antibodies was established by western blot analysis with the use of diluted IgG fraction. Lactoferrin levels in the tear fluids collected from healthy individuals ($n = 4$) and patients with non-penetrating corneal injures ($n = 6$) were determined immunochemically with the use of purified antibodies. The results of western blot of lactoferrin levels in the tear fluids of healthy individuals and patients with corneal wounds were analysed using Mann-Whitney $U$-test. The difference between group mean values was considered significant at $P < 0.05$.

*Results.* Using affine chromatography on Protein A-sepharose, antibodies against human lactoferrin were purified as IgG fraction from blood serum of lactoferrin-immunized rabbits. Western blot analysis showed that obtained antibodies recognize the antigen as a 75-kDa band, which corresponds to the intact human lactoferrin polypeptide. The same major polypeptide band was visualized by western blot with enhanced chemiluminescence detection in the tear fluid samples. Densitometry analysis of 75-kDa lactoferrin band showed 3.2-fold decrease in lactoferrin level in the tear fluid samples obtained from patients with non-penetrating corneal traumas as compared with samples collected from healthy persons ($P < 0.05$). Besides, tear fluid of patients with injured corneas contained large amounts of truncated lactoferrin immunoreactive polypeptides as well as high molecular weight bands, which could correspond to lactoferrin complexes with other proteins occurring during inflammation.

*Conclusions.* According to our data, obtained anti-lactoferrin antibodies can be used as a valuable tool for development of advanced tests and procedures for diagnostics of eye diseases associated with the corneal lesions. Reduced lactoferrin concentration might represent a potential prognostic biomarker for diagnosis of ocular diseases including non-penetrating corneal injuries in a simple and non-invasive way.

***Key words:*** lactoferrin; antibodies; western blot analysis; corneal wounds; tear fluid.

Human lactoferrin, 80-kDa glycoprotein of the transferrin family, is a non-heme iron-binding protein, consisting of 691 amino acid residues folded into two globular lobes [1]. Lactoferrin possesses iron-binding capacities, several times greater than the affinity of transferrin [2]. Lactoferrin is a ubiquitous protein, which is synthesized in many organs of the human body and contained in exocrine fluids such as breast milk, nasal exudate, bronchial secretions, saliva, tears, sweat, sperm, and vaginal discharge [3]. The main source of lactoferrin is milk; it is contained in human colostrum in the concentration up to 7 g/l. Lactoferrin is practically absent in blood plasma. Its concentration in blood is normally about 1.0 mcg/ml, while it can increase to 200 mcg/ml during inflammatory processes. Lactoferrin represents approximately 25% of the total tear proteins by weight (1.4–2.2 mg/ml), so it represents one of the main proteins in human tears. Tear lactoferrin levels are not influenced by age or sex [4]. Lactoferrin is recently referred to as a multipotent protein, which plays several biological functions, including antibacterial, antiviral, antifungal, antiparasitic, and antiinflammatory activities to provide innate defence [2, 5]. In addition, lactoferrin is known to have antioxidant, antiangiogenic, and antitumor properties, and plays sufficient roles in neuronal differentiation, osteogenesis, and wound healing [6, 7]. Lactoferrin has been shown to hamper plasmin-mediated pericellular proteolysis through inhibiting plasminogen activation by urokinase, thus suppressing cancer cell motility [8]. Besides, inhibition of platelet formation from megakaryocytes and other antiplatelet effects underlie antithrombotic activity of lactoferrin [9].

Lactoferrin plays an important role in maintaining eye health [10]. This protein was found to be expressed in the cornea and iris, and cells of retinal pigment epithelium of human and mouse eyes. However, it is mostly secreted by the main lacrimal gland, with both epithelial cells and Meibomian acini contributing to its final tear levels [11]. Antimicrobial activity of lactoferrin was the first to be discovered, and to date is also the most widely studied. Lactoferrin has been shown to inhibit the growth of various bacterial species implicated in adverse events in tear surface including *Escherichia coli*, *Haemophilus influenzae*, *Bacillus subtilis*, *Streptococcus spp.*, *Staphylococcus spp.* and *Pseudomonas spp.* [4]. For example, Williams et al. [12] have shown that lactoferrin deposited on the contact lens surface can effectively kill *Pseudomonas aeruginosa* cells that attempt to colonize the surface. Furthermore, lactoferrin has been shown to prevent complement activation and formation of harmful hydroxyl radicals and to affect the functions of monocytes, granulocytes and lymphocytes. These findings suggest that lactoferrin, apart from its antimicrobial effects, may also be involved in the regulation of inflammatory disorders. Evidence has been presented to show that lactoferrin binds to other proteins and may be present in various forms in the composition of tear film [13]. As a multifunctional protein, lactoferrin also exhibits efficacy in the setting of viral infectious processes against human and animal pathogenic viruses. Recent studies have demonstrated the activity of lactoferrin against the most widely spread viral particles, including cytomegalovirus, herpes simplex virus, human immunodeficiency virus (HIV), hepatitis C virus, poliovirus, parainfluenza virus, human papillomavirus, and adenovirus. In particular, the antiviral activity of lactoferrin lies in the early phase of infection, when it prevents virus entry into host cells [14]. Nowadays, antiviral activity of lactoferrin appears to be of special interest, since the current pandemic coronavirus disease 2019 (Covid-19) caused by SARS-CoV-2 virus has ocular diseases, in particular, conjunctivitis, among its known clinical manifestations [15]. Several mechanisms have been proposed for SARS-CoV-2 (Covid-19) infection, for which lactoferrin has additionally shown the capability of inhibiting *in vitro* viral replication [16, 17].

Development of various pathological conditions, such as dry eye of corneal injuries, can lead to significant decrease in lactoferrin's concentration, thus providing less protection [5]. It has been also reported that lactoferrin is one of the important predictors of the stability and volume of tear film. Tear volumes from the lacrimal gland are shown to have a positive correlation with the concentration of this protein. Patients with lower tear production tend to have lower lactoferrin's concentration [18]. It has been shown that keratitis and conjunctivitis of different aetiologies result in a decrease of tear lactoferrin levels, thus exposing patients affected by these conditions to a higher risk of infection [10]. Therefore, current and future studies are warranted for clinical applications of lactoferrin for the treatment option of various ocular diseases. Topical application of lactoferrin may play a crucial role in the maintenance of a healthy ocular surface system

by compensating deficit of this protein in the tear film. Topical application of lactoferrin has been shown to reduce irradiation-induced corneal epithelial damage in mice models, as well as to promote corneal wound healing after alkali-burn injury [19].

From these circumstances, lactoferrin's concentrations can represent a potential diagnostic biomarker for diagnosis of ocular diseases in a simple and non-invasive way. Besides, determination of lactoferrin levels in the tears of patients with various eye diseases will give more insight into the physiological role of this protein in the tear film. Our study represents an example of generation and possible application of polyclonal antibody for detecting lactoferrin content and analysis of its polypeptide composition in the tear fluid by western blot. Thus, the aim of the present study was to produce polyclonal antibodies against human lactoferrin in order to apply them for evaluation of lactoferrin levels in the tear fluid in health and collected after corneal injury.

## Materials and Methods

All experiments on animals were conducted in compliance with the basic rules and principles of EU Directive 2010/63 on the protection of animals, the Declaration of Helsinki (2008) and the requirements of the Law of Ukraine "On Protection of Animals from Cruelty" (№1759-VI of 15.12.2009).

*Rabbit immunization.* Two males of English angora rabbit (weighting 2.27 and 2.72 kg) were injected with emulsion of highly purified lactoferrin (Novax®Pharma, Monaco) with Freund's adjuvant (Sigma Aldrich, USA) subcutaneously in four sites in the back (0.25 ml per site), according to the scheme presented in Fig. 1.

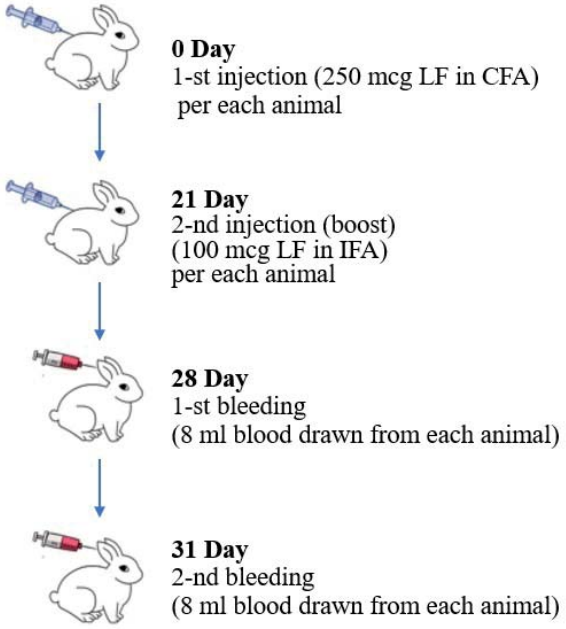


*Fig. 1.* **The scheme of rabbit immunization** (LF — lactoferrin, CFA — complete Freund's adjuvant, IFA — incomplete Freund's adjuvant)

Blood was taken from the ear vein, allowed to form a clot, and then centrifuged at 1,000 *g* for serum collection. Sera from two rabbits were pooled, aliquoted, and stored at −20 °C before further procedures.

*Immunoglobulin G (IgG) isolation and purification.* Isolation of IgG fraction was performed by two-stage fractionation of serum of lactoferrin-immunized rabbits. At the first stage, globulin fraction of serum containing antibodies to lactoferrin was obtained after half saturation with ice-cold ammonium sulphate. Proteins were allowed to precipitate at 4 °C for 12 h. Then, the pellet containing crude globulins was separated by centrifugation at 3,000 *g* for 30 min at 4 °C and dissolved in 0.05 M tris-HCl, pH 7.4, containing 0.15 M NaCl (TBS). Globulin solution was dialyzed against six changes of 100 volumes of the same tris-HCl buffer at 4 °C for 30 min each change till free from ammonium sulphate. After dialysis, p-nitrophenylguanidine benzoate (Sigma Aldrich, USA) was added to globulin solution to the final concentration of 1 mM.

At the second stage, IgG fraction was purified by affinity chromatography on protein A-sepharose (Sigma Aldrich, USA). The column (volume 2.0 $cm^3$) with protein A-sepharose was equilibrated with 10 volumes of TBS. IgG solution was loaded onto column (2:1), and unbound proteins were washed out by TBS. IgG absorbed on protein A-sepharose was eluted by glycine buffer, pH 2.2. Samples were collected, each of 1 ml, and pH was neutralized immediately by 1 M tris-HCl, pH 8.5. Concentration of IgG in eluates was monitored spectrophotometrically at the wavelength of 280 nm. Then, aliquots containing IgG were pooled and dialyzed against three changes of 100 volumes of TBS at 4 °C for 30 min each change with the use of centrifuge ultrafilters Amicon®Ultra $M_m$ 100 kDa (Millipore, Ireland). Further, highly purified IgG fraction was used as a source of specific antibodies against lactoferrin.

*Gel electrophoresis.* The final purity evaluation of immunoglobulin samples was carried out by denaturing electrophoresis

in polyacrylamide gel (SDS-PAGE) in non-reducing conditions. Briefly, the samples of immune serum, globulin fraction, and isolated IgG were dissolved in Laemmli buffer (0.125 M tris-HCl, pH 6.8, containing 4% SDS, 20% glycerol, and 0.005% bromophenol blue) and loaded onto 10% gel. The samples were electrophoresed with the use of tricine-SDS cathode buffer system (0.1 M tris-HCl, 0.1 M tricine, pH 8.3) containing 192 mM glycine and 0.1% SDS, in "Mini-PROTEAN-II" vertical electrophoresis chamber ("BioRad", USA) [20]. After finishing electrophoresis, proteins in gel was fixed in 5% trichloroacetic acid and stained in 0.1% Coomassie Brilliant Blue R-250 dissolved in a mix of 40% ethanol and 10% acetic acid. After destaining, relative molecular weights of the stained protein bands were identified by comparing their migration with the location of coloured trans-blot markers Ruler™ Plus Pre-stained Protein Ladder 10–230 kDa (ThermoScientific, Lithuania). SDS-PAGE was also used for an antigen analysis before rabbit's immunization and as the first step in a western blot procedure of lactoferrin detection in a tear fluid.

*Western blot analysis.* After electrophoresis, proteins were transferred from the gel onto nitrocellulose membranes (GE Healthcare, Amersham Bioscience, UK, RPN 203D) with 0.45 mcm pore diameter by electroblotting using a buffer solution containing 25 mM Tris-HCl, 192 mM glycine, and 25% methanol. Membranes were blocked in a 5% solution of skimmed milk powder in phosphate buffered saline (PBS) and then probed with IgG isolated from the immune serum as a source of anti-lactoferrin antibodies (35 mcg/ml). After incubation with the primary antibodies, the membranes were washed in PBS, containing 0.1% Tween-20 (PBST), and incubated with the secondary antibodies anti-rabbit IgG (H+L)-HRP conjugate diluted 1:8,000 (Bio-Rad Laboratories, Inc., USA). Immunoreactive bands were developed by enhanced chemiluminescence (ECL) and subjected to autoradiography using Kodak X-ray films or using reaction with the chromogen substrate (0.05% diaminobenzidine and 0.03% hydrogen peroxide). Densitometric analysis of blotograms was performed with the use of densitometry software TotalLab TL120 (Nonlinear Inc, USA), signal intensities of the studied proteins were expressed as arbitrary units (a.u.).

*Patients and tear sample preparation.* In order to verify if antibodies isolated from immune rabbit serum are able to recognize and binds lactoferrin, we collected tear fluids from 4 healthy volunteers and 6 patients with non-penetrating corneal injures, which were observed in the clinic "Alexander Clinical Hospital" that is a clinical base of the Bogomolets National Medical University. Informed written consents were obtained from the all participants. The local ethical committee of Bogomolets National Medical University approved the study (protocol no. 138, 10 Nov. 2020) and the research is complied with the last version of Helsinki Declaration. Tear fluid was collected in a sterile plastic Eppendorf tube, mixed with Laemmli sample buffer and stored at −20 °C before laboratory examination. Total protein content in the tear fluid samples was determined spectrophotometrically by Stoscheck method [21]. Proteins of the tear fluids were separated by non-reducing 10% SDS-PAGE, loading 50 mcg total protein per track, and then lactoferrin levels were determined by western blot analysis as described above.

*Statistical analysis.* The results of western blot of lactoferrin levels in tear fluids of healthy individuals and patients with corneal wounds were analysed using Mann-Whitney $U$-test. Values are expressed as the mean ± SD. Difference between group mean values was considered significant at $P < 0.05$.

## Results and Discussion

The major functions of lactoferrin related to the antioxidant, antibacterial, antiviral, antiinflammatory activities have been widely investigated. Lactoferrin is found in faecal, milk, serum, tears and other secretions from human body, and has been reported as a biomarker for several diseases, such as inflammatory bowel disease, Alzheimer's disease, and dry eye disease [22]. The present paper describes the main procedures of production design of polyclonal antibodies against human lactoferrin including immunization protocol and purification of IgG fraction from immune rabbit serum. Then, we addressed here if tear lactoferrin levels and changes in polypeptide composition of this protein could be associated with corneal injury. To do this, we measured relative content of target protein and analysed polypeptide spectrum of lactoferrin immunoreactive bands by western blot with the use of produced antibodies. Proteins of blood plasma of immunized rabbits, desolted globulin fraction and Protein A-eluted fraction containing IgG molecules were analysed by SDS-PAGE (Fig. 2).

Separated proteins of serum and globulin fraction had standard electrophoretic profile. Lanes 1 and 6 correspond to elution peak of rabbit IgG ($M_m$ ~ 140 kDa) collected during affinity chromatography on Protein A-sepharose and concentrated. Electrophoresis showed that the eluted IgG samples contain few impurity proteins, the relative amount of which does not exceed 7-8% of total protein according to densitometry data. It was shown in further experiments that these minor impurities did not influence the immunogenicity of the produced antibodies. Then, we performed electrophoretic analysis of lactoferrin used as an antigen followed by testing produced antibodies by western blot. As shown in Fig. 3*A*, denaturing gel electrophoresis revealed intact lactoferrin polypeptide with apparent molecular weight ~75 kDa. The same major band was visualized by western blot with ECL development of immunoreactive polypeptides or their immunostaining with chromogen substrate (DAB). However, ECL-based detection as more sensitive approach was able to reveal several minor polypeptides bands with lower molecular weights (Fig. 3*B*), in comparison with blot development with the use DAB as chromogenic substrate (Fig. 3*C*).

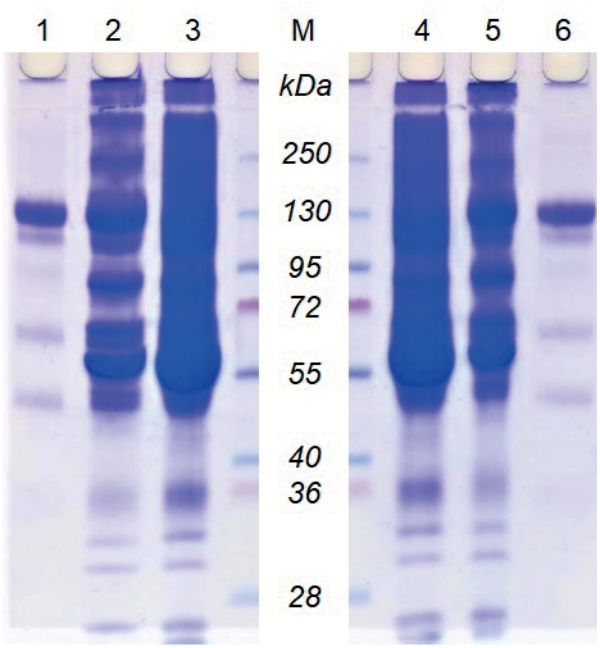


*Fig. 2.* **Coomassie-R250-stained electrophoregram of serum proteins (lanes 3 and 4) of rabbits immunized with lactoferrin, proteins of globulin fraction (lanes 2 and 5), and protein A-purified IgG (lanes 1 and 6) (loading 150, 50, and 10 mcg of total protein, respectively, onto 8% SDS-PAGE). Lane M — standard protein molecular weight markers**

This finding is in agreement with reported evidence that the ECL system is capable of detecting antigens even at a 20-fold increase over chromogenic western blot development [23] and thus is more appropriate technique for detection of lactoferrin in biological materials. Minor immunoreactive bands with $M_m$ lower than 70 kDa may correspond to multiple forms of lactoferrin molecules presented in biological material or its truncated polypeptides since lactoferrin is relatively susceptible to limited proteolysis by various proteases including trypsin, chymotrypsin, pepsin, subtilisin and proteinase K [24], and even by itself owing catalytic activity [25].

Coomassie-stained electrophoregram of tear fluid proteins showed significant difference in protein profile between samples obtained from control individuals and patients with corneal injury (Fig. 4).

Comparison of the band profile of control and patient's tear samples showed a characteristic difference in intensity of two major bands, with apparent $M_m$ 70 kDa, which corresponds to lactoferrin, and 55 kDa, which corresponds to human serum albumin. It is obviously seen that tear fluid

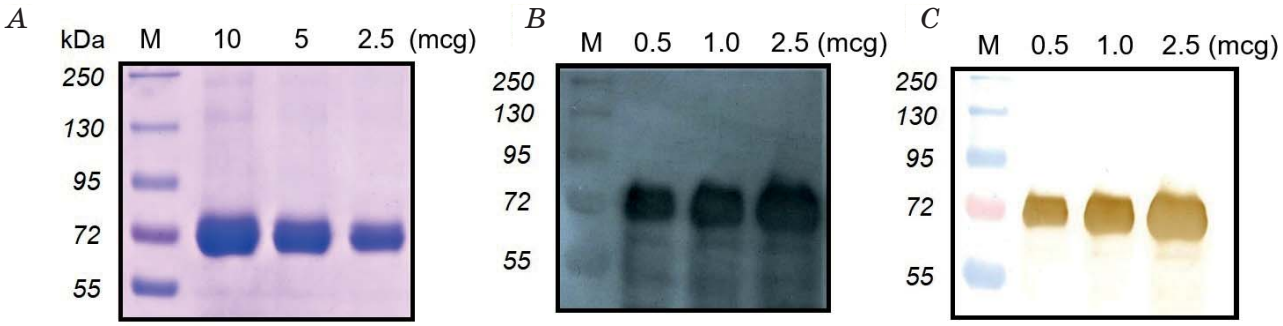


*Fig. 3.* **Electrophoregram of lactoferrin (*A*) and its western blot analysis with ECL detection (*B*) or staining with chromogenic substrate (*C*)**

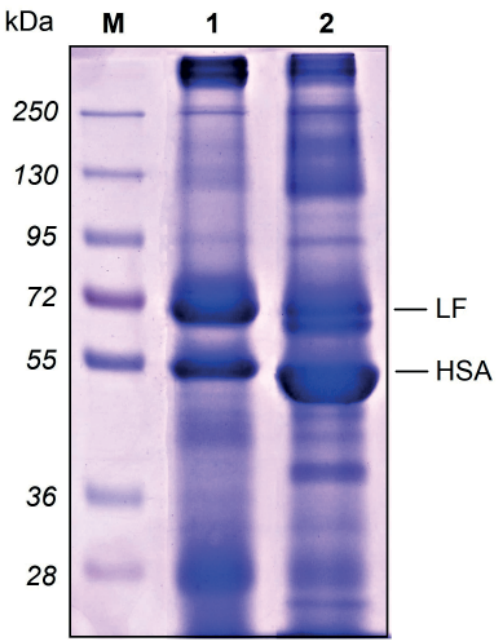


*Fig. 4.* **Typical example of the band profile in tear fluid samples obtained from healthy person (1) and patient with corneal non-penetrating trauma (2)** (SDS-PAGE data, loading 50 mcg of total protein per track, LF — lactoferrin, HSA — human serum albumin)

collected from intact eye contains higher levels of lactoferrin than samples obtained from traumatic eye, whereas in patients with corneal wounds, albumin level appeared to be increased in comparison with healthy persons. The high albumin level in patient's samples might be a result of a leakage of albumin from the inflamed conjunctival capillaries since albumin is a plasma-derived protein.

