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Sapropel Exploration and Characterisation for the Development of Sodium Carboxymethylcellulose-Based Bioactive Hydrogels: Formulation and Functional Evaluation

Doctoral Thesis – set of publications – for obtaining the scientific degree "Doctor of Science (*PhD*)"

Sector Group – Medical and Health Sciences

Sector – Basic Medicine

Sub-Sector – Technology of Drug Forms

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Riga, 2026

This Thesis has been developed with the funding from the project "Analysis of characteristics of medical sapropel and its usage for medical purposes and elaboration of industrial extraction methods" was financed by European Regional Development Fund, project No 1.1.1.1/16/A/165.

This Thesis has been developed with financing from the European Social Fund and Latvian state budget within the project No 8.2.2.0/20/I/004 "Support for involving doctoral students in scientific research and studies" at Rīga Stradiņš University.

Abstract

Sapropel, an organic-rich freshwater sediment, has long been used in traditional medicine and cosmetics, however its therapeutic potential remains underexplored by modern science. Nevertheless, the use of sapropel in these traditional ways presents some practical challenges, like requirement for specialised facilities and large quantities of fresh sapropel sediments. Sapropel application, particularly in the form of mud baths, necessitates dedicated spaces equipped for the storage, preparation, application, and removal of the mud. This Doctoral Thesis investigates sapropel as a source of bioactive compounds and its integration into sodium carboxymethylcellulose (Na-CMC) hydrogels for potential biomedical and cosmetic applications.

The study begins with a comprehensive analysis of sapropel's origin, structure, and biologically active constituents, particularly humic and fulvic acids. A critical review of extraction methodologies was conducted, comparing solid–liquid, ultrasound-assisted, and supercritical fluid extraction techniques. Emphasis was placed on establishing quality criteria for sapropel extracts suitable for medical use, addressing the current lack of standardised protocols.

Sapropel samples from Latvian freshwater lakes were systematically collected, layer-wise characterised, and processed to obtain extracts. These were analysed for total organic carbon, humic and fulvic acid content, polyphenol levels, antioxidant status, trace elements, and microbiological indicators. Selected extracts demonstrated significant antioxidant activity and promoted cell regeneration *in vitro*, particularly in fibroblast and keratinocyte models; however cytotoxic effects were observed at prolonged, high concentrations.

In the final phase, the functional incorporation of sapropel extracts into Na-CMC-based hydrogels was achieved. Eight formulations were developed and evaluated for physicochemical properties, stability, pH, and viscosity under various conditions. Hydrogels containing sapropel extract exhibited favourable stability, a biocompatible pH range, and maintained organoleptic qualities, making them suitable as a platform for topical application.

This research supports the potential of sapropel as a valuable natural resource for developing bioactive formulations. The results contribute to the scientific foundation for its evidence-based use in dermal therapeutics and cosmetics, and open avenues for further product development based on local natural resources.

Keywords: Sapropel; hydrogel; antioxidants; fulvic acid; humic acid; sapropel extract; sodium carboxymethylcellulose (Na-CMC); pharmaceuticals; natural compounds; polyacids.

Anotācija

Sapropeļa izpēte un tā integrācija bioaktīvos hidrogelos ar nātrija karboksimetilcelulozi: īpašību analīze un praktiskais potenciāls

Sapropelis, kas ir ar organiskām vielām bagāts saldūdens nogulums, jau izsenis tiek izmantots tradicionālajā medicīnā un kosmētikā, tomēr tā ārstniecisko potenciālu mūsdienu zinātne joprojām nav pietiekami izpētījusi. Sapropeļa izmantošana tradicionālajos veidos ir nepraktiska, it īpaši sapropeļa izmantošana dubļu vannās. Šīm procedūrām ir nepieciešamas speciāli aprīkotas telpas, kurās var uzglabāt, sagatavot, uzklāt un noņemt sapropeli, kā arī šīm procedūrām nepieciešams liels svaiga sapropeļa daudzums. Šajā promocijas darbā tiek pētīts sapropelis kā bioloģiski aktīvo savienojumu avots un tā ekstraktu integrācija nātrija karboksimetilcelulozes (Na-CMC) hidrogelos potenciālai lietošanai medicīnā un kosmētikā.

Pētījums sākās ar visaptverošu sapropeļa izcelsmes, struktūras un bioloģiski aktīvo sastāvdaļu, īpaši humīnskābju un fulvīnskābju, analīzi. Tika veikts ekstrakcijas metožu un procedūru pārskats, salīdzinot pieejamos datus par cietās–šķidrās fāzes, ultraskaņas un superkritiskā šķidruma ekstrakcijas metodēm. Uzsvars likts uz medicīniskai lietošanai piemērotu sapropeļa ekstraktu kvalitātes kritēriju noteikšanu, risinot pašreizējo standartizēto protokolu trūkumu.

Sapropeļa paraugi no Latvijas saldūdens ezeriem tika sistemātiski savākti, veikta ezeru, sapropeļa nogulumu un slāņu atšķirību izpēte un noteikts piesārņojuma līmenis, mikroelementi un mikrobioloģiskie indikatori. Visi paraugi tika apstrādāti pēc vienotas metodes un no tiem iegūti ekstrakti. Ekstraktos tika analizēts kopējais organiskā oglekļa, humīnskābju un fulvīnskābes saturs, polifenolu līmenis, antioksidantu statuss. Izvēlētie ekstrakti uzrādīja nozīmīgu antioksidanta aktivitāti un veicināja šūnu reģenerāciju *in vitro* testos, īpaši fibroblastu un keratinocītu modeļos, lai gan ilgstoši augstās ekstrakta koncentrācijās tika novērota citotoksiska iedarbība.