In order to detect lactoferrin in tear samples and evaluate differences in lactoferrin content between healthy persons and patients with corneal injuries, we used IgG fraction purified from rabbit immune serum in western blot analysis (Fig. 5). As seen in Fig. 5*A*, all tear samples of control persons contain major intact lactoferrin band with $M_m$ ~75 kDa and minor polypeptide of ~55 kDa, while native lactoferrin polypeptide is contained in less number of patient's tear fluid.

Moreover, in addition with decreased level of lactoferrin, all tear film samples of patients with corneal injury contains well-developed band with $M_m$ 30 kDa, which correspond of product of lactoferrin proteolytic degradation. It is also observed that several patient's tear samples contain high molecular weight immunoreactive bands, which may reflect ability of lactoferrin to form complexes with other proteins. This observation is in line with earlier published evidence that showed lactoferrin to bind other proteins including IgA, secretory components, albumin, and lysozyme, and thus it may be present in various forms in the tear film [1]. For example, human lactoferrin has been previously shown to form both *in vitro* and *in vivo* a complex with ceruloplasmin, the copper-containing protein of human plasma, which is present in the tear fluid in increased amounts during corneal inflammation [26, 27]. Densitometry analysis of 75-kDa lactoferrin band showed 3.2-fold decrease in the level of lactoferrin in the tear fluid samples obtained from patients with non-penetrating corneal trauma as compared with samples collected from healthy persons ($P < 0.05$) (Fig. 5*B*). These results of quantitative analysis of lactoferrin content may have clinical application and relevance to ophthalmic traumatology, although further testing of produced antibodies on the larger sized samples is required.

Tear fluid is a complex mixture of proteins, lipids, mucins, water and salts, and a recent study has identified more than 3,000 proteins in human tear samples by proteomic analysis [28], making them more complex (as a body fluid) than serum or plasma. Lactoferrin, also known as lactotransferrin, first isolated from milk (lacto + ferric = milk + iron) is a non-heme iron-binding protein belonging to the transferrin family. It has been estimated that a glass of cow's milk contains about 25-75 mg of this protein [2]. Lactoferrin is one of the main proteins in the tear fluid representing 25% of total tear proteins. Lactoferrin can occur as a holo-protein, which consists of a single polypeptide chain folded into two globular lobes, each with one binding site for iron and apo-protein with less than 5% iron saturation, which is more susceptible to proteolysis due to its molecular conformation that is characterized by lobes that are more 'open'. Lactoferrin performs several biological functions, including antimicrobial and immunomodulatory activities. Thanks to its multifunctional protective character, this glycoprotein is ubiquitous and it is present in different mucosal secretions such as tears, saliva, milk, and nasal secretions, among others. Lactoferrin found in most secretions is almost entirely as an apo-form and thus has the ability to tightly bind any free iron and effectively compete with bacteria for this essential cofactor [4]. Besides, lactoferrin displays broad-spectrum antiviral activity both *in vitro* and *in vivo*.

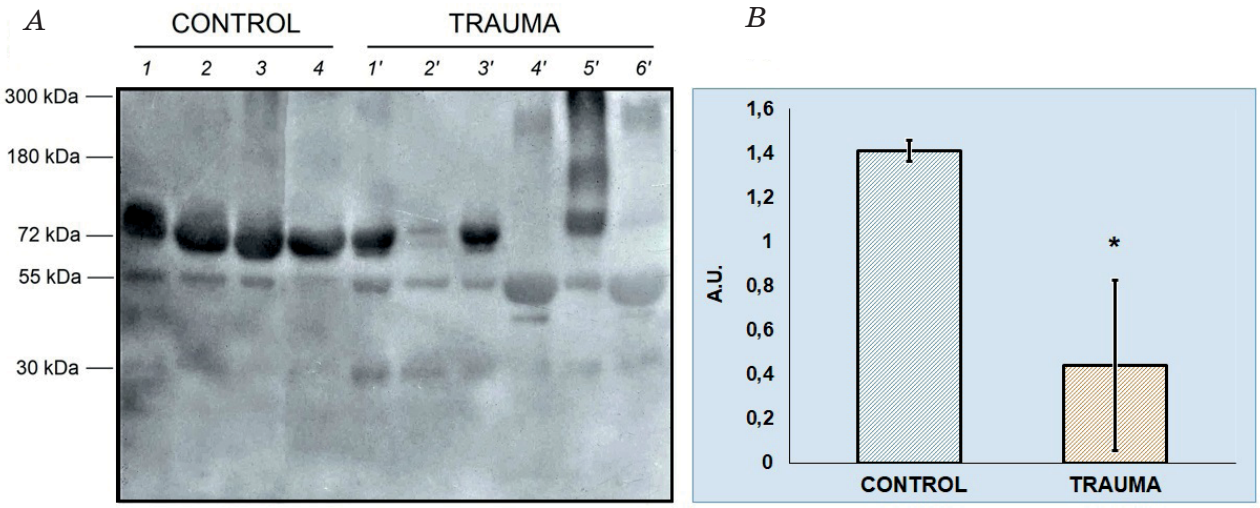


*Fig. 5.* **Detection of lactoferrin in samples of the tear fluid by western blot (*A*) and the results of its densitometry analysis (*B*)** (a.u. — arbitrary units)

*In vitro* results demonstrate that lactoferrin inhibits respiratory syncytial virus, influenza A virus (H3N2, H1N1, H3N2), as well as avian influenza A virus (H5N1), rotavirus, adenovirus, poliovirus, echovirus, herpes simplex virus (HSV-1, HSV-2), and other viruses. Orally administered bovine lactoferrin has been shown to improve the severity of viral infections including rotavirus and norovirus [14, 29]. Lactoferrin is able to bind to receptors, such as angiotensin-converting enzyme 2 (ACE2) and heparan sulfate proteoglycans (HSPGs), used by SARS-CoV-2 virus as an anchor sites in the cell membrane and thus inhibits the adsorption of the pathogen to the cell. In addition, lactoferrin is able to block the pathogen's surface receptors and prevent it from binding to the target cell [30]. It has been established that lactoferrin has not only strong *in vitro* efficacy against SARS-CoV-2, but also alleviates ocular manifestations of Covid-19 [10, 31].

Numerous earlier publications have reported a significant correlation between low levels of tear lactoferrin and the development of some ocular diseases such as dry eye disease, chronic meibomitis, and keratokonus in comparison with healthy subjects [32, 33]. Therefore, the level of lactoferrin in tears of patients with various ocular diseases has great potential to be considered as a valuable biomarker for determining, diagnosis, and prognosis of ophtalmo-pathological condition development. Other studies have also reported reduced lactoferrin tear levels with increased age and in certain diseases such as Sj gren syndrome (an autoimmune disease of the lacrimal gland), idiopathic dry eye, myotonic muscular dystrophy, vernal conjunctivitis, contact lens-induced giant papillary conjunctivitis, trachoma, herpes simplex keratitis, chronic irritative conjunctivitis keratocon-junctivitis sicca, ocular pemphigoid when it occurs concomitantly with dry eye, patients suffering cutaneous pemphigus and clinical dry eye with a marked keratopathy, post-operative cataract surgery, asymptomatic HIV-positive patients, in patients with chronic hepatitis C, and patients suffering Type 2 reactions in leprosy [reviewed in 34].

In general, patients with ocular diseases seem to have lower levels of lactoferrin compared with healthy subjects that is in complete agreement with our results, which indicate significantly decreased lactoferrin content in the tear fluid of patients with corneal injuries. Along with quantitative comparison of lactoferrin tear levels between healthy persons and patients with ocular diseases, differences in its polypeptide heterogeneity analysed by western blot may represent additional diagnostic indices. Many methods have been developed to detect lactoferrin levels during the last decades and different techniques have been used to measure concentration of this protein in tears, such as gel electrophoresis, high-performance liquid chromatography (HPLC), mass spectrometry, enzyme-linked immunosorbent assay (ELISA), or diagnostic test kits [35]. We highlight here that western blot analysis of lactoferrin in the tear fluid with the use of produced polyclonal antibodies allows performing quantitative comparison of lactoferrin levels between healthy individuals

and patients with traumatic ocular pathologies, as well as detecting differences in polypeptide composition of the studied protein in the tear samples.

## Conclusion

Applicability of the produced and tested antibodies against human lactoferrin in diagnostics of ophthalmic pathology (non-penetrating corneal wound) has been clearly demonstrated. We suggest that reduced lactoferrin concentration might represent a potential diagnostic biomarker for diagnosis of ocular diseases including non-penetrating corneal injury in a simple and non-invasive way, thanks to the accessibility of tears and the convenience of tear sampling. Besides, obtained antibodies may become valuable tools for fundamental immunological research, immunohistochemistry, diagnostic testing, and drug quality control.

*Conflicts of interest.* Authors declare no conflict of interest.

*Funding.* The present research was conducted without any external financial support.

*Acknowledgments.* The authors gratefully thank to Andriy Wenger and Liliya Shimboretska for their kind help in animal care.

## REFERENCES

1. *Kell D. B., Heyden E. L., Pretorius E.* The biology of lactoferrin, an iron-binding protein that can help defend against viruses and bacteria. *Front. Immunol.* 2020, 11, 1221. https://doi: 10.3389/fimmu.2020.01221
2. *Kowalczyk P., Kaczyńska K., Kleczkowska P., Bukowska-Ośko I., Kramkowski K., Sulejczak D.* The lactoferrin phenomenon — a miracle molecule. *Molecules*. 2022, 27 (9), 2941. https://doi:10.3390/molecules27092941
3. *Alexander D. B., Iigo M., Yamauchi K., Suzui M., Tsuda H.* Lactoferrin: an alternative view of its role in human biological fluids. *Biochem. Cell Biol*. 2012, 90 (3), 279-306. https:// doi:10.1139/o2012-013
4. *Flanagan J. L., Willcox M. D.* Role of lactoferrin in the tear film. *Biochimie*. 2009, 91 (1), 35-43. https://doi:10.1016/j.biochi.2008.07.007
5. *Rageh A. A., Ferrington D. A., Roehrich, H., Yuan, C., Terluk, M. R., Nelson, E. F., Montezuma, S. R.* Lactoferrin expression in human and murine ocular tissue. *Curr Eye Res*. 2016, 41 (7), 883-889. https://doi:10.31 09/02713683.2015.1075220
6. *Li Y.-Q. Guo C. A* Review on Lactoferrin and Central Nervous System Diseases. *Cells.* 2021, *10,* 1810. https://doi.org/10.3390/ cells10071810
7. *Bielecka M., Cichosz G., Czeczot H.* Antioxidant, antimicrobial and anticarcinogenic activities of bovine milk proteins and their hydrolysates — A review. *Int. Dairy J*. 2022, 127, 105208. https://doi.org/10.1016/j. idairyj.2021.105208.
8. *Zwirzitz A., Reiter M., Skrabana R., Ohradanova-Repic A., Majdic O., Gutekova M., Cehlar O., Petrovčíková E., Kutejova E., Stanek G., Stockinger H., Leksa, V.* Lactoferrin is a natural inhibitor of plasminogen activation. *J. Biol. Chem.* 2018, 293 (22), 8600–8613. https://doi.org/10.1074/jbc.RA118.003145
9. *Matsumura-Takeda K., Ishida T., Sogo S., Isakari Y., Taki T., Sudo T., Kiwada H.* Lactoferrin inhibits platelet production from human megakaryocytes *in vitro*. *Biol. Pharm. Bull*. 2008, 31 (4), 569–573. https://doi. org/10.1248/bpb.31.569
10. *Vagge A., Senni C., Bernabei F., Pellegrini M., Scorcia V., Traverso C. E., Giannaccare G.* Therapeutic effects of lactoferrin in ocular diseases: from dry eye disease to infections. *Int. J. Mol. Sci*. 2020, 21 (18), 6668. https://doi.org/10.3390/ ijms21186668
11. *Rusciano D., Pezzino S., Olivieri M., Cristaldi M., Gagliano C., Lupo G., Anfuso C.D.* Age-related dry eye lactoferrin and lactobionic acid. *Ophthalmic Res.* 2018, 60 (2), 94–99. https:// doi.org/10.1159/000489093
12. *Williams T. J., Schneider R. P., Willcox M. D.* The effect of protein-coated contact lenses on the adhesion and viability of gram-negative bacteria. *Curr. Eye Res*. 2003, 27 (4), 227–235. https://doi.org/10.1076/ ceyr.27.4.227.16602
13. *Hanstock H. G., Edwards J. P., Walsh, N. P.* Tear lactoferrin and lysozyme as clinically relevant biomarkers of mucosal immune competence. *Front. Immunol*. 2019, 10, 1178. https://doi.org/10.3389/ fimmu.2019.01178
14. *Wakabayashi H., Oda H., Yamauchi K., Abe F.* Lactoferrin for prevention of common viral infections. *J. Infect. Chemother.* 2014, 20 (11), 666–671. https://doi.org/10.1016/j. jiac.2014.08.003
15. *Sen M., Honavar S.G., Sharma N., Sachdev M.S.* COVID-19 and eye: a review of ophthalmic

manifestations of COVID-19. *Indian J. Ophthalmol*. 2021, 69 (3), 488-509. https://doi:10.4103/ijo.IJO_297_21
16. *Briana D. D., Papadopoulou A., Syridou G., Marchisio E., Kapsabeli E., Daskalaki A., Papaevangelou V.* Early human milk lactoferrin during SARS-CoV-2 infection. *J. Matern. Fetal. Neonatal. Med.* 2021, 1–4. https://doi.org/10.1080/14767058.2021.1920010
17. *Zimecki M., Actor J. K., Kruzel M. L.* The potential for Lactoferrin to reduce SARS-CoV-2 induced cytokine storm. *Int. Immunopharmacol*. 2021, 95, 107571. https://doi.org/10.1016/j.intimp.2021.107571
18. *Yolton D. P., Mende S., Harper A., Softing, A.* Association of dry eye signs and symptoms with tear lactoferrin concentration. *J. Am. Optom. Assoc*. 1991, 62 (3), 217–223.
19. *Pattamatta U., Willcox M., Stapelton, F., Garrett, Q.* Bovine lactoferrin promotes corneal wound healing and supresses IL-1 expression in alkali wounded mouse cornea. *Curr. Eye Res*. 2013, 38 (11), 1110–1117. https://doi.org/10.3109/02713683.2013.811259
20. *Schägger H., Von Jagow G.* Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem*. 1987, 166 (2), 368–379. https://doi.org/10.1016/0003-2697(87)90587-2
21. *Stoscheck C. M.* Quantitation of protein. *Methods Enzymol*. 1990, 182, 50–68. https://doi.org/10.1016/0076-6879(90)82008-p
22. *Zhang Y., Lu C., Zhang J.* Lactoferrin and its detection methods: a review. *Nutrients*. 2021, 13 (8), 2492. https://doi.org/10.3390/nu13082492
23. *Mruk D. D., Cheng C. Y.* Enhanced chemiluminescence (ECL) for routine immunoblotting: an inexpensive alternative to commercially available kits. *Spermatogenesis*. 2011, 1 (2), 121–122. https://doi.org/10.4161/spmg.1.2.16606
24. *Hopp T. P., Spiewak K., Matthews M. H., Athanasiou Z., Blackmore R. S., Gelbfish, G. A.* Characterization of proteolytic degradation products of vaginally administered bovine lactoferrin. *PloS One*. 2022, 17 (5), e0268537. https://doi.org/10.1371/journal.pone.0268537
25. *Massucci M. T., Giansanti F., Di Nino G., Turacchio M., Giardi M. F., Botti D., Ippoliti R., De Giulio B., Siciliano R. A., Donnarumma G., Valenti P., Bocedi A., Polticelli F., Ascenzi P., Antonini, G.* Proteolytic activity of bovine lactoferrin. *Biometals*. 2004, 17 (3), 249–255. https://doi.org/10.1023/b:biom.0000027700.90
26. *Sabatucci A., Vachette P., Vasilyev V. B., Beltramini M., Sokolov A., Pulina, M. Salvato B., Angelucci C. B., Maccarrone M., Cozzani I., Dainese, E.* Structural characterization of the ceruloplasmin: lactoferrin complex in solution. *J. Mol. Biol*. 2007, 371 (4), 1038–1046. https://doi.org/10.1016/j.jmb.2007.05.089
27. *Pulina M. O., Zakharova E. T., Sokolov A. V., Shavlovski M. M., Bass M. G., Solovyov K. V., Kokryakov V. N., Vasilyev, V. B.* Studies of the ceruloplasmin-lactoferrin complex. *Biochem. Cell. Boil*. 2002, 80 (1), 35–39. https://doi.org/10.1139/o01-206
28. *Jones G., Lee T.J., Glass J., Rountree G., Ulrich L., Estes A., Sezer M., Zhi W., Sharma S., Sharma A.* Comparison of different mass spectrometry workflows for the proteomic analysis of tear fluid. *Int. J. Mol. Sci*. 2022, 23 (4), 2307. doi: 10.3390/ijms23042307.
29. *Valenti P., Antonini G.* Lactoferrin: an important host defense against microbial and viral attack. *Cell. Mol. Life Sci.* 2005, 62 (22), 2576–2587. https://doi.org/10.1007/s00018-005-5372-0
30. *Wotring J. W., Fursmidt R., Ward L., Sexton J. Z.* Evaluating the in vitro efficacy of bovine lactoferrin products against SARS-CoV-2 variants of concern. *J. Dairy Sci*. 2022, 105 (4), 2791–2802. https://doi.org/10.3168/jds.2021-21247
31. *Alpogan O., Karakucuk S.* Lactoferrin: the natural protector of the eye against coronavirus-19. *Ocul. Immunol. Inflamm.* 2021, 29(4), 751–752. https://doi.org/10.1080/09273948.2021.1954202
32. *Kuo M. T., Fang P. C., Chao T. L., Chen A., Lai Y. H., Huang Y. T., Tseng C.Y.* Tear proteomics approach to monitoring sjögren syndrome or dry eye disease. *Int. J. Mol. Sci.* 2019, 20 (8), 1932. https://doi.org/10.3390/ijms20081932
33. *Ohashi Y., Ishida R., Kojima T., Goto E., Matsumoto Y., Watanabe K., Ishida N., Nakata K., Takeuchi T., Tsubota K.* Abnormal protein profiles in tears with dry eye syndrome. *Am. J. Ophthalmol*. 2003, 136 (2), 291–299. https://doi.org/10.1016/s0002-9394(03)00203-4
34. *Ponzini E., Scotti L., Grandori R., Tavazzi S., Zambon A.* Lactoferrin concentration in human tears and ocular diseases: a meta-analysis. *Invest. Ophthalmol. Vis. Sci*. 2020, 61 (12), 9. https://doi.org/10.1167/iovs.61.12.9
35. *Zhang Y, Lu C, Zhang J.* Lactoferrin and its detection methods: a review. *Nutrients*. 2021, 13 (8), 2492. https://doi:10.3390/nu13082492

# ОТРИМАННЯ АНТИТІЛ ДО ЛАКТОФЕРИНУ ТА ЇХНЄ ВИКОРИСТАННЯ В АНАЛІЗІ СЛІЗНОЇ РІДИНИ У НОРМІ ТА ЗА ПОШКОДЖЕННЯ РОГІВКИ

*А. Тихомиров*[1], *О. Юсова*[1], *Л. Капустянецько*[1], *В. Білоус*[1], *Т. Дроботько*[1], *І. Гавриляк*[2], *Н. Гребень*[2], *Дж. А. Агджа*[3]

[1]Інститут біохімії ім. О. В. Палладіна НАН України, Київ, Україна
[2]Національний медичний університет ім. О. О. Богомольця, Київ, Україна
[3]Бінгольський Університет, Бінгол, Туреччина

*E-mail: artem_tykhomyrov@ukr.net*

Лактоферин є мультифункціональним протеїном, що синтезується різними тканинами організму та володіє антимікробною та імуномодуляторною активністю. Лактоферин відіграє провідну роль у підтриманні нормального функціонування ока.