Noslēgumā tika panākta sapropeļa ekstraktu funkcionāla iekļaušana Na-CMC bāzes hidrogelos. Tika izstrādāti astoņi sastāvi un novērtētas to fizikāli ķīmiskās īpašības, stabilitāte, pH un viskozitāte. Sapropeļa ekstraktu saturošie hidrogeli uzrādīja atbilstošu stabilitāti pēc izturēšanas stresa apstākļos, tiem bija ar ādas pH līmeni bioloģiski saderīgs pH diapazons, un tie saglabāja organoleptiskās īpašības, padarot tos piemērotus kā zāļu formu lokālai lietošanai uz ādas.

Uz pierādījumiem balstītie rezultāti sniedz zinātnisku pamatu hidrogelu ar sapropeļa ekstraktu lietošanai dermatoloģijā un kosmētikā, kā arī paver iespējas turpmākai produktu izstrādei, izmantojot vietējos dabas resursus.

Atslēgvārdi: sapropelis; hidrogels; antioksidanti; fulvīnskābe; humīnskābe; sapropeļa ekstrakts; nātrija karboksimetilceluloze (Na-CMC); farmaceitiskie līdzekļi; dabiskie savienojumi; poliskābes.

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Abbreviations used in the Thesis

HS	Humic Substances
HA	Humic Acid
FA	Fulvic Acid
HMA	Hymatomelanic Acid
LOI	Loss-on-ignition
DDT/DDE	Dichlorodiphenyltrichloroethane / Dichlorodiphenyldichloroethylene
CFU	Colonies Forming Units
ISO	International Standard Organisation
CMC	Carboxymethyl Cellulose
Na-CMC	Sodium Carboxymethyl Cellulose Salt
SEM	Scanning Electron Microscopy
SCCS	The Scientific Committee on Consumer Safety
SLE	Solid-Liquid Extraction
UAE	Ultrasound-assisted Extraction
SFE	Supercritical Fluid Extraction
HPLC	High Pressure Liquid Chromatography
TPC	Total Phenolic Content
TAS	Total Antioxidant Status
HaCaT	Human Adult Low-calcium High-temperature Keratinocytes
BALB/c 3T3	Bagg Albino mouse line developed by S.A. Aaronson and G.T. Todaro in 1968
UV	Ultraviolet Light
XRD	X-ray Diffraction
NRU	Neutral Red Uptake

Introduction

The inherent human desire to maintain health, enhance beauty, and combat illnesses is a timeless pursuit. Dating back to the 5th century BC, ancient Greek intellectuals like Herodotus (484–425 BC) pioneered the therapeutic use of mineral waters. Hippocrates (460–370 BC), widely regarded as the father of medicine, also documented the healing properties of saltwater in his writings.

In the 19th century, balneology, a new scientific discipline focusing on the therapeutic use of mineral and thermal waters and mud, emerged thanks to the work of English doctor J. Currie and Austrian doctor V. Priessnitz, the founder of modern hydrotherapy. Balneology aims to improve health and treat various ailments using these natural resources. Between the 17th and 19th centuries, mud therapy experienced a surge in popularity across Europe, coinciding with the rapid advancement of balneology. This period saw the establishment of balneological clinics in several countries, including Germany, France, Italy, Austria, and Romania.

Review by H. Routh (Routh, 1996) describe numerous experiments by various scientist to understand the therapeutic potential of peloids, mud and mineral waters. These investigations focused on understanding how different factors influenced the effectiveness of these treatments. Review paper on thermal muds by Veniale and colleagues (Veniale et al., 2007) found out that temperature fluctuation from during application, duration of application periods and chemical composition, particularly the levels of sulphur and nitrous oxides, play the key role in the treatments. The reviews highlight that these experiments aimed to refine the application of mud and mineral water therapies for optimal therapeutic benefit. Mud remedies also gained popularity in cosmetology and cosmetic surgery of the time, with mud being used to speed up the regeneration and renewal of skin.

In the early 20th century, hydrotherapy was increasingly used alongside other therapies to enhance patient health. These complementary therapies included: Peloidal therapy (mud therapy), massage, iontophoresis, phonophoresis physiotherapy, physical exercise. This combined approach proved effective in addressing a range of health issues, notably – rheumatological disorders, osteoarthritis, fibromyalgia, spondylosis, various musculoskeletal disorders.

While muds, mineral waters and peloids, including sapropel, have a long history of use, the scientific evidence supporting their effectiveness remains an area of ongoing research. The growing interest in non-pharmaceutical medical treatments has highlighted the need for robust scientific evidence supporting the health benefits of sapropel to promote the safe and effective use of sapropel in medicine and cosmetics. This research should focus on establishing

a strong evidence base for its applications, especially in balneology. By doing so, the use of sapropel, a locally sourced natural resource, can be expanded in healthcare and medical cosmetics.

While sapropel might appear as a mysterious substance hidden in the depths of lakes, seas and swamps, it is a valuable material with a long history of use in health improvement and treatments. Found commonly in the lakebeds, Latvian sapropel is a fine-graded sediment rich in organic matter that is produced by sedimentation and transformation of residues from aquatic plants and various living organisms together with mineral particles.

One of the most notable features of sapropel is its high content of humic substances (HSs), specifically humic acid (HA) and fulvic acid (FA). These acids are believed to be responsible for many of the health benefits associated with sapropel, including antioxidant, anti-inflammatory, and antibacterial effects. Latvian freshwater sapropel is particularly interesting due to its high concentration of HA and FA, showing promise for use in pharmaceutical and cosmetic products.

Traditionally, sapropel is used therapeutically in two main ways: thermal mud baths and direct application to the skin. A typical mud bath involves adding 1 kg of sapropel to 10 L of water, maintaining a temperature of 37°C, and having the patient soak for 15–20 minutes. This method has shown very positive results in treating skin conditions like eczema and dermatitis, as well as hand osteoarthritis. Handling large quantities – traditional sapropel applications often require significant amounts of the material, which can be cumbersome to manage. The process of storing, applying, and cleaning up after sapropel treatments can be labour-intensive and potentially messy, making it challenging to perform in settings more convenient for the patient, such as their homes. This limits treatment to specific locations like balneotherapy centres, clinics or sanatoriums, which might not be easily accessible to all patients.