*Метою* роботи було одержати поліклональні антитіла до лактоферину людини для їхнього використання у визначенні вмісту лактоферину в слізній рідині, отриманої за норми та з очей з ушкодженою рогівкою.

*Матеріали та методи.* Фракцію імуноглобулінів G (IgG) було виділено з сироватки крові кролів, імунізованих лактоферином, за допомогою афінної хроматографії на протеїн А-сефарозі. Ступінь чистоти протеїну на кожній стадії очищення перевіряли за допомогою денатуруючого гель-електрофорезу (SDS-PAGE). Розпізнавання цільового антигену отриманими антитілами оцінювали за допомогою вестерн-блот аналізу з використанням розчину фракції IgG. Рівень лактоферину в слізній рідині, отриманої із здорового ока ($n = 4$) та з ока пацієнтів з непроникаючим пошкодженням рогівки ($n = 6$) визначали імунохімічно з використанням отриманих антитіл. Результати визначення вмісту лактоферину в сльозі за умов норми та за травмування рогівки аналізували з використанням $U$-тесту Манна-Уітні. Міжгрупова різниця вважалася статистично достовірною за $P < 0{,}05$.

*Результати.* Антитіла до лактоферину людини у вигляді фракції IgG було виділено на протеїн А-сефарозі з сироватки крові імунізованих кролів. Вестерн-блот аналізом було показано, що отримані антитіла розпізнають відповідний антиген як зону 75 кДа, яка відповідає за молекулярною масою інтактному поліпептиду лактоферину людини. Та ж сама поліпептидна зона була визначена вестерн-блотом з підсиленою хемілюмінісцентною детекцією у зразках слізної рідини. Денситометричний аналіз поліпептиду 75 кДа уможливив встановити, що вміст лактоферину в сльозі, зібраної в пацієнтів з непроникаючими травмами рогівки є у 3,2 рази нижчим за цей показник у нормі ($P < 0{,}05$). Крім того, слізна рідина пацієнтів з пошкодженнями рогівки у значних кількостях містила також імунореактивні продукти розщеплення лактоферину, а також високомолекулярні поліпептиди, які можуть відповідати комплексам лактоферину з іншими протеїнами, що утворюються за розвитку запальних процесів.

*Висновки.* Згідно з наведеними даними, отримані антитіла до лактоферину можуть бути використані як корисний інструмент для створення удосконалених тестів та підходів для діагностики очних хвороб, пов'язаних з ушкодженням рогівки. Зниження вмісту лактоферину може слугувати прогностичним біомаркером перебігу ранового процесу в оці, зокрема, за непроникаючих травм рогівки, та є зручним до визначення в простий та неінвазійний спосіб.

***Ключові слова:*** лактоферин; антитіла; вестерн блот аналіз; травми рогівки; слізна рідина.

UDC 57.088.2 **https://doi.org/10.15407/biotech15.05.041**

# APPROBATION OF CALIX[4]ARENE AS AN ANTITHROMBOTIC AGENT *in vivo*

*V. A. Didkivskyi*[1]
*A. O. Pavlenko*[1, 2]

[1]Palladin Institute of biochemistry of the National Academy of Sciences of Ukraine, Kyiv
[2]ESC "Institute of biology and medicine"of Taras Shevchenko National University of Kyiv, Ukraine

*E-mail: didkivskyivalik@gmail.com*

Received 20.07.2022
Revised 09.10.2022
Accepted 31.10.2022

Intravascular thrombosis is one of the main causes of mortality in the working-age population of the world. There are no antithrombotic drugs that act directly on the final stage of thrombosis — fibrin polymerization. However, a new compound of the calix[4]arene series, calix[4]arene C-145, which directly interacts with the fibrin polymerization site 'A-knob' thus blocking formation of polymeric fibrin and preventing thrombosis.

So, the *purpose* of this work was to study the calix[4]arene C-145 series as antithrombotic agents *in vivo* using different animals and types of administration.

*Materials and methods*. Laboratory animals (rats, mice and rabbits) were used for C-145 testing *in vivo*. Activated partial thromboplastin time and platelet aggregation were measured to determine the anticoagulant action after intravenous or *per os* administration.

*Results*. Per os way of administration was selected as the optimal one. We showed the substantial prolongation of clotting time in APTT test that was observed starting from the 2nd hour after the *per os* administration, reached the maximum on 6th hour and eliminated in 24 hours. The effect of C-145 on platelets reached maximum on 4–6 hours and eliminated in 12 hours.

*Conclusions*. C-145 was proven to be prospective antithrombotic drug that can be administered *per os*. Further investigations must be focused on the study of C-145 pharmacodynamics and metabolism. Such data would allow fast implementation of the tested compound into practice.

***Key words:*** calix[4]arene, blood plasma, platelets, thrombosis, blood coagulation, noncovalent complex.

Intravascular thrombosis is one of the main causes of mortality in the working-age population of the world. It consists in the formation of fibrin-platelet thrombus, which blocks the lumen of the vessel, preventing blood supply to tissues and organs and causing severe pathologies such as myocardial infarction, thrombotic brain stroke, pulmonary embolism, etc.

Therefore, the search for ways to effectively prevent thrombus formation in the vessel is an important issue of modern medicine and biochemistry. The antithrombotic drugs are used for rapid and controlled inhibition of the process of activation of the blood coagulation system. According to the direction of action there are anticoagulants, inhibiting blood clotting at different stages, and fibrinolytics, aimed at accelerating the destruction of the fibrin clot (t-PA, streptokinase, urokinase). The most common among anticoagulants are acting directly on thrombin (dabigatran), factor Xa (rivaraxaban), factors VIIIa and V (drotrekogin), blocking the blood coagulation cascade at the stages of activation of factor X, prothrombin or conversion of fibrinogen to fibrin. A separate class of compounds that block the formation of fibrin-platelet thrombus are platelet aggregation inhibitors [1].

As of today, there are no antithrombotic drugs that act on the final stage of thrombosis, that is fibrin polymerization. As a direct inhibitor of fibrin polymerization, silver

nanoparticles or inhibitor peptides (in particular, GPRP) conjugated with albumin have been proposed. No such agents have passed preclinical trials [2].

A new compound of the calix[4]arene series [3] created at the Institute of Organic Chemistry of the National Academy of Sciences of Ukraine, calix[4]arene C-145, which directly interacts with the fibrin polymerization site 'A-knob' due to its hydrophobic cup, was characterized in the Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine [4]. Therefore, calix[4] arene C-145 directly inhibits the formation of fibrin protofibrils ($IC_{50}$ = $2.5\times10^{-6}$), preventing the formation of polymeric fibrin [5]. Its efficacy both *in vitro* and *in vivo* has been shown [6].

This work was aimed to the study of the calix[4]arene C-145 series as antithrombotic agents *in vivo* using different animals and types of administration.

## Materials and Methods

*Materials*. Chemicals: APTT (activated partial thromboplastin time) reagent and $CaCl_2$ solution were purchased from Siemens (Germany), ADP was purchased from Sigma-Aldrich (USA).

Calix[4]arene C-145 was syntesized by the reaction of tetraformylcalixarene with sodium salt of diisopropylphosphite with formation of tetrakisbisphosphonate, which after subsequent dealkylation due to treatment with trimetylbromosilane and methanol gives C-192 and its sodium salt C-145 [7]. Sample of calix[4]arene C-145 was kindly provided by Institute of Organic Chemistry of National Academy of Sciences of Ukraine. It was dissolved in 0.9% NaCl solution.

*Animal keeping*. Male Wistar rats (170 ± 4 g, $n$ = 60) and Balb mice (30 ± 1 g, $n$ = 30) were kept in the animal house of Palladin Institute of biochemistry of NAS of Ukraine. The animals were kept in standard cages (5 animals in each cage) under controlled conditions (22 °C ± 2, 12-h/12-h light/dark cycle) with unlimited access to drinking water and food. Outbreed male rabbits (3.5 ± 0.5 g, $n$ = 9) were kept in the animal house of Bila Tserkva National Agrarian University on a standard diet. This study was carried out with the approval of animal care and use committee of the Palladin Institute of Biochemistry of NAS of Ukraine (Protocol #10/11-2019).

*Administration of calix[4]arene C-145*. Solution of C-145 was administered per os to mice (in a volume of 0.1 ml) and to rats (in a volume of 0.5 ml). Solution of C-145 was also injected into the rat's lateral tail vein and into rabbit's marginal vein of the ear. The dosage was standard in all experiments (12 mg/kg).

*Blood sampling*. Samples of rat or mice blood were collected by heart puncture. Pentobarbital (Nembutal) anesthesia (dosage was 50 mg/kg of body weight) was used intraperitoneally according to ethical standards and principles. A 3.8 % sodium citrate solution was added to the blood samples immediately after collection. Animals were decapitated immediately after blood collection still being under anesthesia. Rabbit blood samples were collected using Wenflon catheter (Becton Dickinson, USA), G22 (0.8 mm). 3.8 % Sodium Citrate added immediately after collection to whole blood in 1:9 ratio was used as an anticoagulant.

*Blood plasma preparation*. Platelet rich plasma (PRP) was prepared by centrifuging blood at 160 g for 20 min at 25 °C. Platelets were spun-down at 300 g for 15 min at 25 °C for obtaining of platelet poor plasma (PPP). PRP was analyzed immediately after collection. PPP was fresh frozen at −20 °C and stored no longer than 2 months.

*Activated partial thromboplastin time*. Activated partial thromboplastin time (APTT) was performed according to the following procedure. 0.1 ml of blood plasma was mixed with an equal volume of APTT-reagent and incubated for 3 min at 37 °C. Then the coagulation was initiated by adding 0.1 ml of 0.025 M solution of $CaCl_2$. Clotting time was monitored by the Coagulometer Solar CGL-2410 (Solar, Minsk, Belarus).

*Platelet aggregation*. Platelet aggregation measurements were based on changes in the turbidity of human PRP. Aggregation was registered for 10 min using the Aggregometer Solar AP2110 (Solar, Minsk, Belarus). Platelet count was estimated using the same device. We measured the initial rate and final level of aggregation at 37 °C. In a typical experiment, 0.25 ml of PRP was incubated with $CaCl_2$ (0.010 mM) activated by platelet agonist ADP (0.012 mM) at 37 °C.

*Statistical data analysis*. Statistical data analysis was performed using Microsoft Excel. All assays were performed in series of three replicates and the data were fitted with standard errors using "Statistica 7". Results are presented as means ± standard deviation. The difference between the groups was analyzed by one way ANOVA. Data were considered significant when $p < 0.05$.

## Results and Discussion

*Intravenous administration.* Intravenous administration is a useful method for drugs that must be administered fast and are used urgently [8]. It is also useful for initial testing as far as this way does not depend on metabolization and delivers drug to the bloodstream directly [9].

In our study we applied C-145 intravenously to rabbits and rats and determined the anticoagulant effect of the compound using APTT test. Prolongation of clotting time in APTT test was an evidence of the presence of C-145 into the bloodstream.

For both rats and rabbits the anticoagulant effect was observed 2–12 h after injection and completely eliminated after 24 h (Fig. 1, 2). However it was more pronounced in the case of rats (Fig. 2).

Analyzing the rat model, we also detected the dramatic decrease of platelet aggregation rate after the C-145 administration (Fig. 3). Platelet aggregation was insulted in 2 h after C-145 injection, aggregation rate was constantly low in 2–12 h and remained decreased after 24 h.

Results of APTT and platelet aggregation testing in rats allowed us to conclude that the used dosage was higher than it was needed for this kind of animals. Also, the anti-platelet effect of C-145 was undesirable. We presume that the per os administration of C-145 would allow to avoid this effect.

*Per os administration.* Administration per os is the best way for the use of deugs of regular administration. As non-invasive method, it could be recommended the preferential method of drug delivery [10].

Testing of per os administration of C-145 was performed on mice and rats models. We showed the substantial prolongation of clotting time in APTT test in the case of both species (Fig. 4, Fig. 5).

Anticoagulant effect was observed starting from the 2$^{nd}$ hour after the administration, reached the maximum on 6$^{th}$ hour and eliminated in 24 hours.

Even more important finding was the moderate effect of C-145 on platelets. In both animal models the effect of C-145 on platelets reached maximum on 4-6 hours and eliminated in 12 hours (Fig. 6, 7).

Also, it should be noted that anticoagulant effect of C-145 was too high. Actually 1.5-times prolongation of clotting time would be enough [11]. So, we expect that C-145 dosage can be decreased substantially in the future studies that also is an important issue in the light of future implementation of this antithrombotic drug.

Antithrombotic action of C-145 through its action of fibrin was reported earlier [12]. This inhibition of fibrin polymerization occurs as a result of direct non-covalent complex formation of C-145 and GPRP motive of fibrin Aα-chain (polymerization site 'A'-knob) [4, 5].

Important outcome of the study is the direct demonstration of C-145 anticlotting activity in blood plasma of animals. While its efficacy after intravenous injection was predictable, high effect during oral administration was a remarkable finding. Prolongation of clotting time of blood plasma of rats after C-145 oral

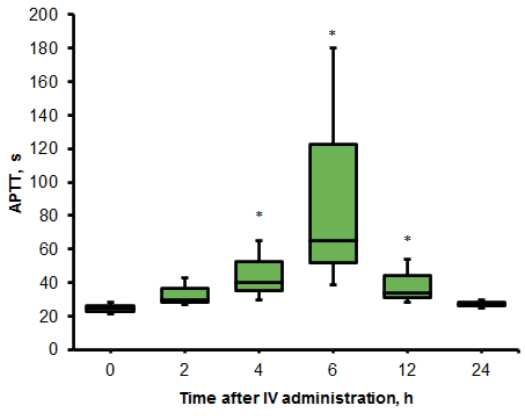


*Fig. 1.* **Activated partial thromboplastin time of blood plasma clotting after intravenous (IV) administration of C-145 to rabbits (12 mg/kg)**
0 — clotting time before the C-145 administration. 2–24 — time of clotting of blood plasma after the C-145 administration. $n = 5$. * $P \le 0.05$

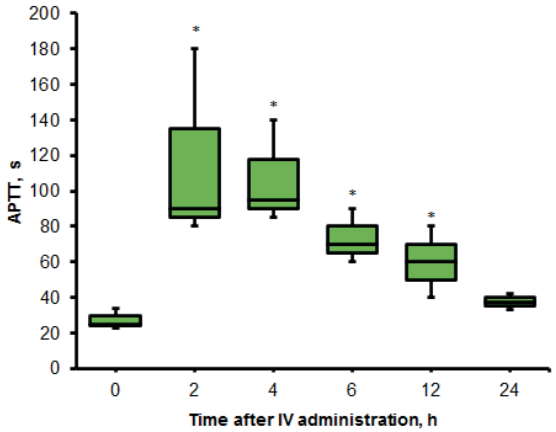


*Fig. 2.* **Activated partial thromboplastin time of blood plasma clotting after intravenous (IV) administration of C-145 to rats (12 mg/kg)**
0 — clotting time before the C-145 administration. 2–24 — time of clotting of blood plasma after the C-145 administration. $n = 5$. * $P \le 0.05$

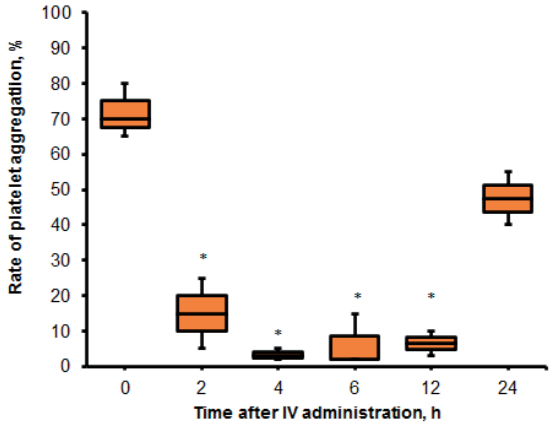


*Fig. 3.* **The rate of platelet aggregation after intravenous (IV) administration of C-145 to rats (12 mg/kg)**
0 — clotting time before the C-145 administration. 2–24 — time of clotting of blood plasma after the C-145 administration. $n = 5$. * $P \leq 0.05$

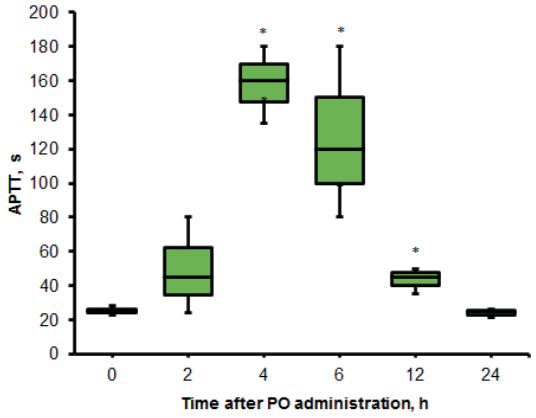


*Fig. 4.* **Activated partial thromboplastin time of blood plasma clotting after per os (PO) administration of C-145 to mice (12 mg/kg)**
0 — clotting time before the C-145 administration. 2–24 — time of clotting of blood plasma after the C-145 administration. $n = 5$. * $P \leq 0.05$

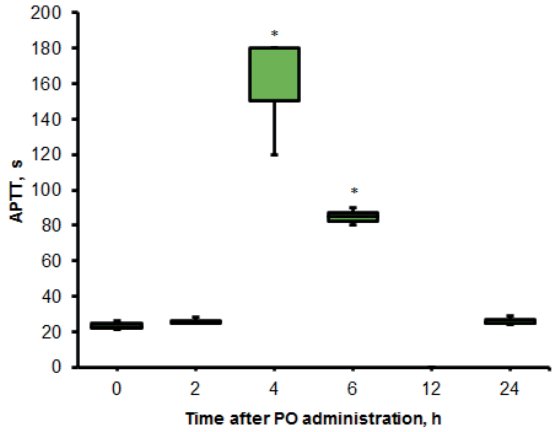


*Fig. 5.* **Activated partial thromboplastin time of blood plasma clotting after per os (PO) administration of C-145 to rats (12 mg/kg)**
0 — clotting time before the C-145 administration. 2–24 — time of clotting of blood plasma after the C-145 administration. $n = 5$. * $P \leq 0.05$

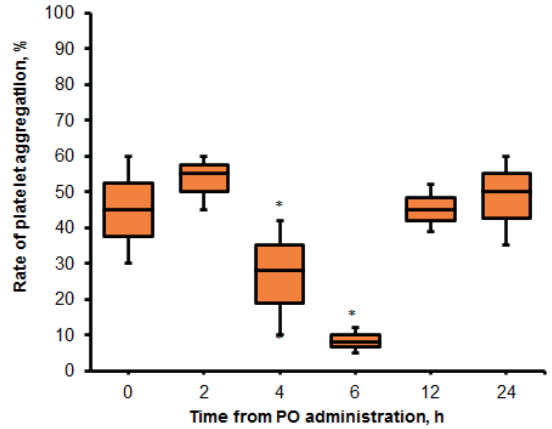


*Fig. 6.* **The rate of platelet aggregation after oral administration of C-145 to mice (12 mg/kg)**
0 — clotting time before the C-145 administration. 2–24 — time of clotting of blood plasma after the C-145 administration. $n = 5$. * $P \leq 0.05$

administration indicates the appearance of active compound into the bloodstream in unchanged active form. Observing the stable anti-clotting effect up to 12 hours after the administration is evidence of the continuing presence of C-145. The normalization of clotting time after 24 hours indicates its clearance from the bloodstream.

In conclusion, C-145 was proven to be a prospective antithrombotic drug that can be administered per oral. Its anticoagulant effect slightly differed for different animal species. We presume that the selected concentration (12 mg/kg) can be decreased substantially. Also, antiplatelet effect that was observed after C-145 administration can be minimized by the dosage selection. Further investigations must be concentrated on the study of C-145 pharmacodynamics and metabolization. Such data would allow fast implementation of the development compound into practice.

*Funding source*

Study was financed by project 0121U110361 of National Academy of Sciences of Ukraine “Development of the prototype of antithrombotic preparation Calix[4]arene C-145 and its pre-clinical studies”.

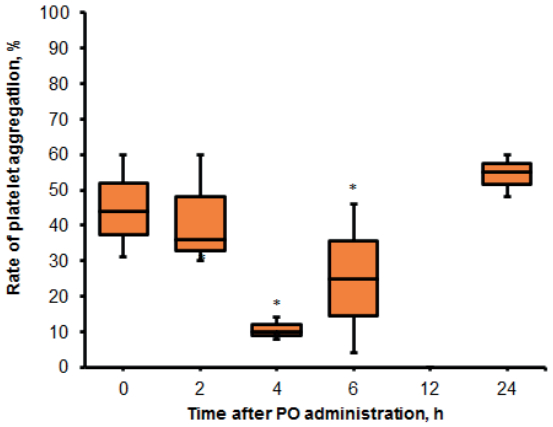


*Fig. 7.* **The rate of platelet aggregation after per oral (PO) administration of C-145 to rats (12 mg/kg)**
0 — clotting time before the C-145 administration. 2–24 — time of clotting of blood plasma after the C-145 administration. $n = 5$. * $P \leq 0.05$

*Acknowledgement*

Authors cordially acknowledge Prof. Kalchenko V. I. (Institute of Organic chemistry of the National Academy of Sciences of Ukraine) for providing the calix[4[arene samples and Prof. Rublenko M. V. (National Agrarian University. Bila Tserkva, Ukraine) for his help with rabbit studies. Authors also thank to Dr. Chernyshenko V.O. (Palladin Institute of biochemistry of NAS of Ukraine) for his supervising and guidance in the preparation of the manuscript.