These challenges point to the need for alternative, user-friendly delivery systems for sapropel, especially its bioactive elements like HA and FA. One potential solution is the development of stable, water-soluble hydrogels containing sapropel extracts. Such formulations could make therapeutic applications easier, manageable and more accessible to patients. Overcoming these practical obstacles is essential for unlocking sapropel's full therapeutic potential and ensuring its safe, effective use in health and cosmetic products.

Aim of the Thesis

To investigate the potential of sapropel and its extracts for incorporation into carboxymethylcellulose-based hydrogels, with the goal of further developing innovative, biologically derived materials for medical applications.

Objectives of the Thesis

The following objectives are set to reach the aim of the Doctoral Thesis:

1 Collect and analyse information on sapropel resources available in Latvia, including acquisition methods, legal regulations, and potential areas of application.
2 Conduct a quality assessment of sapropel, identifying impurities and potential contaminants such as heavy metals and pesticides.
3 Evaluate methods for obtaining sapropel extracts and assess their potential therapeutic effects.
4 Develop sapropel-enriched sodium carboxymethylcellulose hydrogel systems.
5 Conduct stability and persistence tests on the developed hydrogels
6 Perform physical-chemical and persistence tests on the resulting hydrogel and assess their practical applicability in medical contexts.

Hypothesis of the Thesis

1. Sapropel extract demonstrates effective compatibility with carboxymethyl cellulose hydrogel, enhancing its potential applications in medical formulations.

Novelty of the Thesis

This Thesis explores a previously underutilised natural resource - Latvian sapropel – for the development of modern biomedical applications. While sapropel has traditionally been used in balneology, its integration into water-soluble hydrogel systems based on carboxymethylcellulose sodium salt represents a novel and promising approach. The research introduces a new method of delivering the bioactive components of sapropel in a controlled, stable, and more accessible form, potentially overcoming the limitations of traditional mud therapy. Additionally, the study contributes new data on sapropel's composition, safety, and therapeutic potential, offering a scientifically grounded foundation for its incorporation in innovative medical and cosmetic products. In addition, the Thesis explores novel formulations of carboxymethylcellulose-based hydrogels, examining their stability and potential for medical applications.

Discussion

Sapropel: A Resource for Health and Beauty

Sapropel is a rich source of antioxidant compounds such as polyphenols, sulphated polysaccharides, carotenoids, humic and fulvic acids and many other compounds. Sapropel is a fine-graded organic sediment found in freshwater and saltwater resorts that is produced by sedimentation and transformation of residues from aquatic plants and various living organisms together with mineral particles (Stankevica et al., 2016). It is considered a partly renewable resource and literature suggests potential for a wide range of therapeutic applications and a long history of use in traditional medicine and cosmetology, specialty within the field of balneology.

The accumulation of sapropel began following the last ice age, estimated to have occurred around 12,000 to 15,000 years ago, with notable deposits forming during the Holocene period. It is believed that the formation process includes the sedimentation of biomass from the lake or sea and, on occasion, the input of organic matter from rivers flowing into the water resorts. Sapropel has a complex structure influenced by the variety of organisms and compounds involved in its creation. Its colloidal nature allows it to hold a significant quantity of water (Stankevica & Klavins, 2014; Vanadziņš et al., 2022). Freshwater lakes in Latvia can possibly contain both sapropel and peat mud. The primary difference between them lays in their finer structure, acidity, content of organic and humic substances, and the types of sediment-forming organisms.

There are multiple theories about how sapropel develops. A popular theory suggests that it mainly consists of three types of components: mineral substances of allochthonous origin, inorganic compounds of biogenic origin, and organic materials, which include remnants of plants and small aquatic creatures (Stankevica & Klavins, 2014; Vanadziņš et al., 2022).

The historical usage of Sapropel has been traced back to ancient Greece and is connected to the use of mineral waters and muds that lead to the subsequent development of balneology in Europe. This demonstrates long-standing practice of natural substances like sapropel usage in the therapeutic application, predating modern pharmaceutical medicine.

There is documented history of sapropel being used for skin ailments such as eczema and dermatitis, typically in the form of creams or patches that do not dissolve in water (Dolmaa et al., 2011; Drobnik & Stebel, 2020; Pavlovska et al., 2020; Sarlaki et al., 2024; Vanadziņš et al., 2022). This naturally occurring sapropel used in balneology is primarily composed of humic substances (HSs) and non-humic compounds, both of which have shown biological activity (Jarukas et al., 2021; Klavina et al., 2020; Platonova & Adeeva, 2015). Humic substances are divided into four types: humic acid (HA), hymatomelanic acid (HMA), fulvic acid (FA), and humin and were more discussed in the article "*Freshwater Sapropel:*

Biologically Active Components and Methods of Extraction" and will be discussed more in the upcoming chapters.

Additionally, sapropel contains water-soluble vitamins, such as ascorbic acid (C), thiamine (B1), riboflavin (B2), pantothenic acid (B5), pyridoxine (B6), folic acid (B9), and cyanocobalamin (B12), along with fat-soluble vitamins like vitamin D and tocopherol (E) (Klavina et al., 2020; Kļaviņa et al., 2024; Pavlovska et al., 2020; Stankevica & Klavins, 2014; Vanadziņš et al., 2022).Vitamin B12, which is produced by certain bacteria and blue-green algae, is particularly important due to its role in blood formation, the metabolism of amino acids, and nucleic acid synthesis (Luhila et al., 2022).