## REFERENCES

1. *Chernyshenko V.O.* Mechanisms of intravascular and extravascular blood clotting: fundamental studies for clinical practice. *Visn. Nac. Acad. Nauk Ukr.* 2019, 4, 50–56. https://doi.org/10.15407/visn2019.04.050
2. *Chernyshenko V. O, Lugovska N. E.* Molecular mechanisms of intravascular inhibition and stimulation of extravascular thrombosis. *Biotechnologia Acta.* 2021, 14(6), pp. 5–22. https://doi.org/10.15407/biotech14.06.005
3. *Asfari M. Z., Bohmer V., Harrowfield J., Vicens J. (eds).* Calixarenes 2001. *Kluwer academic Publishers.* 2001, 3–684.
4. *Komisarenko S. V., Kosterin S. O., Lugovskoy E. V., Kalchenko V. I.* Calixarene methylene bisphosphonic acids as promising effectors of biochemical processes. *Ukr. Biochem. J.* 2013, 85(6), 106–128. http://dx.doi.org/10.15407/ubj85.06.106
5. *Lugovskoy E. V., Gritsenko P. G., Koshel T. A., Koliesnik I O., Cherenok S. O., Kalchenko O. I., Kalchenko V. I., Komisarenko S. V.* Calix[4]arene methylenebisphosphonic acids as inhibitors of fibrin polymerization. *FEBS J.* 2011, 278(8), 1244–51. https://doi.org/10.1111/j.1742-4658.2011.08045.x
6. *Chernyshenko V. O., Korolova D. S., Dosenko V. E., Pashevin D. O., Kalchenko V. I., Pirogova L. V., Chernyshenko T. M., Lugovska O. E., Kravchenko N. A., Makogonenko Y. M., Lugovskoy E. V., Komisarenko S. V.* Calix[4]arene C-145 Effects on Plasma Haemostasis. *Pharmaceutica Analytica Acta.* 2015, 6(8), 1–5. https://doi.org/10.4172/2153-2435.1000406
7. *Rodik R. V., Boyko V. I., Kalchenko V. I.* Calixarenes in bio-medical researches. *Current Medicinal Chemistry.* 2009, 16(13), 1630–1655. https://doi.org/10.2174/092986709788186219
8. *Bansal S, Sangha KS, Khatri P.* Drug treatment of acute ischemic stroke. *Am J Cardiovasc Drugs.* 2013, 13(1), 57–69. https://doi.org/10.1007/s40256-013-0007-6.
9. *Alsenaidy M.A., Kazi M., Ahmad M.Z.* Advances in Oral Drug Delivery. *Sec. Translational Pharmacology.* 2021, 12, 618411. https://doi.org/10.3389/fphar.2021.618411
10. *Homayun B., Lin X., Choi H.J.* Challenges and Recent Progress in Oral Drug Delivery Systems for Biopharmaceuticals. *Pharmaceutics.* 2019, 11(3), 29. https://doi.org/10.3390/pharmaceutics11030129.
11. *Hartmann S., Biliouris K., Lesko L.J., Nowak-Göttl U., Trame M.N.* Quantitative Systems Pharmacology Model-Based Predictions of Clinical Endpoints to Optimize Warfarin and Rivaroxaban Anti-Thrombosis Therapy. *Front Pharmacol.* 2020, 14(11), 1041. https://doi.org/10.3389/fphar.2020.01041.
12. *Chernyshenko V., Pirogova L., Didkivskyi V., Cherenok S., Dosenko V., Pashevin D., Kalchenko V., Makogonenko E., Lugovskoy E.* Effects of calix[4]arene C-145 on overall haemostatic potential of blood plasma in vitro and in vivo. *Journal of International Research in Medical and Pharmaceutical Sciences.* 2016. 10(3), 146–151.

# АПРОБАЦІЯ КАЛІКС[4]АРЕНУ ЯК АНТИТРОМБОТИЧНОГО ЗАСОБУ *in vivo*

*В. А. Дідківський*[1], *А. О. Павленко*[1, 2]

[1]Інституту біохімії ім. О. В. Палладіна НАН України, Київ
[2]Київський національний університет імені Тараса Шевченка,
ННЦ «Інститут біології та медицини» Україна

*E-mail*: *didkivskiyvalik@gmail.com*

Внутрішньосудинні тромбози є однією з основних причин смертності працездатного населення світу. Антитромботичних препаратів, що впливають безпосередньо на кінцеву стадію тромбозу — полімеризацію фібрину, наразі не створено. Однако раніше було описано нову сполуку калікс[4] аренового ряду — калікс[4]арен С-145, яка безпосередньо взаємодіє з центром полімеризації фібрину «А».

Отже, *метою* цієї роботи було вивчення калікс[4]арену С-145 як антитромботичного агенту *in vivo* з використанням різних видів тварин і способів введення.

*Матеріали та методи*. Для дослідження калікс[4]арену С-145 *in vivo* використовували лабораторних тварин (щурів, мишей і кроликів). Для визначення антикоагулянтної дії після внутрішньовенного або перорального введення вимірювали активований частковий тромбопластиновий час і агрегацію тромбоцитів.

*Результати*. Оптимальним був визначений пероральний спосіб застосування. Показано значне подовження часу згортання в тесті АЧТЧ, яке спостерігалося, починаючи з 2-ї години після перорального прийому, досягало максимуму на 6-й годині та припинялася через 24 години. Дія калікс[4]арену С-145 на тромбоцити досягала максимуму через 4–6 годин і припинялася через 12 годин.

*Висновки*. Було доведено, що калікс[4]арен С-145 є перспективним антитромботичним препаратом, який можна вводити перорально. Подальші дослідження мають бути зосереджені на вивченні фармакодинаміки та метаболізму калікс[4]арену С-145. Такі дані потрібні для якомога швидкого впровадження створено комплексу на практиці.

***Ключові слова:*** калікс[4]арен; плазма крові; тромбоцити; тромбоз; згортання крові; нековалентний комплекс.

UDC 577.27:606.61:617.7 **https://doi.org/10.15407/biotech15.05.047**

# NEUROBEHAVIOURAL AND ANALGESIC EFFECT OF OCIMUM GRATISSIMUM LINN. LEAVES ESSENTIAL OIL IN WISTAR ALBINO MICE

*Onosetale E. Aigbomian*[1]
*Comfort T. Senjobi*[2]
*S. A. Onasanwo*[3]
*M. O. Olatunde*[4]
*Taiye R. Fasola*[1]

[1]Department of Botany, University of Ibadan, Nigeria
[2]Department of Plant Science, Olabisi Onabanjo University, Ago-Iwoye, Nigeria
[3]Department of Physiology, University of Ibadan, Nigeria
[4]Department of Pharmacology and Therapeutics, University of Ibadan, Nigeria

*E-mail: fasolatr@gmail.com*

Received 25.08.2022
Revised 12.10.2022
Accepted 31.10.2022

Studies have shown that pain relieving medications may be neuroprotective. *Ocimum gratissimum* Linn. that is widely used in traditional medicine for debility and many other illnesses neuropharmacologically related has not been fully explored.

*Aim.* This study was designed to investigate the safety of intake, neurobehavioral and analgesic effects of the Essential Oil of *Ocimum gratissimum* Linn leaves (EOOG) in mice.

*Methods.* Acute toxicity of EOOG was determined following standard method while the neurobehavioural properties were assessed using the open field for Novelty-Induced Rearing (NIR), Novelty-Induced Grooming (NIG) and locomotor activity in mice. The hole board apparatus was used for the frequency of head dips. The Y-maze was used for short- working memory. Mechanistic studies were conducted with Atropine (muscarinic blocker, 0.5 mg/kg), Propanolol (non-selective β-adrenoceptor blocker, 0.2 mg/kg), Haloperidol (dopamine receptor blocker, 0.2 mg/kg), Cyproheptadine (Serotoninergic antagonist, 0.5 mg/kg) and Yohimbine ($\alpha$-$_2$ adrenergic blocker, 1 mg/kg). The analgesic activity of *Ocimum gratissimum* was investigated using acetic acid writhing test and thermally-induced pain.

*Results.* The median lethal dose ($LD_{50}$) of *Ocimum gratissimum* was 2449 mg/kg. The EOOG significantly reduced novelty-induced behaviour in a dose-dependent manner. The exploratory activity of animals treated with the EOOG was observed to decrease non-dose dependently with the highest dose (40 mg/kg) showing no activity on the hole board apparatus. The EOOG produced a significant reduction in locomotor activity in all the doses in a non-dose dependent manner but at the lowest dose. In the Y-maze, EOOG did not produce any significant effect on working memory as the percentage alternation produced was not significantly different from the control. The EOOG in hot plate analgesic assay showed increased reaction time suggesting central nervous system analgesic property.

*Conclusions.* The results of the investigation showed that EOOG might possess sedative properties due to its ability to inhibit NIR and NIG, head dips, and locomotor activity. Furthermore, the inhibition of nociception marked in this research advocates antinociceptive activity which might be through the peripheral or central opioid receptor.

***Key words:*** Ocimum gratissimum; neuroprotective; pain; sedative; medicinal plants.

Central Nervous System (CNS) diseases are complex, with multiple symptoms, like the negative symptoms of Schizophrenia, which tend to be resistant to treatment. Furthermore, the complexity of the brain and its neuronal pathways result in a significant risk of side effects, even when the most-biochemically selective agent is administered. The lack of disease-modifying treatments for CNS diseases represents a very significant unmet medical need, and of a high priority. These failures of synthetic drugs and their side effects have necessitated looking into ancient healing methods for alternatives [1].

Pain causes a lot of suffering and discomfort to the victims, lowering the quality

of life and therefore needs to be carefully managed. To suppress pain, non-steroidal anti-inflammatory drugs (NSAIDs) are mostly prescribed [2, 3]. For severe or chronic malignant pain, opioid analgesics are drugs of choice [4]. However, due to adverse side effects like gastric lesions, caused by NSAIDs and tolerance and dependence induced by opiates, the use of these drugs as analgesic agents have not been successful in all cases. Therefore, analgesic drugs lacking those effects are being searched all over the world as alternatives to NSAIDs and opiates.

Many of the thousands of plant species growing throughout the world have a direct pharmacological action on the body [5], and many native African plants are rich in medicinal properties that are well documented in folkloric medicine as a potential source for novel compounds with beneficial therapeutic effects and fewer side effects being natural products. However, we must not assume that since a plant has been used for thousands of years, it is therefore safe and truly effective for its claimed indications, thus, they must be scientifically investigated [6]. *Ocimum gratissimum* Linn. is a native African plant widely used in traditional medicine for health and healings. Pre-clinical studies of brain injuries in experimental rodent animal models have shown neurobehavioral and neurodegenerative abnormalities [7]. Currently, there are only a few literature available for neuropharmacological profile of *Ocimum gratissimum* leaf. Thus, this study aimed to evaluate the safety of consumption, neurobehavioral and analgesic effect of essential oil of *Ocimum gratissimum* leaf (EOOG) in mice.

## Material and Methods

*Toxicity Assay*

The method described by OECD (2002) was used to determine the $LD_{50}$, which is the index of acute toxicity. Male Swiss Albino mice (20–25 g) were used. This method involved an initial dose-finding procedure, in which the animals were divided into three groups of five animals. Doses of 10, 100, and 1000 mg/kg were administered intra-peritoneally (*i.p.*) for each mouse per group. The treated animals were monitored for 24 hours for mortality and general behaviour. Likewise, four different doses of (500, 1000, 2000, and 3000 mg/kg) were chosen and administered intra-peritoneally respectively to four groups of one mouse per group. The treated animals were monitored for 24 hours. The $LD_{50}$ was then calculated as the geometric mean of the highest dose showing no death and the lowest dose showing death.

*Novelty-induced rearing (NIR) and grooming (NIG) in mice*

The behavioural parameters employed in this observational analysis were rearing and grooming [8, 9]. The frequency of rearing and grooming episode was quantified using a manual counter and a stopwatch. The total frequency was summed up for each animal for 30 minutes of observation time. Rearing was taken as the number of times the mouse stood on its hind limbs or with its forelimbs against the wall of the observation cage or in the free air. Grooming was taken as the number of body cleaning with paws, picking of the body, and pubis with mouth and face washing actions.

*Exploratory behaviour on hole-board apparatus*

The effect of the EOOG on the frequency of head dipping was determined in the hole-board with 16 equidistant holes in the floor through which the animal can poke its head. The test is a measure of exploratory behavior that reveals the sedative activity of agents. Thirty animals were divided into six groups ($n$ = 5). Group 1 was given distilled water (10 mL/kg, i.p), while group 2–5 received EOOG (5, 10, 20, and 40 mg/kg, i.p.) respectively and group 6 received Diazepam (2 mg/kg). The animals were placed on top of the wooden board 30 minutes after treatment administration. The number of times that each animal dipped its head into the holes was counted for 5 min [10].

*Locomotor activity in the open field*

Motor activity was measured in an open field apparatus consisting of a white Plexiglas box (28 × 28 × 25 cm) with a painted black grid dividing the floor into 16 (7×7 cm) equal squares. The animals were divided into six groups ($n$ = 5). Group 1 was given the vehicle (10 mL/kg distilled water), while group 2–5 received EOOG (5, 10, 20, and 40 mg/kg, i.p), respectively and group 6 received Diazepam (2 mg/kg). Thirty minutes after a single intraperitoneal (i.p.) injection of EOOG or standard drug, the animals were placed singly in the center of the box; the number of squares crossed with all four paws was counted for 5 min. The cage was cleaned with 70% ethanol at a 5-minute interval when the animal was removed [11].

*Y-maze test*

The animals were divided into six groups ($n$ = 5). Group 1 was given distilled water (10 mL/kg, *i.p*), group 2 - 5 received EOOG (5, 10, 20, and 40 mg/kg, *i.p*) respectively, and group 6 received diazepam (2 mg/kg), all administered 30 minutes before the observation. Each mouse was placed in one of the arm compartments usually arm A for consistency and was allowed to move freely through the maze, and the series of arm entries were recorded for 5 min. An arm entry is defined as the body of a mouse (except for its tail) completely entering into an arm compartment and the sequence of arm entries is manually recorded. An alternation is defined as an entry into all three arms on consecutive devices. The alternation behavior was defined by the successful entry into the three arms, an overlapping triplet sets, and such behaviour (in percentage) was expressed as the ratio of actual alternations to possible alternation (defined as the total number of arm entries minus two) multiplied by 100. Seventy percent (70%) ethanol was used to clean the Y-maze at intervals [12].

*Mechanistic Studies*

Mice were pre-treated (intraperitoneally) with neurotransmitter blockers 15 min before administration of EOOG (10 mg/kg i.p) to evaluate the possible mode of actions of the extracts on novelty-induced rearing behaviours in mice. The following transmitter receptor blockers were used: Atropine (muscarinic blocker, 0.5 mg/kg), Propanolol (non-selective β-adrenoceptor blocker, 0.2 mg/kg), Haloperidol (dopamine receptor blocker, 0.2 mg/kg), Cyproheptadine (Serotoninergic antagonist, 0.5 mg/kg) and Yohimbine ($\alpha_2$ -adrenergic blocker, 1 mg/kg) [30].

*Acetic Acid-Induced Writhing Test*

The method described by [14] was employed. Acetic acid (0.7%) was injected intra-peritoneally and the animal was observed for a specific contraction of the body referred to as writhing. A comparison of writhing was made between positive control (Diclofenac), control, and test sample, given orally 30 minutes before acetic acid injection. Each experimental group consisted of five mice. The animals were divided into five groups, Group 1 received distilled water (10 mL/kg, i.p), Group 2–4 received EOOG (2.5, 5, and 10 mg/kg, *i.p*) respectively while group 5 received Diclofenac (2 mg/kg).

*Hot Plate Latency Assay*

The method described by [15] as modified by [16] was used for this study. Animals were divided into five experimental groups consisting of five mice each: control (10 mL/kg distilled water, *i.p.*), EOOG (2.5, 5, and 10 mg/kg, *i.p.*), and pethidine (10 mg kg, *i.p.*). Animals were fasted for 12 hours with adequate clean water provided *ad libitum*. Each of the mice was placed on a hot plate maintained at the temperature of 55 ± 1 °C and the Pain Reaction Time (PRT) or latency period determined with a stopwatch was recorded which represents the time taken for the mice to react to the pain stimulus. The response to pain stimulus considered included; jumping, raising, and licking of the hind foot. The cut off time was fixed for 20 s.

## Results and Discussion

The Essential Oil of *Ocimum graissimum* (EOOG) leaves did not produce any sign of toxicity until the intra-peritoneal dose of 3000 mg/kg. The median lethal dose ($LD_{50}$) of EOOG in mice was found to be 2449 mg/kg (*i.p.*) body weight. The administration of EOOG (5–40 mg/kg, *i.p.*) reduced novelty induced rearing in mice. A significant [$F(5, 24) = 4.85$, $P < 0.001$] reduction in the frequency of rearing episodes was observed at doses 5 mg/kg and 10 mg/kg when compared to the vehicle (Fig. 1). However, EOOG doses showed significant differences ($P < 0.05$) in the depression of novelty induced rearing, compared to the reference drug (Diazepam, 2 mg/kg).

The administration of EOOG (5–40 mg/kg, *i.p.*) reduced novelty induced rearing in mice. A significant [$F(5, 24) = 4.051$, $P < 0.001$] reduction in the frequency of grooming episodes was observed at doses 5 mg/kg and 10 mg/kg when compared to the vehicle (Fig. 2). Also the studied plant (5–40 mg/kg, *i.p.*) showed a significant reduction [$F(5, 24) = 137.7$, $P < 0.001$] in the locomotor activity of mice at all doses when compared to vehicle (Fig. 3). The EOOG (5–40 mg/kg, i.p.) reduced significantly [$F(5, 24) = 5.71$, $P < 0.001$] entrance of mice in Y-maze which is an indication of a reduction in locomotor activity when compared to control.

The extract shows no percentage alteration at lower doses (5–20 mg/kg, *i.p.*) when compared to the control, hence, has no impairment effect on learning and memory (Fig.4). A significant [$F(5, 24) = 26.50$, $P < 0.001$] reduction in head dips was observed at

lower doses, however, a U-shaped pattern was observed with dose (10 mg/kg, *i.p.*) showing the highest significant reduction in head dips when compared to vehicle (Fig. 5). Pre-treatment with Propranolol, Yohimbine, and Cyproheptadine reversed the inhibitory effect of EOOG on novelty-induced rearing (Table 1). One-way analysis of variance (ANOVA) showed a significant difference [F (7, 34) = 115.0, $P < 0.0001$ (NIR)] and [F (6, 28) =133.5, $P < 0.0001$(NIG)] between EOOG + Antagonists and EOOG alone.

The antagonists alone showed significant ($P < 0.001$) difference in NIR and NIG as compared to EOOG (10 mg/kg), but when administered with EOOG (10 mg/kg after 15 minutes), only Propranolol showed significant ($P < 0.001$) increase in NIR, indicating a reversal of EOOG activity. Pre-treatment with Cyproheptadine and Yohimbine showed no significant effect on the activity of EOOG, but pre-treatment with Atropine and Haloperidol showed significant ($P < 0.001$) further depression activity. As revealed in Table 2, the vehicle-treated animals administered acetic acid showed marked writhing; however, the administration of EOOG 2.5–10 mg/kg significantly antagonized this symptom. At the same time, Diclofenac antagonized these effects.

The result revealed marked writhing count with vehicle-treated animals administered acetic acid; however, the administration of EOOG 2.5–10 mg/kg significantly antagonized the symptoms. As shown in Table 3, the administration of EOOG 2.5–10 mg/kg significantly [F (5, 24) = 12.85, $P < 0.0001$ (NIR)] protect against thermal stimulus compared to vehicle-treated animals. Pethidine also exhibited similar activity.

This work was undertaken to study the neurobehavioural and analgesic effect of essential oil of *Ocimum gratissimum* Linn. leaf in Swiss mice. Some of the neurobehaviour explored in this work are novelty-induced rearing, and grooming, locomotor activity in the open field, percentage correct alternations in the Y-maze, and head dips in the hole board. Mechanistic studies were also carried out to ascertain the pathway through which these effects are been elicited. Afterward, analgesic studies were carried out using the thermal hot plate, and acetic acid methods.

The acute toxicity of EOOG was established by the determination of $LD_{50}$. The $LD_{50}$ is the dose required to cause mortality in half the members of the tested population of the experimental animals. The higher the value of the $LD_{50}$ is for an extract, the relatively safe the extract is assumed to be. The median lethal dose ($LD_{50}$) of EOOG in mice was found to be 2449 mg/kg (i.p., body weight).