The article "*Sapropel – Mining Characteristics and Potential Use in Medicine*" points to several potential mechanisms underlying sapropel's effects. Healing mud and peat that contain sapropel are primarily employed as external treatments (Celik Karakaya et al., 2010; Centini et al., 2015; Fioravanti et al., 2014; Gerencser et al., 2010; Glavas et al., 2017; Odabasi et al., 2007; Tserenpil et al., 2010; Veniale et al., 2007). One of the main properties is high heat capacity and low heat transfer. They are clearly identified as significant contributors to its therapeutic action, facilitating deep tissue heating and influencing circulation and metabolism (Centini et al., 2015; Glavas et al., 2017; L. Ji et al., 2018; Tateo et al., 2009). Sapropel's high thermal capacity and low heat conductivity allow it to retain heat for extended periods and transfer it slowly to tissues. This gentle and deep heat penetration can improve blood circulation, relax muscles, and reduce pain (Balciunas et al., 2016; Centini et al., 2015; Veniale et al., 2007). Sapropel applications can stimulate microcirculation in the skin and muscles, improving oxygen supply and nutrient delivery to tissues (Kļaviņa et al., 2024; Pavlovska et al., 2020; Vanadziņš et al., 2022).

The chemical composition, including humic substances, vitamins, and minerals, is also considered crucial, with potential for dermal penetration and various biological activities like anti-oxidation and chelation. Sapropel's fine texture and colloidal structure facilitate the penetration of its biologically active components into the skin and deeper tissues. These components, such as humic substances – humic and fulvic acid, vitamins, and amino acids exert various therapeutic effects at the cellular level (Pavlovska et al., 2020; Vanadziņš et al., 2022). Humic and fulvic acids found in sapropel exhibit antioxidant properties, protecting cells from damage caused by free radicals (Klavina et al., 2019, 2020). Antioxidants play a crucial role in preventing chronic diseases, reducing inflammation, and slowing down aging processes. A Bellometti et. al. (2000) research that involved 37 arthritis patients found that sapropel baths and other type of mud baths had positive effects both on homeostasis of cartilage and reduction of inflammation, reducing values of NO and myeloperoxidase, while there was

no correlation in increase of GSH peroxidase (Bellometti et al., 2000). Research suggest that sapropel can modulate the immune system, enhancing its ability to fight infections and reduce inflammation. It can stimulate the activity of phagocytes, cells that engulf and destroy harmful microorganisms, thereby contributing to tissue regeneration and overall immune health (Bellometti et al., 1996, 2000, 2002).

Furthermore, the microbial community within sapropel is not simply passive but actively participates in the formation of biologically active compounds and contributes to antimicrobial properties. Many authors describe Sapropel's antimicrobial activity against a range of bacteria and fungi. Suraganova et.al. (2014) in her research describes more sanitary and microbiology properties of sapropel in the Lake Kossor, Akimzhanova et.al. (2024) and Antonelli and Donelli (2018) in their research suggest that this activity is attributed to the presence of various microorganisms that produce antibiotics and other antimicrobial compounds (Akimzhanova et al., 2024; Antonelli & Donelli, 2018; Suraganova et al., 2014). Sapropel's antimicrobial properties can contribute to faster wound healing, reduce inflammation, and combat skin infections (Akimzhanova et al., 2024; Antonelli & Donelli, 2018; Pavlovska et al., 2020; Suárez Muñoz et al., 2015; Suraganova et al., 2014; Vanadziņš et al., 2022).

Sapropel can be effective in treating a variety of skin conditions, such as psoriasis, eczema, acne, and wounds. Its antimicrobial, anti-inflammatory, and regenerative properties contribute to its efficacy in these applications. Sapropel's thermal and anti-inflammatory properties make it beneficial for treating various musculoskeletal conditions, including arthritis, osteoarthritis, muscle pain, and joint stiffness. Enhanced microcirculation can promote tissue regeneration, reduce swelling, and support overall healing processes (Centini et al., 2015; Fioravanti et al., 2014; Gerencser et al., 2010; Odabasi et al., 2007; Tserenpil et al., 2010).

During literature research many articles mentioned potential benefits of sapropel in treating conditions like diabetes, cardiovascular diseases, and respiratory disorders. The exchange between these thermal, chemical, and biological factors accounts for the observed effects on tissue regeneration, inflammation, and immune function. Nevertheless, further studies are needed to confirm these effects and establish optimal treatment protocols (Vanadziņš et al., 2022).

Typically, sapropel is applied through thermal mud baths or external applications. The bath is prepared for 15–20 minutes with a water temperature of 37 °C and a sapropel concentration of 1 kg per 10 L of water. Notable improvements were seen in patients undergoing treatment for eczema, dermatitis, and hand osteoarthritis (Fioravanti et al., 2014; Fortunati et al., 2016; Veniale et al., 2007). However, the use of sapropel has certain constraints

due to its nature; it necessitates stationary facilities, such as baths or specially designed areas where sapropel is applied to patients. A relatively large volume of sapropel is needed for external procedures, which presents challenges for storage, application, and disposal, making it difficult to carry out these treatments in locations convenient for patients (Fioravanti et al., 2014; Odabasi et al., 2007; Tateo et al., 2009; Rensburg van, 2015; Vanadziņš et al., 2022; Veniale et al., 2007).

However, the literature review has proven wide usage possibilities, the precise mechanisms of action are not yet fully clear, and much of the research has been fragmented, lacking modern, systematic investigation. This highlights a gap between traditional practice and robust scientific evidence. While review by Stankevics and Klavins (2014) and review by Isabel Carretero (2020) provided wide information suggesting positive effects on musculoskeletal disorders, skin conditions, and circulation, the call for more scientifically sound medical and cosmetic use-based research with firm evidence is a main point of these theses. The potential for sapropel components to penetrate the skin, while offering therapeutic benefits, also raises concerns regarding potentially toxic elements like heavy metals, requiring further analysis and understanding of their interactions with human tissues (Carretero, 2020; Pavlovska et al., 2020; Stankevica & Klavins, 2014).

In conclusion, the article "*Sapropel – Mining Characteristics and Potential Use in Medicine*" provides a comprehensive overview of sapropel, moving from its definition, formation, and composition to its historical use and potential therapeutic properties. It underscores the promise of sapropel as a natural therapeutic agent with diverse applications, but also critically points to the need for more rigorous scientific investigation to fully understand its mechanisms and ensure its safe and effective evidence-based use in the future.

Geological and Biological profile of Sapropel

The evaluation of primary study findings was more described in the article "*Assessment of sapropel use for pharmaceutical products according to legislation, pollution parameters, and concentration of biologically active substances*". Focusing to answer the questions asked in the article "*Sapropel – Mining Characteristics and Potential Use in Medicine"*.

The article highlights the considerable potential of Latvian freshwater sapropel as a natural resource for pharmaceutical and cosmetic applications, aligning with global trends towards using local and natural materials. The study provides crucial systematic research into the properties and composition of sapropel from specific Latvian lakes, which was previously lacking in the context of biomedical and biopharmaceutical potential.

Sapropel sediment appears in various colours and has a pH that ranges from 5 to 8. Typically, the humidity of sapropel deposits after extraction from lakes ranges between 65 % and 95 % (Vanadziņš et al., 2022). In this research, the sapropel was observed to be brown to greenish yellow, indicating a mixed type of sapropel, derived from the plankton and plants of the lake, and sometimes associated with the presence of peat. Green and yellow sapropel often correlates with high silica content and is usually found in moraine lakes. Black-coloured sapropel, which contains high organic matter, is typically found in lakes with low mineral content. The pH of the sampled sapropel deposits was around 7–8, suggesting high mineral content in these sediments and higher than typical swamp sapropel. The organic matter content was determined using the loss-on-ignition (LOI) method, where a sediment with at least 15 % organic matter qualifies as sapropel. This criterion is essential for differentiating sapropel from other sediment types. The organic matter levels in the tested lakes ranged from 20 to 90 % (Vanadziņš et al., 2022). The organic content varied from 52 to 54 %, with carbonate concentration between 4.40 and 5.00 % (Pavlovska et al., 2020; Stankevica & Klavins, 2014). Generally, a higher level of organic matter suggests a stronger potential for therapeutic effects. These findings align with the classifications made by Stankevica and Klavins (2014) regarding sapropel sediments found in Latvia (Stankevica & Klavins, 2014).

The thickness and depth of the sapropel layer differ based on the lake's depth and the rate at which organic matter decomposes. Layers that extend less than 1.5 m from the surface are typically regarded as underdeveloped and not suitable for sediment extraction (Pavlovska et al., 2020; Vanadziņš et al., 2022). The sapropel layer deemed suitable in this investigation ranged from 2.0 to 9.0 m; for comparison, sapropel layers in studied lakes were observed between 0.9 to 11.4 m from the sediment's surface, with exact depths varying according to the specific lake and exploration site (Pavlovska et al., 2020).

The sapropel's composition exhibited significant variation among lakes due to a variety of factors. Additionally, the composition of sapropel is influenced by the surrounding environment and historical land use in the exploration area (Stankevica & Klavins, 2014; Vanadziņš et al., 2022). For instance, lakes located near agricultural zones may exhibit higher concentrations of pesticides and heavy metals within their sapropel sediments. The inflow from rivers and ditches plays a role in how pollutants and nutrients are distributed throughout the lake, which in turn affects the sapropel's composition and results in differing microelement concentrations among various sapropel types (Pavlovska et al., 2020).

The analysis of pollution parameters, heavy metals and pesticides shows that while these contaminants are present, their levels in the tested samples did not exceed current maximum acceptable levels for cosmetic products. However, the detection of DDT/DDE, persistent

organic pollutants, highlights the environmental history of the lake areas and the importance of ongoing monitoring. The highest levels of DDE/DDT were found in all depth of the Lakes Mazais Kivdalovas and Zeilu, as well as at one exploration point in the Audzelu Lake, and the amount of DDE/DDT was below the limit of quantification in other researched lakes. Limit values for DDT concentrations contained in Republic of Latvia, Cabinet Regulation No. 118, Annex 1, "Environmental Quality Standards for Dangerous Substances in Surface Waters", where the average annual concentration of DDT is 0.025 μg/L or ppb and para-DDT – 0.01 μg/L or ppb. When comparing lakes, the concentrations of DDE/DDT were slightly different. In general, the concentrations of DDE/DDT found in surface water from lakes were lower than those found in samples of sapropel (Pavlovska et al., 2020).

The analysis of heavy metal concentration in the sapropel provides information on the natural and anthropogenic origin of the metal flow in the lake's ecosystem and the influence on sapropel application in medicine. Lead (Pb), Cadmium (Cd), Cobalt (Co), Nickel (Ni) and Copper (Cu) and Antimony (Sb) were present in all samples, but none of them exceeded maximum acceptable level compared with Health Canada or Food and Drug Administration (FDA) guidance on heavy metal impurities in cosmetics that can be tolerated in a different kind of cosmetic products (CFR Title 21 Food and Drugs, Sub-Chapter G – Cosmetics, 2024; Health Canada, 2012).