A compound is considered to have CNS activity if it crosses the blood-brain barrier and modulates the neurotransmitters in the CNS,

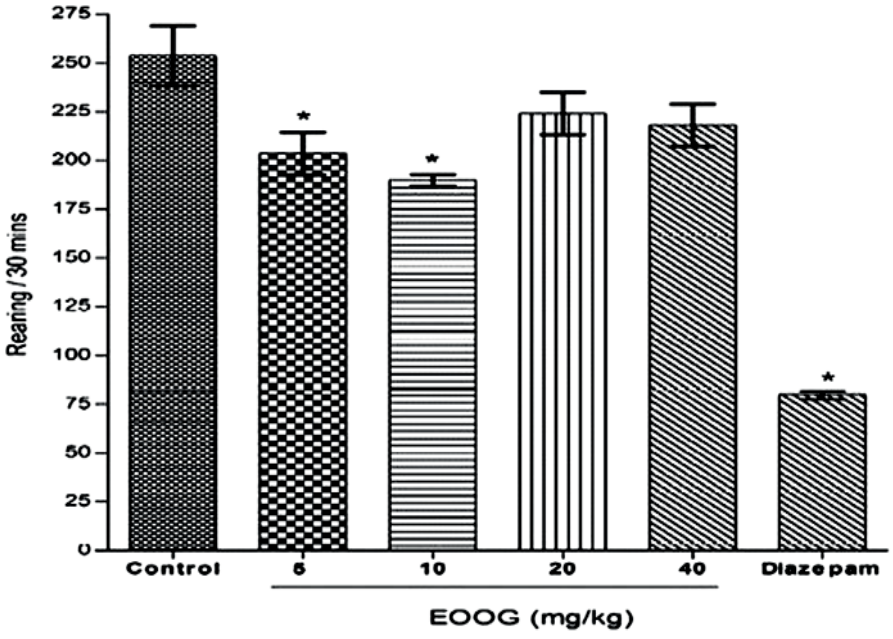


*Fig. 1.* **Effect of EOOG (Essential oil of *Ocimum gratissimum* Leaves) on Novelty-induced rearing behaviour in Swiss mice (*$P < 0.05$)**

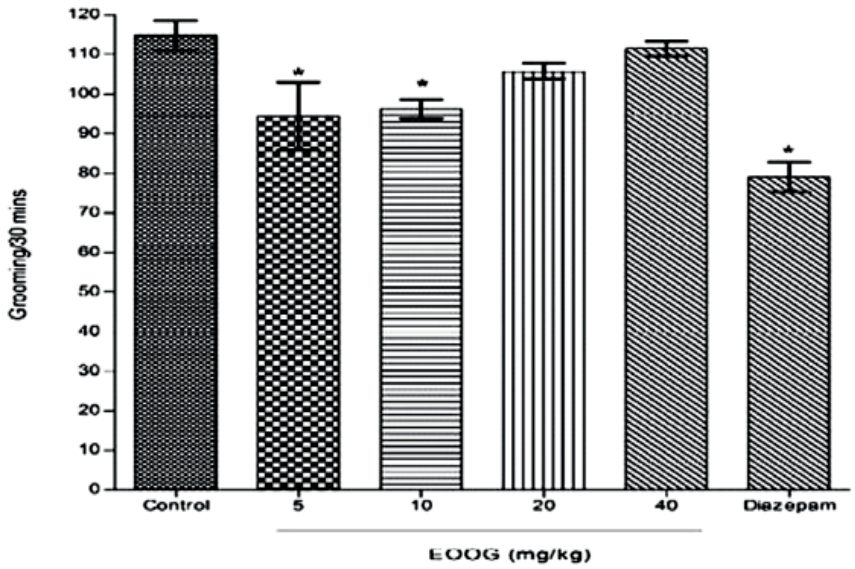


*Fig. 2.* **Effect of EOOG (Essential oil of *Ocimum gratissimum* Leaves) on Novelty-induced grooming behaviour in Swiss mice (*$P < 0.05$)**

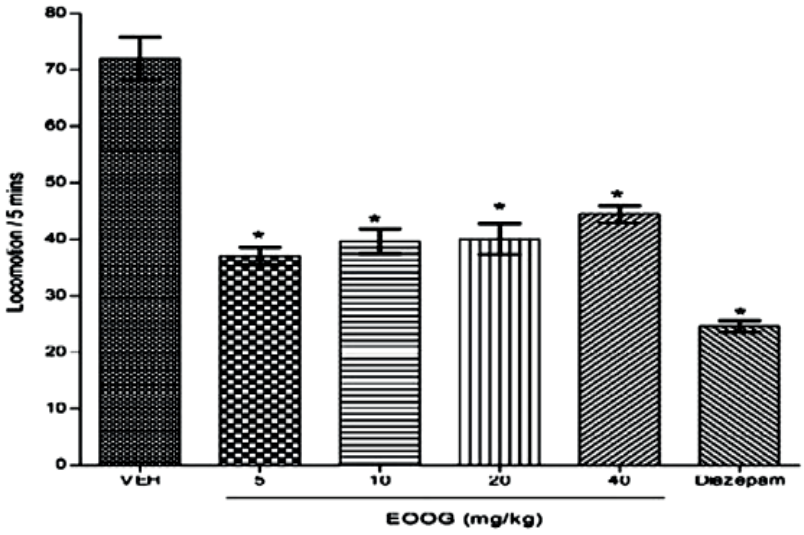


*Fig 3.* **Effect of EOOG (Essential oil of Ocimum gratissimum Leaves) on locomotor activity in the open field in Swiss mice (*$P < 0.001$)**

which eventually causes changes in behavioural expressions in the mice. Neurobehavioural effects of *Ocimum gratissimum* on Novelty-Induced Behaviours (NIB) (rearing, grooming, and locomotor activity) in mice were observed. Rearing is a vertical locomotor activity that involves an animal standing on its hind limbs while raising its forearms in the air or placed on the wall of the cage [8]. It is an indication of explorative behaviour which measures central nervous system excitation [16]. This behaviour has been used to classify test drugs/substances as sedatives or stimulants [17]. The results obtained showed that (EOOG) in 5 and 10 mg/kg inhibited/attenuated the novelty-induced rearing (NIR) behavior in a statistically significant manner comparable to the standard drug diazepam dose-dependently. The ability of an extract to inhibit rearing suggests its sedative effect. This is in agreement with [12],

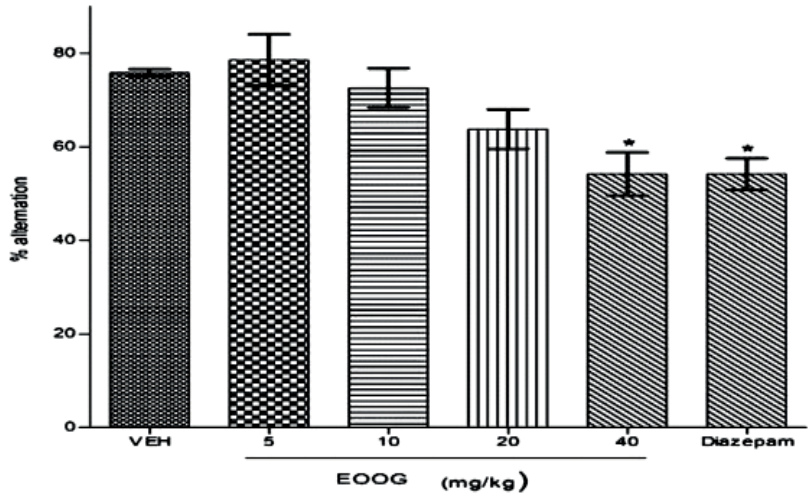


*Fig. 4.* **Effect of EOOG (Essential oil of *Ocimum gratissimum* Leaves) on learning and memory in the Y-maze in Swiss mice (*$P < 0.05$)**

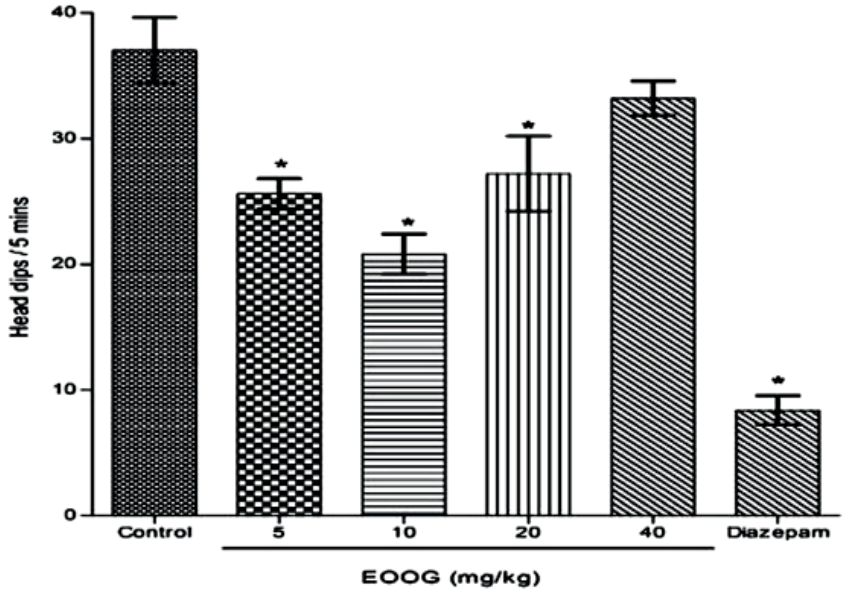


*Fig. 5.* **Effect of EOOG (Essential oil of *Ocimum gratissimum* leaves) on the frequency of head dips in Swiss mice (*$P < 0.05$)**

which reported that CNS stimulants increased rearing while [18], submitted that CNS depressants inhibited this behaviour.

Grooming is a "maintenance" behaviour that is specifically elicited in a situation in which an animal is in stress-induced frustration [10]. It is described as a face or head-important behavioural component in animals, and that plays a deactivating role in restoring homeostasis under stressful situation [19]. The inhibitory effect of an extract on Novelty-Induced Grooming (NIG) suggests its stress-attenuating role in a novel environment. The EOOG at 5 and 10 mg/kg also inhibited novelty-induced grooming behaviour significantly in a dose-dependent manner. At the highest doses (20 and 40 mg/kg), EOOG did not demonstrate any inhibitory activity of the extract compared to the standard drug, Diazepam. This inhibitory effect observed in both Novelty-Induced Rearing (NIR) and Novelty-Induced Grooming (NIG) behaviours suggest the EOOG possess a sedative or central nervous system depression activity at 5 and 10 mg/kg. This is in agreement with some plants that have shown to possess strong

*Table 1.* **Effects of Antagonists on Novelty-induced rearing and Novelty-induced grooming in Swiss mice**

| Treatment group | Rearing (NIR) | Grooming (NIG) |
|---|---|---|
| VEH (10ml/kg) | 225.80 ± 5.74 | 114.80 ± 3.72 |
| EOOG (10 mg/kg) | 189.80 ± 2.81 | 96.20 ± 2.39 |
| Haloperidol (0.2 mg/kg) | 107.06 ± 11.64 | 46.00 ± 0.70 |
| Haloperidol (0.2 mg/kg) + EOOG | 13.00 ± 3.08# | 23.80 ± 1.35# |
| Cyproheptadine (0.5 mg/kg) | 103.00 ± 3.97 | 17.20 ± 1.36 |
| Cyproheptadine (0.5 mg/kg) + EOOG | 171.20 ± 10.07 | 30.20 ± 2.05 |
| Yohimbine (1 mg/kg) | 127.20 ± 14.39 | 38.20 ± 7.94 |
| Yohimbine (1 mg/kg) + EOOG | 153.20 ± 8.68 | 71.60 ± 1.96 |
| Propranolol (0.2 mg/kg) | 96.00 ± 4.03 | 42.60 ± 5.41 |
| Propranolol (0.2 mg/kg) + EOOG | 222.00 ± 9.00** | 59.20 ± 6.33 |
| Atropine (0.5 mg/kg) | 128.80 ± 8.42 | 40.60 ± 2.56 |
| Atropine (0.5mg/kg) + EOOG | 77.20 ± 8.20# | 70.00 ± 2.30 |

$^{\#}P < 0.001$ (significant further depression), $^{**}P < 0.001$ (significant reversal) between EOOG + Antagonists and EOOG alone

*Key:* VEH= Vehicle; EOOG = Essential Oil of *Ocimum gratissimum*

*Table 2.* **Effect of Essential Oil of *Ocimum gratissimum* on Acetic Acid-induced Writhing in Swiss mice**

| Treatment ($n = 5$) | Dose (mg/kg) | Writhing Counts (per 30 min) | % inhibition |
|---|---|---|---|
| Control | 10 mL/Kg | 42.67 ± 7.49 | - |
| Diclofenac sodium | 2 mg/kg | 15.00 ± 2.91* | 64.84 |
| EOOG | 2.5 mg/kg | 21.67 ± 3.19* | 49.21 |
| | 5.0 mg/kg | 18.50 ± 1.69* | 56.64 |
| | 10.0 mg/kg | 16.67 ± 1.26* | 60.93 |

$^{*}P < 0.001$ as compared to vehicle control is significant.

*Key:* EOOG = Essential Oil of *Ocimum gratissimum*

sedative effects such as *Cissus quadrangularis, Spondias mombin, Ficus thoningii, Stachys lavandulifolia, Nigella sativa*, and *Cryptolepsis sanguinolenta* [20–25].

The exploratory activity test is a measure of exploratory behaviour in rodents [26, 27] and has been employed to screen sedative agents. 26 reported that reduction in the frequency of head dips depicts CNS depression. The method has been used also to measure anxiety and test anxiolytic agents. The exploratory test carried out in this study is based on assumption that the head-dipping of animals is inversely proportional to their anxiety state in a moderately aversive environment. Therefore, an increased number of head dips on the hole board means reduced anxiety state [28]. In more aversive situations, when the anxiety level of the animals is high, the holes may represent a possible way to escape from the aversive environment instead of an explorable object. The exploratory activity of animals treated with the EOOG was observed to decrease non-dose dependently with the highest dose (40 mg/kg) showing no activity on the hole board apparatus. In a moderate condition, anxiolytics increase the frequency of head poking, while sedative agents decrease the frequency of head poking. Since a decrease in the frequency of head dips in the hole board

*Table 3.* **Effect of Essential Oil of *Ocimum gratissimum* on Thermally-induced Pain in mice**

| Treatment ($n$ =5) | Dose (mg/kg) | Mean Latency of Response | % Protection against thermal stimulus |
|---|---|---|---|
| Control | 10 mL/Kg | 4.54 ± 0.34 | – |
| EOOG | 2.5 mg/kg | 6.00 ± 0.05* | 32.21 |
| | 5.0 mg/kg | 6.04 ± 0.21* | 33.00 |
| | 10.0 mg/kg | 6.34 ± 0.22* | 39.60 |
| Pethidine | 10.0 mg/kg | 21.74 ± 0.56* | 379.10 |

*$P < 0.001$ as compared to Vehicle treated mice.

by the EOOG was observed in all (but not 40 mg/kg) the doses used for this experiment, it is therefore suggested that EOOG has sedative properties; in accordance with (29).

Spontaneous motor activity is a parameter used to measure central nervous system excitability in animals. However, this behaviour (motor activity) has been shown to be mainly governed by motor area of frontal cortex, corpus striatum and brainstem. Any morphological changes or change in the level of brain amines in these areas is expected to cause neurotoxicity which may be in the form of motor deficit. Dopamine is one of the main amines found in the substantial nigra, ventral tegmental area, and hypothalamus of the brain, and has been implicated in the locomotor and exploratory activity. Drugs that enhance dopaminergic transmission produce increased locomotor activity (10), and are said to be stimulants, while agents that reduce dopaminergic transmission suppress locomotor activity. The EOOG produced a significant reduction in locomotor activity in all the doses in a non-dose dependent manner, but at the lowest dose, the reduction was more significant. This decrease in locomotor activity marked in the entire doses buttresses the fact that EOOG may be an index of the central nervous system depressant effect.

Spontaneous alternation is a behavioural test for measuring the willingness of rodents to explore new environments. Rodents typically prefer to explore a new arm of the Y-maze rather than returning to one that was previously visited. The EOOG at the three lower doses used for this experiment has no effect on working memory as the percentage alternation produced was not significantly different from the control. Conversely, at the highest dose (40 mg/kg), a statistically significant reduction in percentage correct alternation was marked, advocating the point that EOOG may have a memory impairing effect as the dose is increased.

In this study, the neural mechanism of action of the extract was investigated by interacting EOOG with the antagonists of the systems that regulate neurobehaviours in rodents. The administration (intraperitoneally, 15 minutes prior) of atropine and haloperidol did not reverse the inhibitory effect of the extract on rearing and grooming, rather propanolol, potentiated the effect of the EOOG, thus excluding involvement of cholinergic, dopaminergic, and α-adrenergic systems. In another experiment, pre-treatment with β-adrenergic and serotonergic antagonists reversed the inhibitory effect of the extract on the observed parameters. This shows that the EOOG may contain a compound(s) that has an affinity for β-adrenergic and serotonergic receptors, thus suggesting the participation of β-noradrenergic and serotonergic systems in the inhibitory effect of the EOOG on novelty-induced behaviours.

The thermally-induced pain (hot plate) test responses are believed to be spinally and supra-spinally mediated reflex and are to assess centrally acting analgesics. The effectiveness of analgesics agents in the hot plate pain model is highly correlated with the relief of human pains (30). In this investigation, EOOG significantly increased reaction time to pain stimuli at all the doses in the 30$^{th}$ minute compared to the normal control, similar to pethidine. These results are indicative that thermal stimulation is associated with central neurotransmission. The result further confirms the central analgesic effect of EOOG since the test is predominantly spinal reflex mediated and is considered to be selective for opioids like and centrally acting analgesics. Janssen et al. [31] and [32] submitted that peripheral analgesics are known to be inactive on thermal stimuli,

and the receptors are mostly implicated in central mediation of anti-nociceptive response are the opioid receptors.

In the acetic acid-induced abdominal constriction test, EOOG dose-dependently and significantly reduced the abdominal writhing level, which was comparable with the standard drug diclofenac sodium at 2 mg/ kg in this investigation. Inhibition of abdominal constrictions by EOOG is thus suggestive of its anti-nociceptive action, which may be due to inhibitory action against the synthesis and release of inflammatory mediators. Although this test of nociception has been successfully employed to screen peripheral acting analgesics, centrally acting analgesics (such as opioid agonists without peripheral action) have effectively attenuated nociception in this model. This phenomenon is attributed to a lack of specifics between peripheral and central pain effects [33]. Hence, the need for a model that can discriminate pains in the peripheral components from pains in the central components.

## Conclusion

The acute toxicity test showed that the essential oil of *Ocimum gratissimum* leaves could be utilized as documented in traditional use. The inhibitory effects demonstrated on neurobehavioural parameters suggest that essential oil possesses a sedative or central nervous system depressant activity. The mechanistic study indicates that the β-adrenoceptors and serotonergic systems may be responsible for the inhibitory effects observed.

The essential oil of *Ocimum gratissimum* produced a significant reduction in locomotor activity in all the doses in a non-dose dependent manner but at the lowest dose. In the Y-maze, oil did not produce any significant effect on working memory as the percentage alternation produced was not significantly different from the control. The oil in hot plate analgesic assay showed increased reaction time suggesting central nervous system analgesic property. In conclusion, the results of the investigation showed that essential oil of *Ocimum gratissimum* might possess sedative properties due to its ability to inhibit NIR and NIG, head dips, and locomotor activity. Furthermore, the inhibition of nociception marked in this research advocates antinociceptive activity which might be through the peripheral or central opioid receptor.

*Source of funding*

The research was carried out and sponsored by the researchers.

## REFERENCES

1. *Maliym, Barrett. The handbook of clinically tested herbal medicines CBS publishers, New Delhi, 1st edition* I. 2007, 232–255.
2. *Boursinos L. A., Karachalios T., Poultisides L., Malizos M.* Do steroids, conventional non-steroidal anti-inflammatory drugs, and selective Cox-2 inhibitors adversely affect fracture healing? *Journal of Musculoskeletal and Neuronal Interactions.* 2009, 9, 44–52. http://dx.doi.org/10.4172/2329-6836.1000255
3. *Sparkes A., Heiene R., Lascelles B. D., Malik R., Sampietro L. R.* NSAIDs and cats- it's been a long journey. *Journal of Feline Medicine and Surgery.* 2010, 12, 519–538.
4. *Richard F., Michelle A. C., Luigi X. C. Lippincott's Illustrated Reviews. Pharmacology. Lippincott Williams & Wilkins, Philadelphia, 4th edition*. 2008, P. 564.
5. *Nandan K., Pandey K.* An herbal formulation for psychiatric (*Nigella sativa*), *Phytopharmacology.* 2008, 9, 37–38.
6. *Castren E.* Is mood chemistry? *Nature Reviews Neuroscience.* 2005, 6,241–246.
7. *Prins M. L., Hales A., Reger M., Giza C. C, Hovda D. A.* Repeat traumatic brain injury in the juvenile rat is associated with increased axonal injury and cognitive impairments. *Dev Neurosci.* 2010, 32, 510–518. http://dx.doi.org/10.1159/000316800
8. *Onigbogi O., Ajayi A., Ukponmwan O.* Mechanisms of Chloroquine- Induced Body Scratching Behaviour in Rats: Evidence of involvement of endogenous opioid peptides. *Journal of Pharmacology Biochemistry and Behaviour.* 2000, 65, 333–337. https://doi.org/10.1016/S0091-3057(99)00221-X
9. *Dorr M., Joycee D., Porsolt R. D., Steinberg H., Summerfield A., Tomkiewiez M.* Persistence of dose-related behaviour in mice. *Nature.* 1971, 231, 121–123. http://dx.doi.org/10.1038/231121a0
10. *Akanmu M. A., Adeosun S. O., Ilesanmi O. R.* Neuropharmacological effects of oleamide in male and female mice. *Behavioural Brain Research.* 2007, 182, 88–94. http://dx.doi.org/10.1016/j.bbr.2007.05.006
11. *Akanmu M.A., Olowookere T.A., Atunwa S.A., Ibrahim B. O., Lamidi O. F., Adams P. A., Ajimuda B. O., Adeyemo L. E.* Neuropharmacological effects of Nigerian honey in mice.

*African Journal of Traditional Complementary Alternative Medicine*. 2011, 8, 230–249. http://dx.doi.org/10.4314/ajtcam.v8i3.65285
12. *Aderibigbe A., Agboola O.I.* Neuropharmacological profile of *Struchiums parganophora* Linn. in mice. *Asian Journal of Traditional Medicines*. 2011, 6(3), 104–111.
13. *Koster R., Anderson M., De Beer, E. J.* Acetic Acid for Analgesic Screening. *Federation Proceedings*. 1959, 18, 412–417. http://dx.doi.org/10.12691/ajbr-3-4-2
14. *Shetty S. N., Anika S. M. Laboratory manual of pharmacology and toxicology*. *Fourth Dimension Publishing co. Ltd, Enugu*. 1982, pp 30–37.
15. *Franzotti E. M., Santos C. V., Rodrigues H. M., Mour o R. H., Andrade M. R., Antoniolli A. R.* Anti-inflammatory, analgesic activity and acute toxicity of *Sida cordifolia* L. (Malva-Branca). *Journal of Ethnopharmacology*. 2000, 72(1-2), 273–277. http://dx.doi.org/10.1016/s0378-8741(00)00205-1
16. *Labella F. S., Punsky C., Havlicek V.* Morphine derivatives with diminished opiate receptor potency show enhanced control excitatory activity. *Brain Research*. 1979, 174, 263–271. http://dx.doi.org/10.1016/0006-8993(79)90849-7
17. *Blanchard D. C., Griebel G., Blanchard R. J.* Mouse defensive Behaviours: Pharmacological and Behavioural Assays for Anxiety and Panic. *Neuroscience and Behavioral Reviews*. 2001, 25, 205–218. http://dx.doi.org/10.1016/S0149-7634(01)00009-4
18. *Hellion-Ibarrola M. C., Ibarrola D. A., Montalbetti I., Villalba D., Heinichen O., Ferro E. A.* Acute toxicity and general pharmacological effect on the central nervous system of the crude rhizome extract of *Kyllinga brevifolia* Rottb. *Journal of Ethnopharmacology*. 1999, 66, 271–276. https://doi.org/10.1016/S0378-8741(99)00002-1
19. *Gispen W. H., Isaacson R. L.* ACTH-induced excessive grooming in the rat. *Pharmacology & Therapeutics*. 1981, 12, 209–246. doi: 10.1016/0163-7258(81)90081-4
20. *Al-Naggar T. B., Gomez M. P., Carretero M. E., Villar A. M.* Neuropharmacological activity of Nigella sativa L. extract. *Journal of Ethnopharmacology*. 2003, 88, 63–68.
21. *Rabbani M., Sajjadi S. E., Zarett H. R.* The anxiolytic effect of *Stachys lavandulifolia* Vahl on the elevated plus-maze model in mice. *Journal of Ethnopharmacology*. 2003, 89, 271–276. https://doi.org/10.1016/J.JEP.2003.09.008
22. *Viswanatha A., Thippeswamy A., Manjula D., Mehendra K.* Some neuropharmacological effects of the methanolic root extract of *Cissus quadrangularis* in mice. *African Journal of Biomedical Research*. 2006, 9, 69–75. https://doi.org/10.4314/ajbr.v9i2.48777
23. *Musa A., Yaro A., Danjuma, N.* Preliminary phytochemical screening and central nervous system depressant activity of the stem bark of *Ficus thoningii* blume. *Biological and Environmental Science Journal for the Tropics*. 2006, 3, 1–6. https://doi.org/10.21203/rs.3.rs-512523/v1
24. *Ayoka A. O., Akomolafe R. O., Iwalewa E. O., Akanmu M. A., Ukponwan O. E.* Sedative, antiepileptic, and antipsychotic effect of *Spondias mombin* L. (Anacardiaceae) in mice and rats. *Journal of Ethnopharmacology*. 2006, 103, 166–175. https://doi.org/10.1016/j.jep.2005.07.019
25. *Ansah C., Mfoafo E. A. Woode E., Duwiejua M.* Anxiogenic effect of an aqueous extract of *Cryptolepiss anquinolenta*. (Periplocaceae) in mice. *International Journal of Pharmacology*. 2008, 4, 20–26.
26. *File S. E., Wardill A. G.* Validity of head-dipping as a measure of exploration in a modified hole-board. *Psychopharmacology*. 1975, 44, 53–59. https://doi.org/10.1007/BF00421184
27. *Crawley J. N.* Exploratory behaviour models of anxiety in mice. *Neuroscience & Biobehavioural Reviews*. 1985, 9, 37–44. https://doi.org/10.1016/0149-7634(85)90030-2
28. *Bilkei-Gorzo A., Gyertyan I.* Some doubts about the basic concept of the hole board test. *Neurobiology* (Bp). 1996, 4, 405-415. PMID: 9200132.
29. *Aderibigbe A. O., Iwalewa E. O., Adesina S. K., Agboola I. O.* Studies of Behavioural and Neural Mechanisms of Aridanin Isolated from *Tetrapleura tetraptera* in Mice. *International Journal of Pharmacology*. 2010, 6(4), 480–486. https://dx.doi.org/10.3923/ijp.2010.480.486
30. *Dharmasiri M. G., Jayakody J. R., Galhena G., Liyanage S. S., Ratnasooriya W. D.* Anti-inflammatory and analgesic activities of mature fresh leaves of *Vitex negundo*. *Journal of Ethnopharmacology*. 2003, 87(2-3), 199–206. https://doi.org/10.1016/S0378-8741% 2803% 2900159-4
31. *Janssen P. A. J., Niemegeers C. J. E., Dony J. G. H.* The inhibitory effect of fentanyl and other morphine-like analgesics on the warm water induced tail withdrawal reflex in rats. *Arzneimittelforschung. Drug Research*. 1963, 13, 502–507. https://doi.org/10.4236/pp.2011.23019
32. *Srinivasan K., Shanmugam M., Lal J., Chandra S., Tandan S., Raviprakash V., Kumar D.* Anti-nociceptive and antipyretic activities of *Pongamia glabra* leaves. *Phytotherapy Research*. 2003, 17, 259–264. http://dx.doi.org/10.1155/2012/576086

33. *S nchez-Mateo C. C., Bonkanka C. X., Hern ndez-P rez M., Rabanal R. M.* Evaluation of the analgesic and topical anti-inflammatory effects of *Hypericum reflexum* L. fil. *Journal of Ethnopharmacology*. 2006, 107(1), 1–6. https://doi.org/10.1016/j.jep.2006.01.032

## НЕЙРОПОВЕДІНКОВИЙ І ЗНЕБОЛЮЮЧИЙ ЕФЕКТ *Ocimum gratissimum* Linn. ЕФІРНА ОЛІЯ ЛИСТЯ У МИШЕЙ Wistar albino

*Onosetale E. Aigbomian*[1], *Comfort T. Senjobi*[2], *S. A. Onasanwo*[3], *M. O. Olatunde*[4], *Taiye R. Fasola*[1]

[1]Department of Botany, University of Ibadan, Nigeria
[2]Department of Plant Science, Olabisi Onabanjo University, Ago-Iwoye, Nigeria
[3]Department of Physiology, University of Ibadan, Nigeria
[4]Department of Pharmacology and Therapeutics, University of Ibadan, Nigeria

*E-mail: fasolatr@gmail.com*

Дослідження показали, що знеболюючі препарати можуть бути нейропротекторними. *Ocimum gratissimum* Linn. який широко використовується в традиційній медицині для лікування слабкості та багатьох інших нейрофармакологічно пов'язаних захворювань, до кінця не вивчений.

*Мета*. Це дослідження було розроблено з метою вивчення безпечності споживання, нейроповедінкових і знеболювальних ефектів ефірної олії листя *Ocimum gratissimum* Linn (EOOG) у мишей.

*Методи*. Гостру токсичність EOOG визначали за стандартним методом, тоді як нейроповедінкові властивості оцінювали за допомогою відкритого поля для виховання, викликаного новизною (NIR), догляду, викликаного новизною (NIG) і рухової активності мишей. Для визначення частоти падіння голови використовувався апарат hole board. Y-подібний лабіринт використовувався для короткочасної пам'яті. Механічні дослідження проводили з атропіном (мускариновий блокатор, 0,5 мг/кг), пропанололом (неселективний блокатор β-адренорецепторів, 0,2 мг/кг), галоперидолом (блокатор дофамінових рецепторів, 0,2 мг/кг), ципрогептадином (серотонінергічний антагоніст, 0,5 мг/кг). і йохімбін (α-2-адреноблокатор, 1 мг/кг). Анальгетичну активність *Ocimum gratissimum* досліджували за допомогою тесту на звивання з оцтовою кислотою та термічного болю.

*Результати*. Середня летальна доза ($LD_{50}$) *Ocimum gratissimum* становила 2449 мг/кг. EOOG значно зменшив поведінку, спричинену новизною, залежно від дози. Спостерігалося, що дослідницька активність тварин, які отримували EOOG, зменшувалася незалежно від дози, причому найвища доза (40 мг/кг) не виявляла активності на апараті з отворами. EOOG викликав значне зниження рухової активності у всіх дозах незалежно від дози, але при найнижчій дозі. У Y-лабіринті EOOG не справляв жодного суттєвого впливу на робочу пам'ять, оскільки вироблена зміна у відсотках істотно не відрізнялася від контролю. EOOG в анальгетичному аналізі на гарячій пластині показав збільшення часу реакції, що свідчить про аналгетичну властивість центральної нервової системи.

*Висновки*. Результати дослідження показали, що EOOG може мати седативні властивості завдяки своїй здатності пригнічувати NIR та NIG, падіння голови та рухову активність. Крім того, інгібування ноцицепції, відзначене в цьому дослідженні, свідчить про антиноцицептивну активність, яка може здійснюватися через периферичний або центральний опіоїдний рецептор.

***Ключові слова:*** *Ocimum gratissimum*; нейропротектор; болезаспокійливий; лікарські рослини.

UDC 616.72-002-074::616.153:[616.153.96:577.175.4]
https://doi.org/10.15407/biotech15.05.058

# EFFICIENCY OF DETERMINATION OF ACUTE PHASE PROTEINS AND PROCALCITONIN UNDER THE CONDITIONS OF EXPERIMENTAL INFECTIOUS ARTHRITIS IN MICE

*S. Magomedov*[1]
*I.G. Litovka*[2]
*N.P. Hrystai*[1]
*V.I Sabadosh*[1]
*L.V. Polishchuk*[1]
*T.A. Kuzub*[1]

[1] Institute of Traumatology and Orthopedics of the National Academy of Sciences of Ukraine, Kyiv

[2]Bogomoletz Institute of Physiology of the National Academy of Sciences of Ukraine

*E-mail: alexander@magomedov.kiev.ua*

Received 24.08.2022
Revised 27.10.2022
Accepted 31.10.2022

Acute phase proteins, i.e. ceruloplasmin, haptoglobin, C-reactive protein (CRP) and procalcitonin are the markers that characterize the inflammatory process. C-reactive protein is one of the central components of the acute phase (AF), and is a generally accepted indicator of inflammatory processes.

*Aim.* To establish the level and the efficiency of determination of acute-phase proteins (CRP, haptoglobin, ceruloplasmin), as well as procalcitonin under the conditions of modeled infectious arthritis.

*Materials and methods.* Experimental studies were conducted on 52 white male Wistar rats. A model of infectious arthritis was created for seven days by daily injection of 0.02 ml of *S. aureus* 108 No. 209 into the knee joint of a rat. The animals were divided into groups and vivarium control (group I). The following dosage scheme was used for the experimental groups: a single daily injection of 0.02 ml of flosteron into the knee joint for seven days (group II); daily single administration for seven days of 0.02 ml of S. aureus 108 No. 209 (III group); daily one-time intermittent (every other day) administration for seven days of 0.02 ml of flosteron and 0.02 ml of *S. aureus* 108 No. 209 into the knee joint (group IV). The effectiveness of the drugs was observed 3 and 14 days after administration.

*Results.* It was established that the concentration of haptoglobin was significantly increased in the blood serum of rats after 3 and 14 days in all studied groups of animals compared to the control. The greatest increase compared to control values was noted 3 days after the seven-time injection of *S. aureus* 108 #209 into the knee joint. However, after 14 days it was already not so evident, and was significantly lower (by 85.33%) compared to the measurement after three days. Only the rats receiving intermittent (every other day) injections of 0.02 ml of flosteron and 0.02 ml of *S. aureus* 108 No. 209 into the knee joint demonstrated a statistically significant increase in the level of haptoglobin (by 775.08%, $P < 0.05$) compared to the control, and 77.78% reduced compared to the measurement after three days. The concentration of ceruloplasmin in blood serum increased in all experimental rats during the entire observation period and differed little between 3 and 14 days. The content of C-reactive protein in blood serum increased in all studied groups of rats without exception, which proves its high specificity for detecting inflammatory processes of various severity. The concentration of procalcitonin was most significantly increased by 235.0% 3 days after alternating (every other day) administration of 0.02 ml of flosterone and 0.02 ml of *S. aureus* 108 No. 209. It was slightly lower (by 120.0%) under the same conditions experiment after 14 days. This indicator significantly increased (by 65%) 14 days after the 7-time introduction of *S. aureus* 108 #209. In the experimental animals of other groups, the PCT concentration did not change.

*Conclusions.* The determination of haptoglobin reflects the initial activation of the inflammatory process, which was enhanced by the hormonal drug flosteron. However, its determination over a longer period of time can be efficient as well, because numerous factors lead to a bacterial infection, reinforcing each other. At the same time, the synthesis of ceruloplasmin is being activated exactly during the first three days of the infectious process, which makes it an informative marker for detecting early infectious complications. The dynamics of changes in the level of C-reactive protein in blood serum showed the highest correlation with the activity of the infectious process, which proves its high efficiency for detecting inflammatory processes of various severities, choosing adequate treatment and predicting the course of the disease.

***Key words:*** haptoglobin; ceruloplasmin; C-reactive protein; procalcitonin; infectious arthritis.

An important role in the pathogenesis of inflammatory diseases of the joints is attributable to the changes in biochemical indicators, namely, the increase of acute-phase proteins content in blood. There is a direct relationship between the change in their level, the severity and dynamics of clinical manifestations of inflammation. In this way, they have advantage over the determination of the number of leukocytes, platelets, leukocyte formula, leukocyte intoxication index, ESR. Although the effectiveness of modern microbiological tests is highly specific, the time required to obtain results may not be acceptable. However early diagnosis of inflammatory joint diseases is vital to prevent devastating complications.

Acute-phase proteins, i.e. ceruloplasmin, haptoglobin, C-reactive protein (CRP) are markers that characterize the inflammatory process and show a high correlation with disease activity. C-reactive protein is one of the central components of the acute phase (AF) and is a generally recognized indicator of inflammatory processes. An increase in the concentration of CRP precedes the increase in ESR and the increase in the number of peripheral blood neutrophils in bacterial infections. Its content increases rapidly in the first 6–8 hours (by 20–100 times, sometimes by 1000 times) when inflammation increases and also decreases quickly with amelioration of the inflammation. That is why this indicator is one of the most specific and sensitive clinical and laboratory indicators of inflammation, and it is widely used to monitor and control the effectiveness of therapy for bacterial and viral infections, chronic inflammatory diseases.

Ceruloplasmin is intensively synthesized during the first two days from the beginning of the infectious process. Its level nearly doubles in response to inflammation, injury, or infection. Haptoglobin is an acute phase protein that binds free hemoglobin and has anti-inflammatory properties but can have a pro-inflammatory effect on the joint. It plays a role in the inflammatory process of bone destruction through bradykinin and thrombin, stimulating the formation of prostaglandin E2, which leads to bone resorption [1, 2].

Procalcitonin protein (PCT) is an important biochemical marker for the diagnosis of inflammatory processes in the joints. This indicator makes it possible to assess the degree of development of the inflammatory process and sepsis and distinguish bacterial infection from non-bacterial infection [3–5]. In the presence of viral infections, the concentration of PCT demonstrate only minor increase, while in the presence of a significant bacterial infection, it increases. In patients without infectious complications, the PCT level quickly returns to normal values. Moreover, a high concentration that does not decrease, or a secondary increase in PCT are considered to be the predictors of sepsis [6]. The synthesis of PCT is induced by endotoxins – bacterial toxic substances, which are structural components of typical bacteria and are released only during lysis, that is, during the breakdown of a bacterial cell [7–9].

There is no doubt that acute-phase proteins and PKT take a direct part in the development of the inflammatory process [1, 2, 6, 8]. An increase in their level in the blood serum of patients, in most cases, indicates the destruction of the cartilage and bone tissue of the joints [10, 11]. However, the inconsistency of biochemical data in certain inflammatory joint diseases and their treatment protocols, as well as the lack of comparability of results complicate both the diagnosis of the disease and the effectiveness of further treatment. There is currently no consensus as to which biochemical markers are most reliable for tracking the level of inflammation and/or infection. Therefore, studies on monitoring the level of acute-phase proteins ceruloplasmin, haptoglobin, C-reactive protein and procalcitonin in order to determine the most effecient marker of them are relevant for the detection of early infectious complications and strategies for its elimination.

The aim of the study was to evaluate the level and efficiency of determination of acute-phase proteins (CRP, haptoglobin, ceruloplasmin), as well as procalcitonin under the conditions of modeling infectious arthritis.

## Materials and Methods

The experiment was conducted on 52 white male Wistar rats. An infectious arthritis model was created for seven days by daily injection (once a day) of 0.02 ml of *S. aureus* 108 No. 209 into the knee joint of a rat. The animals were divided into experimental groups — and vivarium control. The following drug administration model was used for the experimental groups: daily administration (once a day) for seven days of 0.02 ml of flosterone into the knee joint (II group); daily administration (once a day) for seven days of 0.02 ml of *S. aureus* 108 No. 209 (III group); daily administration (once a day) alternately (every other day) for seven days of 0.02 ml

of flosterone and 0.02 ml of *S. aureus* 108 No. 209 into the knee joint (IV group). Animals were decapitated under ether anesthesia after 3 and 14 days of the experiment after drug administration. All animals were under the supervision of a veterinarian in the standard conditions of the accredited vivarium of the Bohomolets Institute of Physiology of the National Academy of Sciences of Ukraine, in compliance with the general principles of bioethics in accordance with the international principles of the European Convention on the Protection of Vertebrate Animals Used for Research and Other Scientific Purposes (Strasbourg, 1986) under the natural light/dark cycle. Animals had free access to water.

Determination of haptoglobin, ceruloplasmin and CRP was carried out on a Cobas 311 biochemical analyzer using Roche Diagnostics test systems. The concentration of procalcitonin (PCT) was determined on a Cobas 411 analyzer using Roche Diagnostics test systems. Statistical processing of the obtained results was carried out using the Origin Pro 8.5 program package. The average values of the obtained indicators (x) with standard deviations (SD) were determined. The probability of difference between the control and experimental samples was estimated by the Student's t test. At $P < 0.05$, changes were considered significant.

## Results and Discussion

The analysis of the results of changes in the concentration of haptoglobin in the blood serum of rats showed its statistically significant increase in relation to the control values after three doses in all experimental groups by 226.18% (II group), 801.85% (III group) and 172.24% (IV group), respectively. The concentration was the highest after three days under the conditions of 7-time injection of 0.02 ml of *S. aureus* 108 No. 209 into the knee joint. Compared with rats of II and IV groups, the increase was 177.46% and 231.28%, respectively (Table 1 ).

Under the same experimental conditions, after 14 days only in rats after alternating (every other day) injection of 0.02 ml of flosterone and 0.02 ml of *S. aureus* 108 No. 209 into the knee joint (group IV), demonstrated the greatest increase in the level of haptoglobin by 775, 08% ($P < 0.05$) compared to the control. Whereas in rats of the II group, its content remained almost at the same level as after the measurement three days later. The concentration of haptoglobin in blood serum of animals of the III group was higher than the control values by 117.67%. However, it was significantly lower (by 85.33%) compared to the measurement the third day of administration (Table 2).