The presence of lead (Pb) in lakes was from 2.6 to 5.7 mg/kg and for Cadmium (Cd) from 0,13 to 0.24 mg/kg (in dried sapropel samples.) The limits in regulatory guidelines for cosmetics are Pb – 10 mg/kg and for Cd – 3 mg/kg (Health Canada, 2012). The identified concentration serves as an indicator of anthropogenic impact, highlighting the need for careful exploration of site selection (Pavlovska et al., 2020). The most common way of being exposed to heavy metals is through consuming contaminated food. In the case of sapropel sediment, the main route of exposure is expected to be through the skin. The potential for lead and cadmium to be absorbed through the skin is relatively low, and the concentrations found are not considered to be a health threat. However, due to the properties of heavy metals that cause them to accumulate, the concentration levels need to be regularly monitored during the phase of extracting the sapropel to ensure that the allowed concentration in the end product is not exceeded (Alqahtani et al., 2024; Borowska & Brzóska, 2015; Witkowska et al., 2021).

The identification of biologically active substances like humic acids, fulvic acids, phenolic content, and antioxidant activity supports the traditional and perceived health benefits of sapropel. The levels of biologically active compounds, such as humic and fulvic acids, can change based on the age of the sapropel layers (Melo de et al., 2016; Jarukas et al., 2021; Levinsky, n.d.). In the analysed sapropel sediments, concentrations of 22–28 g of humic acids

and 5–9 g of fulvic acids were identified in each kilogram of dried sapropel. The variation in the diversity and activity of microorganisms within the sediment layers impacts the breakdown of organic matter and the generation of bioactive compounds, leading to differences in composition. Furthermore, microorganisms can also facilitate the regeneration and preservation of certain organisms and substances within the sediment. While the presence of microorganisms contributes to the properties and regeneration of the mud, it also presents a significant challenge for its use in pharmaceutical and cosmetic products. The activity level of microorganisms in sapropel is influenced by the sediment depth and prevailing climatic conditions. One possible explanation for the differing levels of microbiological activity in the sediment layers of lakes is the availability of oxygen and the depth, which affect the rate of organic substance decomposition and the formation of bioactive compounds (Klavina et al., 2020; Pavlovska et al., 2020).

A key finding was that while raw sapropel samples did not identify active pathogens, the total bacterial counts (CFU/g) were very high, exceeding regulatory limits for cosmetic products by a considerable margin. Furthermore, some isolated species, such as *Aeromonas sobria* and *Serratia fonticola*, are identified as opportunistic pathogens. A high bacterial count in sapropel sediment samples underscores the critical need for processing steps, such as sterilisation or the addition of preservatives, to reduce the microbial load before sapropel can be safely incorporated into topical applications. The presence of *Serratia fonticola* in the Zeilu Lake particularly indicates potential pollution and necessitates sterilisation (Pavlovska et al., 2020).

The legislative framework in Latvia governing sapropel extraction is comprehensive, covering environmental protection, impact assessment, pollution control, species and habitat conservation, spatial planning, protection zones, taxation, and waste management. Adherence to these laws is essential for ensuring that industrial-scale extraction is conducted responsibly and sustainably, minimising adverse environmental effects. Two guidelines for sapropel extraction developed was a part of the study and will be valuable for potential industrial sapropel miners. First guidelines "*The sapropel extraction guidelines*" describes the sapropel sediment exploration from lakes and second "*Guidelines for stability tests, use and preservation of therapeutic properties of sapropel"* looks deeper in valuable medical properties of sapropel sediments and extracts (Vanadziņš, et al., 2020; Vanadziņš, et al., 2020).

In conclusion, the article "Assessment of sapropel use for pharmaceutical products according to legislation, pollution parameters, and concentration of biologically active substances" confirms that Latvian freshwater sapropel contains valuable biologically active substances and does not exceed current limits for heavy metal or pesticide content in the tested samples, supporting its potential as a raw material for pharmaceuticals and cosmetics. However, the high microbial load necessitates mandatory sterilisation or preservation to meet safety

standards for such applications. Further research is needed to fully identify the specific biome and microflora of sapropel and their roles. The variations observed in chemical composition, antioxidant levels, and microbiological profiles between different lakes and depths highlight the complexity of this natural material and the importance of establishing quality criteria and standard methods for extraction and processing (Pavlovska et al., 2020).

In order to harness the potential of sapropel sediments, it is essential to establish a comprehensive approach for selecting lakes and extracting sapropel samples, considering various factors such as the lake's bottom structure, average depth, the presence of landfills, and proximity to potential pollution sources, which should be included in a sapropel exploration protocol. Although there is no standardised quality control testing procedure for sapropel sediments, this study applied generally accepted principles to enhance our understanding of them. Organoleptic evaluations were conducted to assess characteristics such as colour, texture, odour, and the presence of any inclusions. Chemical analyses were performed to determine aspects like dry matter content, organic matter content, and carbonate levels. Active component assessments were carried out to gauge the antioxidant activity of the sapropel sediments. Microbiological testing was utilised to evaluate the microbiological quality of the sapropel sediments and to identify various types of bacteria, fungi, and yeasts (Klavina et al., 2020; Pavlovska et al., 2020).

Until legal guidelines or regulation for using and testing sapropel sediments are established using the International Organization for Standardization (ISO) standard "ISO 21426:2018 Annex D: Guidelines for Control Analysis of Peloids and Monitoring" can be used as a reference for evaluating and ensuring the safety of sapropel for medicinal use. The SCCS guidelines, particularly those related to cosmetic ingredient safety testing, also are valuable resources for assessing the safety and toxicity of sapropel extracts and its use in medicine (Scientific Committee on Consumer Safety (SCCS), 2021).

The potential of sapropel as a local natural resource, particularly in regions like Latgale in Latvia where significant deposits exist, was noted. Developing evidence-based applications could not only enhance healthcare and medical cosmetics but also promote the utilisation of these local resources and develop them.

Extraction and Analysis of Sapropel bioactive components

The organic components of sapropel originate from freshwater flora and fauna transformed under anaerobic, waterlogged conditions and exhibiting biological activities. Naturally formed sapropel primarily consists of HSs and non-humic substances like carbohydrates, amino acids, lipids, and proteins. HSs are formed from the microbiological

decomposition of plant and animal waste and are characterised by high molecular weight, redox activity, and antioxidant properties. HSs are divided into fractions based on solubility: humic acid (HA), hymatomelanic acid (HMA), fulvic acid (FA), and humin (Klavina et al., 2019, 2020).