The concentration of ceruloplasmin in the blood serum of rats of the II group compared to the control increased almost equally by 77.27% and 81.82%, respectively ($P < 0.001$) 3 and 14 days after the last administration of flosteron. It increased by 286.36% and 122.73% after 3 and 14 days after 7-time injection of 0.02 ml of *S. aureus* 108 No. 209 into the knee joint. Whereas three days after alternating (every other day) administration of 0.02 ml of flosterone and 0.02 ml of *S. aureus* 108 No. 209 increased by 613.64% and even more after 14 days – by 640.91% after the last administration 0.02 ml of *S. aureus* 108 No. 209 (Table 1, 2).

The highest concentration of C-reactive protein was 3 and 14 days after the introduction of 0.02 ml of *S.aureus* 108 No. 209 in rats of the III group. After 3 days, it differed from the control values by 2363.04% and 1890.91% after 14 days . These values were somewhat lower in animals of the II group 3 and 14 days after administration of 0.02 ml of flosteron 1118.18% and 1163.04%, respectively compared to the control. After 3 and 14 days of alternating (every other day) administration of 0.02 ml of flosterone and 0.02 ml of *S.aureus* 108 No. 209, the level of C-reactive protein was 1754.55% and 1727.27%, respectively, of the control values (Table. 1, 2).

The largest increase (by 235.0%) in the PCT level occurred 3 days after alternating (every other day) administration of 0.02 ml of flosteron and 0.02 ml of *S.aureus* 108 No. 209. It was slightly lower (by 120.0%) after 14 days for the same conditions of the experiment. This indicator probably increased by 65% 14 days after the 7-time administration of *S.aureus* 108 No. 209 (Tables 1, 2). In other experimental groups, the PCT concentration in animals did not change.

It follows from the above data that the concentration of haptoglobin probably significantly increased in the blood serum of rats both after 3 and 14 days in all studied groups of animals compared to the control. The greatest increase relative to the control values was noted 3 days after the seventh injection of *S. aureus* 108 No. 209 into the knee joint. However, after 14 days it was already not so significant and significantly lower (by 85.33%) compared to the measurement after three days. Only in

*Table 1.* **The content of acute-phase proteins and procalcitonin in the blood serum of experimental animals with 7-fold local administration of the hormonal drug flosteron and *S. aureus*108 N 209**

| Indicators | Control $n = 10$ | Hormone (flosterone) | | Infection (*S. aureus*108 N 209) | |
|---|---|---|---|---|---|
| | | 3 days, $n = 7$ | 14 days, $n = 7$ | 3 days, $n = 7$ | 14 days, $n = 7$ |
| Haptoglobin, g/l | 0.317±0.004 | 1.034±0.011 | 1.016±0.006** | 2.859±0.047 | 0.690±0.021** |
| Ceruloplasmin, g/l | 0.022±0.001 | 0.039±0.003** | 0.040±0.001** | 0.085±0.021 | 0.043±0.005 |
| C-reactive protein, ng/ml | 0.011±0,003 | 0.134±0.013 | 0.139±0.011** | 0.271±0.018 | 0.329±0.042* |
| Procalcitonin, ng/ml | 0.020±0.001 | 0.023±0.,001 | 0.020±0.001** | 0.019±0.002 | 0.033±0.005 |

*$P < 0,05$ : **$P < 0.001$.

*Table 2.* **The content of acute-phase proteins and procalcitonin in the blood serum of experimental animals after the 7th local administration of the hormonal drug flosteron+ *S. aureus*108 N 209**

| Indicators | Control $n = 10$ | Flosteron + *S. aureus* 108 N 209 | |
|---|---|---|---|
| | | 3 days, $n = 7$ | 14 days, $n = 7$ |
| Haptoglobin, g/l | 0.317±0.004 | 0.863±0.028** | 2.774±0.052 |
| Ceruloplasmin, g/l | 0.022±0.001 | 0.157±0.013** | 0.163±0.015 |
| C-reactive protein, ng/ml | 0.011±0,003 | 0.104±0.014 | 0.201±0.023 |
| Procalcitonin, ng/ml | 0.020±0.001 | 0.067±0.002 | 0.044±0.004 |

*$P < 0,05$ : **$P < 0.001$.

rats after a 14-day alternating (every other day) administration of 0.02 ml of flosterone and 0.02 ml of *S. aureus* 108 No. 209 into the knee joint, a probable increase in the level of haptoglobin by 775.08% ($P < 0.05$) was observed compared to the control and decreased by 77.78% compared to the measurement after three days. We believe that this indicator reflects, first of all, the primary activation of the inflammatory process, which was enhanced by the hormonal drug flosteron. Therefore, its determination can be effective over a longer period of time, as bacterial infections lead to several processes, reinforcing each other.

The concentration of ceruloplasmin in blood serum increased in all experimental rats during the entire observation period with minor differences between 3 and 14 days. Probably, the synthesis of ceruloplasmin increases precisely during the first three days of the infectious process, which leads to an increase in its content. The abruption of further increase of this indicator is most likely due to the depletion of the enzyme antioxidant reserve of the serum, of which ceruloplasmin is a component, due to its ability to bind free oxygen radicals and inactivate them.

Among all acute-phase proteins, the concentration of C-reactive protein in the blood demonstrated the highest increase in all studied groups of rats without exception, which proves its high efficiency in detecting inflammatory processes of various severity.

The concentration of procalcitonin in the conditions of our experiment significantly changed in the blood serum of rats 14 days after the introduction of *S. aureus* 108 #209 and after 3 and 14 days under the combined influence of flosterone and *S. aureus* 108 #209.

Therefore, with increasing bacterial infection, this indicator is more specific.

Thus, according to our data, the determination of haptoglobin reflects, first of all, the primary activation of the inflammatory process, which was enhanced by the hormonal drug flosteron. Therefore, its definition can be effective over a longer period of time, as typical, multiple factors lead to bacterial infection, reinforcing each other. At the same time, the synthesis of ceruloplasmin increases exactly during the first three days of the infectious process, which turns it into an effective marker for detecting early infectious complications. The dynamics of changes in the level of C-reactive protein in blood serum showed the highest correlation with the activity of the infectious process, which proves its high efficiency for detecting inflammatory processes of various severity, choosing adequate treatment and predicting the course of the disease.

*Prospects*

Further study of longer use of a hormonal drug (flosterone) is in an experiment.

The study was funded under the theme according the State registration No. 0119U003163.

*Conflict of interest*

The authors declare no conflict of interest during the preparation of the article.

## REFERENCES


1. *Zeller L., Tyrrell P. N., Wang S., Fischer N., Haas J.-P., H gle B.* α2-fraction and haptoglobin as biomarkers for disease activity in oligo- and polyarticular juvenile idiopathic arthritis. *Pediatr Rheumatol Online J.* 2022, 20(1), 66–73. https://doi.org/10.1186/s12969-022-00721-7
2. *Magomedov A. M., Gerasimenko S. I., Boychuk B. P., Krynytskaya O. F., Kravchenko E. N., Arshulyk M. A.* Metabolism of acute phase proteins in patients with trauma to the long bones of the lower extremities. *Visnik ortopedii, travmatologii ta protezuvannya*. 2014, (1), 24–28. (In Russian).
3. *Buhanova D. V., Belov B. S., Tarasova G. M., Dilbaryan A. G.* Procalcitonin test in rheumatology. *Klinitsist*. 2017, 11(2), 16–23. (In Russian).
4. *Lapin S. V., Maslyanskiy A. L, Lazareva N. M., Vasileva E. Yu., Totolyan A. A.* The value of quantitative determination of procalcitonin for the diagnosis of septic complications in patients with autoimmune rheumatic diseases. *Klinicheskaya laboratornaya diagnostika*. 2013, (1), 28–33. (In Russian).
5. *Tsujimoto K, Hata A, Fujita M, Hatachi S, Yagita M.* Presepsin and procalcitonin as biomarkers of systemic bacterial infection in patients with rheumatoidar thritis. *Int. J. Rheum. Dis*. 2018, Jul, 21(7), 1406–1413. https://doi.org/10.1111/1756-185X.12899.
6. *Shipitsyina I. V., Osipova E. V., Lyulin S. V., Sviridenko A. S.* Diagnostic value of procalcitonin in the post-traumatic period in patients with polytrauma. *Politravma*. 2018, (1), 47–59. (In Russian).
7. *Mahomedov S, Kravchenko O. M., Kolov H. B., Shevchuk A. V.* Procalcitonin as a biochemical marker in the diagnosis of inflammatory processes (literature review). *Visnyk ortop., travmat. ta protezuv*. 2018, (1), 63–67. (In Ukrainian).
8. *Fuchs T., Stange R., Schmidmaier G., Raschke M. J.* The use of gentamicin-coated nails in the tibia: preliminary results of a prospective study. *Arch. Orthop. Trauma Surg.* 2011, 131(10), 1419–1425.
9. *Wallbach M., Vasko R., Hoffmann S., Niewold T. B., M llerG.A., Korsten P.* Elevated procalcitonin levels in a severe lupus flare without infection. *Lupus* 2016, 25(14), 1625–1626. https://doi.org/10.1177/0961203316651746.
10. *Magomedov S., Chereshchuk I. A., Krynytskaya O. F. Kuzub T. A., Gerasimenko A. S., Polulyakh D. M.* Metabolism of acute phase proteins and their diagnostic value in patients with rheumatoid arthritis and ankylosing spondylitis. *Bulletin of orthopedics, traumatology, and prosthetics*. 2016, 2(2), 46–48. (in Ukrainian).
11. *Magomedov S., Kravchenko E. M., Kolov G. B., Shevchuk A. V.* Procalcitonin as a biochemical marker in the diagnosis of inflammatory processes (literature review) *Bulletin of Orthop., Traumat. and prosthetics*. 2018, 1, 63–67.
12. *Jevsevar D. S., Brown G. A., Jones D. L, Matzkin E. G., Manner P. A., Mooar P.* TheAmericanAcademyofOrthopaedic Surgeonsevidence-basedguidelineon: treatmentofosteoarthritisoftheknee, 2nd edition. *Journal of Bone and Joint Surgery-American*. 2013, 95(20), 1885–1886.
13. *Shen C. J., Wu M. S., Lin K. H., Lin W. L., Chen H. C., Wu J. Y.* The use of procalcitonin in the diagnosis of bone and joint infection: a systemic review and meta-analysis. *Eur. J. Clin. Microbiol. Infect. Dis*. 2013, 32(6), 807–814. https://doi.org/10.1007/s10096-012-1812-6.

# ЕФЕКТИВНІСТЬ ВИЗНАЧЕННЯ ГОСТРОФАЗНИХ ПРОТЕЇНІВ І ПРОКАЛЬЦИТОНІНА ЗА УМОВ МОДЕЛЮВАННЯ ІНФЕКЦІЙНОГО АРТРИТУ У МИШЕЙ

*С. Магомедов*[1], *І. Г Літовка*[2], *М. П. Грицай*[1], *В. І. Сабадош*[1], *Л. В. Поліщук*[1], *Т. А. Кузуб*[1]

[1]ДУ Інститут травматології та ортопедії НАМН України, Київ
[2]Інститут фізіології ім.О.О. Богомольця НАНУ, Київ

*E-mail: alexander@magomedov.kiev.ua*

Гострофазні протеїни церулоплазмін, гаптоглобін, С-реактивний протеїн (СРП) і прокальцитонінє маркерами, які характеризують запальний процес. С-реактивний протеїн — один із центральних компонентів гострої фази (ГФ) і є загальновизнаним показником запальних процесів.

*Мета*. Визначити рівень і ефективність визначення гострофазних протеїнів (СРП, гаптоглобіну, церулоплазміну), а також прокальцитоніну за умов моделювання інфекційного артриту.

*Матеріали і методи*. Експериментальні дослідження було проведено на 52 білих щурах-самцях лінії Вістар. Модель інфекційного артриту створювали протягом семи діб, щоденним уведенням 0,02 мл *S. aureus* 108 № 209 у колінний суглоб щура. Тварин було розподілено на групи — I віварний контроль. Для експериментальних груп було застосовано таку модель уведення препарату: щоденне одноразове введення протягом семи діб по 0,02 мл флостерону в колінний суглоб (II група); щоденне одноразове введення протягом семи діб по 0,02 мл *S. aureus* 108 № 209 (III група); щоденне одноразове поперемінне (через день) уведення протягом семи діб по 0,02 мл флостерону і 0,02 мл *S. aureus* 108 № 209 у колінний суглоб (IV група). Ефективність дії препаратів спостерігали через 3 і 14 діб після введення.

*Результати*. Встановлено, що концентрація гаптоглобіну вірогідно зросла у сироватці крові щурів як через 3, так і 14 діб у всіх досліджуваних групах тварин порівняно з контролем. Найбільше підвищення відносно контрольних значень спостерігалось через 3 доби після семиразового введення *S aureus* 108 №209 у колінний суглоб. Однак через 14 діб воно вже було не таким суттєвим і значно нижчим (на 85,33%) порівняно з вимірюванням через три доби. Лише у щурів після 14-добового поперемінного (через день) уведення по 0,02 мл флостерону та 0,02 мл *S. aureus* 108 № 209 у колінний суглоб спостерігали вірогідне зростання рівня гаптоглобіну на 775,08% ($P < 0,05$) порівняно з контролем і на 77,78% зниженим порівняно з вимірюванням через три доби. Концентрація церулоплазміну в сироватці крові зростала у всіх експериментальних щурів за весь період спостереження і мало відрізнялася між 3 і 14 добами. Вміст С-реактивного протеїну в сироватці крові зростав у всіх без винятку досліджуваних групах щурів, що доводить його високу специфічність для виявлення запальних процесів різної важкості. Концентрація прокальцитоніну найбільше — на 235,0% вірогідно підвищувалася через 3 доби після поперемінного (через день) уведення по 0,02 мл флостерону та 0,02 мл *S. aureus* 108 № 209. Дещо нижче на 120,0% за тих самих умов експерименту через 14 діб. На 65% цей показник вірогідно зріс через 14 діб після 7-разового введення *S. aureus* 108 №209. У решти експериментальних тварин концентрація ПКТ не змінювалася.

*Висновки*. Визначення гаптоглобіну відображає, перш за все, первинну активацію запального процесу, посиленню якої сприяв гормональний препарат флостерон. Проте його визначення може бути ефективним через більш тривалий термін, оскільки до бактеріальної інфекції призводять декілька чинників, посилюючи один одного. В одночас синтез церулоплазміну посилюється саме впродовж перших трьох діб інфекційного процесу, що перетворює його у результативний маркер для виявлення раннього інфекційного ускладнення. Динаміка змін рівня С-реактивного протеїну в сироватці крові продемонструвала найвищу кореляцію з активністю інфекційного процесу, що доводить його високу ефективність для виявлення запальних процесів різної важкості, вибору адекватного лікування та прогнозу перебігу захворювання.

***Ключові слова:*** гаптоглобін; церулоплазмін; С-реактивній протеїн; прокальцитонін; інфекційний артрит.

UDC 674.032.477.62 https://doi.org/10.15407/biotech15.05.064

# PHYTOCHEMICAL CONSTITUENTS AND ANTILEUKEMIC EFFECTS OF *Juniperus oxycedrus* EXTRACT

*Berfin Tugba Turak*[1, 2]
*Deniz İrem Acar*[2]
*Seda Kusoglu Gultekin*[2]
*İrem Gulfem Albayrak*[2]
*Emine Akalin*[3]
*Belkıs Atasever Arslan*[2]

[1]Department of Bioengineering, Faculty of Engineering and Natural Sciences, Üsküdar University, Istanbul, Turkey
[2]Department of Molecular Biology and Genetics, Faculty of Engineering and Natural Sciences, Üsküdar University, Istanbul, Turkey
[3]Department of Pharmaceutical Botany, Faculty of Pharmacy, İstanbul University, İstanbul, Turkey

*E-mail: belkisatasever.arslan@uskudar.edu.tr*

Received 04.08.2022
Revised 07.10.2022
Accepted 31.10.2022

*Aim.* Many genetic and environmental factors can be effective in the process of cancerization. Preventing the progression of leukemia may be possible by controlling the pathways involving mechanisms such as apoptosis and autophagy. When the literature is examined, there are studies showing the effects of various types of juniper on various cancer cell lines, including human chronic myeloid leukemia cells, but the signal pathways in which they act are not fully known. In this study, the anticancer effects of *Juniperus oxycedrus* extract on K-562 human chronic myeloid leukemia cells were investigated.

*Method.* After the cells were treated with the *Juniperus oxycedrus* extract, cytotoxicity and gene expression analyzes were performed. Changes in the expression of Akt, the member of the PI3K/Akt/mTOR signaling pathway; caspase 3, which is one of the main effective genes in the pathways regulating apoptosis; and the apoptosis suppressor BCL-2 gene, which is an oncogene, were investigated.

*Results.* According to the MTT test results, *Juniperus oxycedrus* extract showed over approximately 50% cell viability in K-562 cells at all doses. The most appropriate dose of *Juniperus oxycedrus* fruit extract in this research was determined as 50 µg/mL considering cell viability. After the gene expression analysis, it was observed that BCL-2 expression decreased approximately 3.3 times, and caspase 3 expression increased 1.2 times. Although Akt gene expression increased 1.092 times, it was not statistically significant.

*Conclusions.* Constituents of *Juniperus oxycedrus* plant may have apoptotic effects on chronic myeloid leukemia cells.

***Key words:*** *Juniperus oxycedrus*; leukemia; apoptosis.

Chronic myeloid leukemia (CML) is a hematological disease that develops mainly as a result of a translocation and oncogenic fusion of BCR and ABL1 genes. The abnormal tyrosine kinase activity exhibited by this fusion protein causes activity changes in various signaling pathways with which it interacts. Although the developed tyrosine kinase inhibitors are standard therapeutics used in the treatment of CML, resistance to BCR-ABL tyrosine kinase inhibitors is a clinically unresolved problem [1, 2].

In the field of cancer biology, it is seen that there is an increasing interest in studies investigating the anticancer effects of compounds obtained from plants. It is known that the fruit and essential oils of the juniper tree were used in digestive system disorders in the Middle Ages, the *Juniperus oxycedrus* species was consumed in Turkey and used in different sectors such as medicine, cosmetics and food [3]. In the literature, there are studies examining the anti-inflammatory, antinociceptive, antibacterial and anticandidal effects of the extracts of the juniper plant, which has many species, prepared with various solvents [4, 5]. Huyan et al. stated that *Juniperus sabina* extract exerts an inhibitory

effect on HepG-2 and K-562 cells by reducing cell viability and inducing apoptosis through upregulation of the expression of apoptosis-related genes FasL, caspase 3 and caspase 9 [6].

The aim of the project is to determine the anticancer effects of methanolic extracts prepared from the fruit parts of *J. oxycedrus* plant collected from Niksar district of Tokat, Turkey, in cells where chronic myeloid leukemia can be modeled.

Within the scope of this study, phytochemical analyzes of *J. oxycedrus* plant extract, determination of cytotoxic dose range with MTT (3-(4,5 dimethylhiazol-2yl)-2,5-diphenyl tetrazolium bromide) in K-562 and ECV-304 cells, and gene expression analysis at determined doses were performed. ECV-304 cells were used as control group.

Changes in expression of Akt, BCL-2 and Caspase 3 genes selected as target genes were examined by Real-Time PCR. As a result of the studies, the anticancer effects of *J. oxycedrus* extract on chronic myeloid leukemia cells were investigated through signal pathways.

## Materials and Methods

*Preparation of Juniperus oxycedrus Extracts*

The plants were collected from Tokat Niksar Akıncı Village in October (Turkey). Identification of species was done by Prof. Dr. Emine Akalın Urusak from İstanbul University, Faculty of Pharmacy. The fruits of the plants were separated from each other and air-dried at room temperature. Fifteen grams of fruit were extracted twice with 150 mL of methanol separately. Extraction was carried out in a shaking hot water bath at 50 °C for 8 hours and then by maceration at room temperature for 16 hours. The amount of extract was determined by taking the first and last weighing of the balloon. The extract was stored at +4 °C to be used in the next experimental stages.

*Phytochemical Screening*

The *J. oxycedrus* extract was analysed for the presence of the phytochemicals. For the screening of alkaloids, saponins, tannins, glycosides, phenols, carbohydrates, proteins, and flavonoids; the method of Khuda et al. was used [7].

*Test for Alkaloids*

Hager's test is used for determination of alkaloid presence in the extract. In this test, 3–4 drops of Hager's reagent (picric acid) were added to 2 mL plant extract. The appearance of a yellow precipitate shows the presence of alkaloids.

*Test for Saponins*

In a test tube, 2 mL plant extract and distilled water was mixed and shaked vigorously for 5 minutes. The formation of a thick foam at the top of the tube reveals the presence of saponins.

*Test for Flavonoids*

Test for detection of flavonoids was performed by adding a few drops of ferric chloride to 2 mL plant extract. The appearance of blakish-red precipitates confirmes the presence of flavonoids.

*Test for Tannins*

Alkaline reagent test is used for determination of tannin presence in the extract. In this test, 2 mL of plant extract and 1N sodium hydroxide was mixed thoroughly. The formation of red or yellow precipitates shows the presence of tannins.