Humic acid (HA) is a major organic component of sapropel. It is a complex aromatic polymer with various functional groups and an average molecular weight of 6500 Daltons. HA is soluble in water at higher pH but insoluble under acidic conditions. HA molecules can interact with cell walls and receptors, penetrate tissues, and exert therapeutic effects, including nervous tissue regeneration, stimulation of macrophage defence, tissue repair, and anti-inflammatory action. Animal trials have shown no toxic effects of HA within a wide dosage range. However, the use of HA in medicine is complicated by its heterogenic structure, polydispersity, and properties varying with the source material. The diverse anti-inflammatory, immunomodulatory, and radioprotective benefits of natural substances appear to be linked to their antioxidant and antiradical properties (García-Villén et al., 2018; Klavina et al., 2020; Mirza et al., 2011; Noon et al., 2020; Obuka et al., 2018). For instance, the neuroprotective effect of HA in a focal cerebral ischemia model in rats is likely attributed to the antioxidant capabilities of HA (Alexandrova et al., 2013; Canellas et al., 2015; Melo de et al., 2016; Jurcsik, 1994; Mirza et al., 2011; Yan et al., 2013). The therapeutic actions of HAs have also been connected to the mitigation of oxidative stress (Noon et al., 2020; C. Wang et al., 1996).

Fulvic acid (FA) is another important HS fraction, composed of weak aliphatic and aromatic acids soluble at any pH level. FA molecules are smaller than HA, with an average molecular weight of 1200 Daltons. Despite their smaller size, FAs have a significantly higher absorption capacity (2-20 times greater than HA) due to their functional groups and carbon content. Their smaller size allows FA to penetrate deeper tissue levels and potentially carry trace elements. FAs have also shown bioactivity, including influencing smooth muscle contractile activity (Aiken & Malcolm, 1987; Canellas et al., 2015; Klavina et al., 2020; Klucakova, 2018; C. Wang et al., 1996; Winkler & Ghosh, 2018).

Extracting bioactive compounds from sapropel involves various techniques such as Solid-Liquid Extraction (SLE), Ultrasound-Assisted Extraction (UAE), Supercritical Fluid Extraction (SFE), and others. These methods aim to maximise yield, avoid impurities, and achieve pharmaceutical quality. A crucial first step is often cell disruption, typically achieved by drying samples and applying an alkaline solution. Water or alkaline solutions are common extractants. The review of extraction methods (SLE, UAE, SFE) demonstrates that while several techniques exist, each comes with its own set of advantages and disadvantages concerning efficiency, cost, environmental impact, and potential impact on the extracted compounds (Klavina et al., 2019, 2020).

Classical methods for extracting HA and FA involve using a strong basic substance like sodium hydroxide (NaOH) solution, followed by separation steps based on solubility. NaOH extraction is widely used method that yields high amounts of humic and fulvic acids. It is simple, conventional, and requires no sophisticated instruments. However, it can be time-consuming, uses large amounts of solvents, and can potentially break down high molecular weight HA components at boiling temperatures, although following standard procedures mitigates this. Sodium pyrophosphate is another extractant option, particularly for sapropel with high calcium content, but may lead to heavy metal contamination (Caseldine et al., 2000; Klavina et al., 2019, 2020; Zanin et al., 2018).

Other techniques like Ultrasound-Assisted Extraction (UAE) are rapid, non-thermal, and efficient, reducing solvent consumption and altering HS structure and activity. However, UAE may not be suitable for high-water content samples, has high energy consumption and instrumentation costs, and is difficult to scale up. Supercritical Fluid Extraction (SFE), often using CO_2, is presented as an environmentally friendly alternative with low toxic, recyclable solvents, offering a continuous extraction process free of inorganic salts and heavy metals. Disadvantages include high instrumentation costs and complex operation (Chen et al., 2019; Hidayah & Abidin, 2017; Ivanovs & Blumberga, 2017; Vinatoru et al., 2017; K. Wang & Luo, 2017).

Analysis and characterisation of sapropel extracts face a challenge as there are no commonly accepted standard methods. The minimal quality criteria suggested by 11th Edition of the Pharmacopoeia for plant extracts include moisture, microbial count, heavy metal residue, and density (The European Pharmacopoeia 11th Edition, 2023). The choice of extraction method, alongside sediment pre-treatment and characteristics, significantly influences the extract's composition and the efficiency of the process. Storage conditions can vary based on aggregate state of extract. Liquid extracts and dried HA are stable at 4 °C, while FA is often frozen or freeze-dried and stored at −4 °C. Extracts are reported to maintain biological activity for months in aqueous solutions (Beer et al., 2000; Klucakova, 2018; Lamar et al., 2014; Winkler & Ghosh, 2018).

The use of sapropel extract in medicine and pharmacy is complicated by its variable, heterogeneous, and polydisperse composition. There is no common standard for obtaining or analysing sapropel extract for pharmaceutical use. Factors like the sediment's origin, type, composition, and extraction parameters significantly influence efficiency (Lamar et al., 2014). Pre-treatment and storage methods are also important. While NaOH extraction with sonication is suggested as suitable, and HPLC for analysis, sufficient experimental data for many pharmacopoeia parameters is lacking (Hoang et al., 2021). Despite the current challenges, sapropel extract holds significant commercial potential in cosmetics and pharmaceuticals,

potentially driving the development of more sophisticated and standardised methods in the future.