*Test for Glycosides*

Keller Killiani test is performed for determination of glycoside presence in the plant extract. This test was performed by addition of 1 mL of the extract and glacial acetic acid in a test tube. After cooling the mixture 2–3 drops of ferric chloride added to the tube. Finally, 0.5 mL sulfuric acid was added through the sides of the test tube. The formation OD reddish-brown ring at the junction of the two layers confirmed the presence of glycosides.

*Test for Carbohydrates*

Benedict's test was used for analysis of the carbohydrates in the extract. A few drops of Benedict's Reagent were mixed with the extract, followed by boiling. The formation of reddish-brown precipitates shows the presence of carbohydrates.

*Test for Proteins*

Xanthoproteic test was performed by the addition of few drops of concentrated nitric acid to about 1 mL of the extract. The appearance of yellow color revealed the presence of proteins in the extract.

*Mammalian Cell Culture and Cell Viability Analysis*

K-562 (ATCC® CCL-243™) and ECV-304 cells were passaged as suggested by Atasever-Arslan, 2016 [8]. MTT test was performed

for cell viability analysis. *J. oxycedrus* stock extracts were prepared with methanol at concentrations of 50, 20, 10 and 5 µg/mL and made ready for MTT assay. Cells were treated with the extracts for 48 hours. ECV-304 cells incubated with the medium were used as the control group [9]. The cytotoxicity index was calculated by comparing the cytotoxicity values of the extracts with the cytotoxicity values of the control group.

*RNA Isolation*

Total RNA was isolated from K-562 cells treated with the substance at the concentration determined by the cytotoxicity test for 24 hours, with BIO BASIC Total RNA Miniprep as recommended by the manufacturer.

*c-DNA Synthesis and Real-Time PCR*

cDNA synthesis from total RNA was performed with OneScript cDNA Synthesis Kit according to the manufacturers protocol. After incubation of K-562 cell line with *J. oxycedrus* extract, changes in expression of Bcl-2, AKT, caspase 3 and β-actin genes were analyzed by Real-Time PCR (qPCR) method using BrightGreen 2X qPCR MasterMix. The primer sequences of the target genes are shown in Table 1. β-actin was used as the reference gene. Gene expression changes were calculated using the 2-ΔΔCt method.

## Results and Discussions

*Phytochemical Screening Results*

The results obtained after the phytochemical evaluation of *J. oxycedrus* extract are shown in Table 2.

*Cytotoxicity Analysis Results*

The cytotoxic effect of *J. oxycedrus* microalgae fruit extract against K562 cells was investigated using the MTT cytotoxicity test. Non-cancer ECV304 cells were used as controls. It showed that 50 µg/mL concentration had the highest cytotoxic effect against K562 and ECV304 cells at a rate of 49±7.23% and 64±2.62 respectively. Other concentration of the extract (20, 10 and 5 µg/ mL) showed 28±1.26%, 24±3.26% and %0±2.83 cytotoxicity against K562 cells, respectively and 39±12.97%, 4±1.69% and 17±19.84%. The most appropriate dose of *J. oxycedrus* fruit extract for all experiments in this research was determined as 50 µg/mL considering cell viability (Fig. 1).

*Real-Time PCR Results*

After determining the appropriate dose (50 µg/mL) K-562 cells were incubated with the extract for 24 hours, RNA isolation was performed. cDNA was synthesized from the isolated total RNA. qPCR experiments were carried out using the obtained cDNA and specific primers. The primers used are BCL-2 and Caspase 3, which are involved in signaling pathways associated with apoptosis, and Akt, which is a member of the PI3K/Akt/mTOR pathway.

Akt is an important signaling molecule in tumor formation and progression. It is involved in cell proliferation and survival. A 1.092-fold change in gene expression was observed in K-562 cells incubated with *J. oxycedrus* fruit extract, close to control (Fig. 2).

BCL-2, the main regulator of the intrinsic pathway of apoptosis, is an antiapoptotic gene and is responsible for suppressing apoptosis. As a result of the experiments, it was observed that *J. oxycedrus* fruit extract decreased BCL-2 gene expression by 0.208 times in K-562 cells (Fig. 3).

Caspase 3 is one of the effector caspases located at the junction of the intrinsic and extrinsic apoptotic signaling pathways. It plays a role in apoptosis by being induced by

*Table 1*. **Primer Sequences of the Target Genes**

| Target Gene | Primer Sequence |
|---|---|
| Bcl-2 | Forward: 5′-GGTGGGGTCATGTGTGTGG-3′<br>Reverse: 5′-CGGTTCAGGTACTCAGTCATCC-3′ |
| AKT1 | Forward: 5′-AGCTCAGCCCACCCTTCAA-3′<br>Reverse: 5′-GCTGTCCACACACTCCATGCT-3′ |
| Caspase 3 | Forward: 5′-CAAACTTTTTCAGAGGGGATCG-3′<br>Reverse: 5′-GCATACTGTTTCAGCATGGCAC-3′ |
| β-aktin | Forward: 5′-GGACATCCGCAAAGACCTGTA-3′<br>Reverse: 5′-ACATCTGCTGGAAGGTGGACA-3′ |

*Table 2.* **Phytochemical Screening Test Results of *Juniperus oxycedrus* extract**

| Phyto-chemicals | Chemical Tests | Results |
|---|---|---|
| | | ***Juniperus oxycedrus*** |
| Alkaloids | Hager's test | – |
| Saponins | Foam test | – |
| Flavonoids | General Test | + |
| Tannins | Alkaline Reagent Test | + |
| Glycosides | Keller Killiani Test | + |
| Carbo-hydrates | Benedict's Test | +** |
| Proteins | Xanthoproteic Test | N |

** Green (0,1–0,5 % sugar), N: Not detected

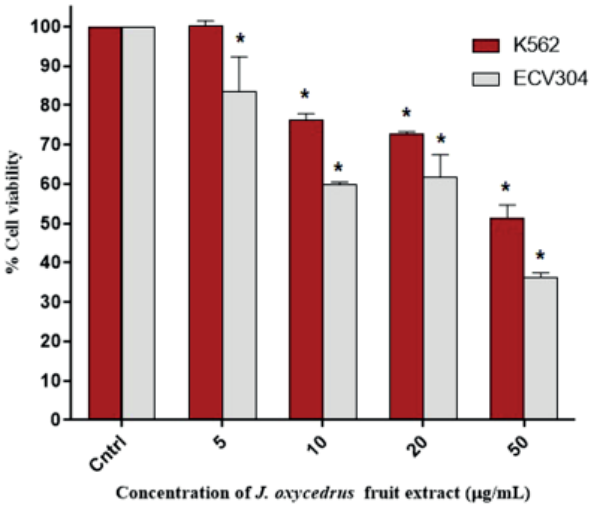


*Fig. 1.* **Cytotoxic effects of *J. oxycedrus* fruit extract against K562 and ECV304 cells**
The control was accepted as 100% and considered as baseline, then the amount of decrease in cell viability was shown at 5, 10,20, and 50 µg/mL, respectively (* $P < 0.05$)

various members of the BCL-2 family. While *J. oxycedrus* fruit extract decreased BCL-2 expression, a 1.218-fold increase in Caspase 3 expression was observed (Fig. 4).

Acute myeloid leukemia is the most common among adult leukemia and has the lowest survival rate. Genetic mutations are found in 97% of cases. Although the cause that induces the mutations is unclear, exposure to carcinogenic agents are risk factors [10].

In chronic myeloid leukemia, oncogenic fusion of BCR-ABL1 genes occurs as a result of translocation between chromosomes 9 and 22. The abnormal tyrosine kinase activity

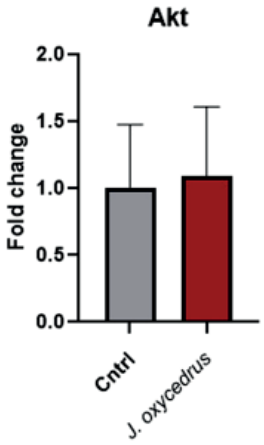


*Fig. 2.* **Effect of *J. oxycedrus* fruit extract on Akt gene expression**
(* $P > 0.05$)

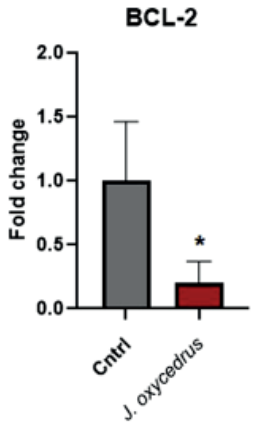


*Fig. 3.* **Effect of *J. oxycedrus* fruit extract on BCL-2 gene expression**
(* $P < 0.05$)

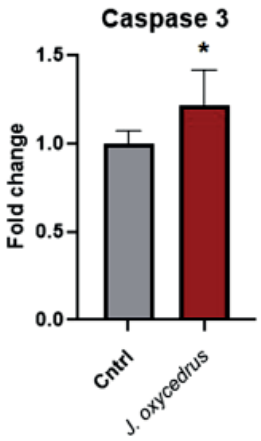


*Fig. 4.* **Effect of *J. oxycedrus* fruit extract on Caspase 3 gene expression**
(* $P < 0.05$)

exhibited by the BCR-ABL1 fusion protein leads to uncontrolled proliferation of cells and malignancy of pluripotent stem cells by activation of Ras/Raf/mitogen-activated protein kinase (MAPK) pathways [11]. In the treatment of the disease, the use of tyrosine kinase inhibitors, which block the ATP binding site of ABL1, is generally preferred. However, when drug resistance or drug sensitivity is observed in patients, additional therapies are needed [12]. In addition, due to the side effects of anticancer drugs, it is important to elucidate the anticancer activities of various natural products to support the treatment and to determine the supportive properties of chemotherapy [11, 12].

The bioactive metabolites contained in the juniper plant, which has many species, have been used in public health since ancient times. In one of the studies examining the effects of various cytotoxic juniper extracts with high antiproliferative effect, high efficiency activity was observed in K-562 human chronic myeloid leukemia cells. Studies show that juniper species, which can perform efficient biosynthesis of antiproliferative agents, are a natural source of drug precursors in the pharmaceutical industry [13].

In the scope of this study, the phytochemical structure and cytotoxic effect of *J. oxycedrus* extract was investigated; and the changes in the expressions of Akt1, the member of the PI3K/Akt/mTOR signaling pathway, caspase 3, which is one of the main effective genes in the pathways regulating apoptosis, and the apoptosis suppressor BCL-2 gene, which is an oncogene, were investigated.

As a result of the MTT tests, it was observed that the extracts obtained from the fruit parts of the *J. oxycedrus* plant showed over approximately 50% cell viability in K-562 cells at all doses. It is seen that the viability of K-562 cells decreases as the dose increases in the fruit extract. The viability of ECV-304 cells is also very low at a dose of 50 µg/mL, where the fruit extract has the highest lethality. ECV-304 cells are intravascular endothelial cell line. Angiogenesis plays an important role in metastasis [14]. The fact that the selected 50 µg/mL dose in these cells is cytotoxic indicates that the extract reduces metastasis and vascularization in the cells. This 50 µg/mL dose was chosen for the further experimental stages in order to investigate the intracellular signaling mechanisms.

After the gene expression analysis, it was observed that Akt expression increased 1.092 times (not statistically significant, $P > 0.05$), BCL-2 expression decreased approximately 3.3 times, and caspase 3 expression increased 1.2 times.

Akt1 is an important signaling molecule in tumor formation and progression. Dysregulation of Akt causes diseases that are difficult to treat clinically, such as diabetes, neurological and cardiovascular diseases, including cancer. Akt1 is widely found in tissues and is involved in cell proliferation and survival [15]. Its overactivation may mediate cellular events that promote tumorigenesis through its downstream effectors. Therefore, it is one of the most overactivated protein kinases in human cancers [16].

BCL-2 is a proapoptotic gene and suppresses elements that induce apoptosis. The decrease in its expression may pave the way for cancer cells to turn to apoptotic pathways. Caspase 3 is one of the essential elements of the apoptotic pathway. It is a determinant gene in the pathway to apoptosis when activated by other apoptotic signals [17]. A 1.2-fold increase in its expression may produce a similar effect to the decrease in BCL-2, resulting in apoptosis and programmed death of cells.

The obtained results revealed that the compounds contained in the *J. oxycedrus* plant were considered worthy of investigation in terms of their anticancer effects on chronic myeloid leukemia cells, but more research is still needed.

*Grant Support*

Authors declare that there is no grant support.

*Conflict of Interest*

Authors declare that there is no conflict of interest.

## REFERENCES


1. *Calabretta B., Salomoni P.* Inhibition of autophagy: A new strategy to enhance sensitivity of chronic myeloid leukemia stem cells to tyrosine kinase inhibitors. *Leukemia and Lymphoma*. 2011, p 54–59. https://doi.org/10.3109/10428194.2010.546913
2. *Ciaffaglione V., Consoli V., Intagliata S., Marrazzo A., Romeo G., Pittalà V., Greish K., Vanella L., Floresta G., Rescifina A., Salerno L., Sorrenti V.* Novel Tyrosine Kinase Inhibitors to Target Chronic Myeloid Leukemia. *Molecules* 2022, 27(10):3220. https://doi.org/10.3390/molecules27103220
3. *Öztürk M., Tümen I., Uğur A., Aydoğmuş-Öztürk F., Topçu G.* Evaluation of fruit extracts of six Turkish Juniperus species for their antioxidant, anticholinesterase and antimicrobial activities. *Journal of the Science of Food and Agriculture*. 2011, 91(5):867–876. https://doi.org/10.1002/jsfa.4258
4. *Akkol EK., Güvenç A., Yesilada E.* A comparative study on the antinociceptive and anti-inflammatory activities of five Juniperus taxa. *Journal of Ethnopharmacology*. 2009, 125(2):330–336. https://doi.org/10.1016/j.jep.2009.05.031
5. *Karaman I., Şahin F., Güllüce M., Öğütçü H., Şengül M., Adigüzel A.* Antimicrobial activity of aqueous and methanol extracts of *Juniperus oxycedrus*. L. *Journal of Ethnopharmacology*. 2003, 85(2–3), 231–235. https://doi.org/10.1016/s0378-8741(03)00006-0
6. *Huyan T., Li Q., Wang YL., Li J., Zhang J.Y., Liu Y.X., Shahid M.R., Yang H., Li H.Q.* Anti-tumor effect of hot aqueous extracts from *Sonchus oleraceus* (L.) L. and *Juniperus sabina* L — Two traditional medicinal plants in China. *Journal of Ethnopharmacology*. 2016, 185, 289–299. https://doi.org/10.1016/j.jep.2016.03.044
7. *Khuda F., Alam N., Khalil AAK., Jan A., Naureen F., Ullah Z., Alotaibi A., Ullah R., Ullah S., Shah Y., Shah SI., Büyüker SM.* Screening of Rhamnus Purpurea (Edgew.) Leaves for Antimicrobial, Antioxidant, and Cytotoxic Potential. *ACS Omega*. 2022, 7(26), 22977–22985. https://pubs.acs.org/doi/10.1021/acsomega.2c03094
8. *Atasever-Arslan B., Yilancioglu K., Kalkan Z., Timucin A.C., Gür H., Isik F.B., Deniz E., Erman B., Cetiner S.* Screening of new antileukemic agents from essential oils of algae extracts and computational modeling of their interactions with intracellular signaling nodes. *European Journal of Pharmaceutical Sciences*. 2016, 83:120-31. https://doi.org/10.1016/j.ejps.2015.12.001
9. *Atasever B., Akgün D.K., Kuruca S., Turan N., Seyhanli V. M.A.* Effects of flavonoids obtained from Cynara syriaca on leukemic cells. *Ankara University Faculty of Pharmacy Journal*. 2003, 32(3), 143–150.
10. *De Kouchkovsky I., Abdul-Hay M.* 'Acute myeloid leukemia: a comprehensive review and 2016 update'. *Blood Cancer J.* 2016, 6(7),441. https://doi.org/10.1038/bcj.2016.50.
11. *Wu P.S., Wang C.Y., Chen P.S., Hung J.H., Yen J.H., Wu M.J.* 8-Hydroxydaidzein Downregulates JAK/STAT, MMP, Oxidative Phosphorylation, and PI3K/AKT Pathways in K562 Cells. *Biomedicines. 2021,* 9(12), 1907. https://doi.org/10.3390/biomedicines9121907
12. *Baccarani M., Cortes J., Pane F., Niederwieser D., Saglio G., Apperley J., Cervantes F., Deininger M., Gratwohl A., Guilhot F., Hochhaus A., Horowitz M., Hughes T., Kantarjian H., Larson R., Radich J., Simonsson B., Silver R.T., Goldman J., Hehlmann R.* Chronic myeloid leukemia: An update of concepts and management recommendations of European LeukemiaNet. *Journal of Clinical Oncology*. 2009, p 6041–6051. https://doi.org/10.1200/JCO.2009.25.0779
13. *Ivanova D.I., Nedialkov P.T., Tashev A.N., Olech M., Nowak R., Ilieva Y.E., Kokanova-Nedialkova Z.K., Atanasova T.N., Angelov G., Najdenski H.M.* Junipers of various origins as potential sources of the anticancer drug precursor podophyllotoxin. *Molecules*. 2021, 26(17), 5179. https://doi.org/10.3390/molecules26175179
14. *Sanna B., Debidda M., Pintus G., Tadolini B., Posadino AM., Bennardini F., Sava G., Ventura C.* The anti-metastatic agent imidazolium trans-imidazoledimethylsulfoxide-tetrachlororuthenate induces endothelial cell apoptosis by inhibiting the mitogen-activated protein kinase/extracellular signal-regulated kinase signaling pathway. *Archives of Biochemistry and Biophysics*. 2002, 403(2), 209–218. https://doi.org/10.1016/s0003-9861(02)00218-7
15. *Hers I., Vincent E.E., Tavaré J.M.* Akt signalling in health and disease. *Cellular Signalling*. 2011, p 1515–1527. https://doi.org/10.1016/j.cellsig.2011.05.004
16. *Altomare D.A., Testa J.R.* Perturbations of the AKT signaling pathway in human cancer. *Oncogene*. 2005, p 7455–7464. https://doi.org/10.1038/sj.onc.1209085
17. *Khan N., Kahl B.*Targeting BCL-2 in Hematologic Malignancies. *Targeted Oncology*. 2018, p 257–267. https://doi.org/10.1007/s11523-018-0560-7

# ФІТОХІМІЧНІ СКЛАДОВІ ТА ПРОТИЛЕЙКЕМІЧНІ ВЛАСТИВОСТІ ЕКСТРАКТУ *Juniperus oxycedrus*

*Berfin Tugba Turak*[1, 2], *Deniz İrem Acar*[2], *Seda Kusoglu Gultekin*[2], *İrem Gulfem Albayrak*[2], *Emine Akalin*[3], *Belkıs Atasever Arslan*[2]

[1]Department of Bioengineering, Faculty of Engineering and Natural Sciences, Uskudar University, Istanbul, Turkey
[2]Department of Molecular Biology and Genetics, Faculty of Engineering and Natural Sciences, Uskudar University, Istanbul, Turkey
[3]Department of Pharmaceutical Botany, Faculty of Pharmacy, İstanbul University, Turkey

*E-mail: belkisatasever.arslan@uskudar.edu.tr*

*Мета.* Багато генетичних факторів і факторів навколишнього середовища можуть бути ефективними в процесі виявлення та лікування онкологічних захворювань. Запобігти прогресуванню лейкемії можна, контролюючи шляхи, що включають такі механізми, як апоптоз і аутофагія. Є дослідження, які демонструють вплив різних видів ялівцю на різні лінії ракових клітин, включаючи клітини хронічного мієлоїдного лейкозу людини, але сигнальні шляхи, за якими вони діють, не повністю відомі. У цій роботі було досліджено протипухлинну дію екстракту *Juniperus oxycedrus* на клітини хронічного мієлоїдного лейкозу людини К-562.

*Матеріали й методи.* Після обробки клітин екстрактом *Juniperus oxycedrus* було проведено аналіз цитотоксичності та експресії генів. Зміни в експресії Akt, члена сигнального шляху PI3K/Akt/mTOR; каспаза 3, яка є одним з основних ефективних генів у шляхах регуляції апоптозу; і досліджено ген супресора апоптозу BCL-2, який є онкогеном.

*Результати.* Згідно з результатами тесту MTT, екстракт ялівцю оксицедрового показав приблизно 50% життєздатності клітин К-562 у всіх дозах. Найбільш відповідна доза екстракту плодів *Juniperus oxycedrus* у цьому дослідженні була визначена як 50 мкг/мл з урахуванням життєздатності клітин. Після аналізу експресії генів було виявлено, що експресія BCL-2 зменшилася приблизно в 3,3 рази, а експресія каспази 3 зросла в 1,2 рази. Хоча експресія гену Akt зросла в 1,092 рази, вона не була статистично значущою.

*Висновки.* Компоненти рослини *Juniperus oxycedrus* можуть мати апоптотичний ефект на клітини хронічного мієлоїдного лейкозу.

***Ключові слова:*** *Juniperus oxycedrus*; лейкоз; апоптоз.