In conclusion there is the significant potential of freshwater sapropel as a source of biologically active compounds, particularly humic substances like humic acid (HA) and fulvic acid (FA), for applications in medicine, pharmacy, and cosmetics (Klavina et al., 2020; Pavlovska et al., 2020). However, a central challenge is generally accepted standard methods for both extracting these bioactive compounds from sapropel and for analysing and characterising the resulting extracts. This absence of standardisation is a significant challenge for the consistent and reliable use of sapropel extracts in regulated industries such as the pharmaceutical industry. The literature research explicitly states that sapropel's composition is not stoichiometric and varies, leading to heterogeneity and polydispersity in extracts. This inherent variability, coupled with the diversity of sapropel sediments themselves, makes developing universal standard procedures difficult(Klavina et al., 2019, 2020; Obuka et al., 2018).

Antioxidant Properties of Sapropel Extract

Sapropel is attributed with having an antioxidant effect that is believed to improve skin structure, smoothen and prevent wrinkles, remove swelling, strengthen nails and hair, and normalise sebaceous gland secretion (Vanadziņš et al., 2022). This multifunctional effect is attributed to the complex chemical and biological structure, which includes humic acids, fulvic acids, heratomelic acids, various vitamins, and microorganisms (Klavina et al., 2019; Kļaviņa et al., 2024, 2025). Among the important organic acids in sapropel are humic acids (HA) and fulvic acids (FA), which are naturally resistant, high-molecular heterogeneous compounds. These substances consist of both aromatic structures and aliphatic circuits with different functional groups. The polyphenols in humus substances found in sapropel can be used as antioxidants in cosmetics and medicine (Ahmed et al., 2019; Alara et al., 2019; Puangbanlang et al., 2019; Tarnawski et al., 2006; Xu et al., 2017).

The assessment of antioxidant activity in this study was discussed throughout all articles and tested across all study periods. To assess the antioxidant properties in the study, the sapropel extracts was characterised based on for their Total Phenolic Content (TPC), Total Antioxidant Status (TAS), and DPPH radical scavenging activity.

The highest TAS values (1.08 ± 0.03 mmol/L) and TPC (146.26 ± 1.16 µg GAL/mL) were observed in extracts from the Audzelu Lake, which also had high HA content. The lowest TAS (0.31 ± 0.01 mmol/L) and TPC (42.07 ± 0.55 µg GAL/mL) were found in the Ivusku Lake. There was a strong correlation (R2 = 0.90) between TAS and HA concentrations. A substantial correlation (R2 = 0.93) was also found between TPC and TAS. The findings indicate a strong

correlation between antioxidant activity (measured as TAS and TPC) and HA concentration, but no correlation with FA concentration. This suggests that polyphenols, which contribute significantly to antioxidant properties, are responsible for the antioxidant activity observed in the extracts, and their presence correlates with HA content.

Humic acid and fulvic acid concentrations were also measured using a spectrometric method. These concentrations varied significantly between lakes. For HA, the levels ranged from a minimum of 113.1 mg/ml (Ivusku) to a maximum of 167.8 mg/ml (Mazais Kivdalovas). For FA, the range was from 44.5 mg/ml (Dunaklu) to 76.5 mg/ml (Ivusku). Results revealed that the antioxidant activity is dependent on the concentration of the carbon fraction in FA and corelates with organic matter in sapropel. It was found that antioxidant levels are considerably higher in organic sapropel extracts from the Lakes Audzelu, Mazais Kivdalovas, and Zeilu. This broadly aligns with their TPC values being among the highest measured.

One notable tendency was that the Dunaklu Lake had considerably lower levels of both antioxidants and humic and fulvic acids. However, the Ivusku Lake, which had the lowest antioxidant levels, showed a high FA level, indicating that while FA is important, other factors like polyphenols in extract or the carbon fraction concentration within FA also influence antioxidant activity. Lim and colleagues (2019) in their study about *Sargassum serratuifolium* and Zykova et.al. (2017) in their study about HA have found the similar correlation between antioxidant components and what contributes to their activity (Lim et al., 2019; Zykova et al., 2018). High TPC in sapropel extracts suggests potential for treating skin diseases, complex wounds, and chronic non-healing wounds (Aeschbacher et al., 2012; Hoang et al., 2021; Neha et al., 2019).

In summary, the study confirmed the presence of compounds associated with antioxidant activity, such as humic acids, fulvic acids, and total phenolics, in Latvian freshwater sapropel extracts. The levels of these components and the measured antioxidant activity were found to vary significantly depending on the lake from which the sapropel was extracted. Lakes like Audzelu, Mazais Kivdalovas, and Zeilu appeared to have higher levels of compounds contributing to antioxidant potential.

Cytotoxicity Assessment (Neutral Red Uptake – NRU)

The NRU test was used to check the concentration-dependent cytotoxicity of sapropel extracts on mouse fibroblasts (BALB/c 3T3) at FA concentrations of 17.5, 70.0, and 140.0 μg/mL. Assessing NRU data relative to both standard medium (S10) control and solvent control revealed that results correlated well only at the lowest concentration (17.5 μg/mL) (The Food and Drug Administration, 2015). At higher concentrations, the data comparison

diverged, suggesting potential toxic effects from the solvent itself. For instance, at 140 µg/mL, NRU decreased significantly compared to the S10 control but increased significantly compared to the solvent control, indicating the solvent's toxicity at high extract concentrations (Kļaviņa et al., 2024).

Sapropel extract from the Audzelu Lake showed a significant decrease in NRU compared to S10 control at all tested concentrations, but an increase compared to solvent control at 70.0 and 140.0 µg/mL, further highlighting the solvent's potential toxicity. Extracts from the Lakes Ivusku and Zeilu at 17.5 µg/mL showed no harmful effects compared to the S10 control and potentially beneficial effects, with Ivusku showing better NRU than the solvent control. The Lakes Dunaklu and Mazais Kivdalovas showed moderate results at low concentrations, still suggesting potential solvent toxicity. At 70 µg/mL, most extracts showed lower NRU than both controls, though some were slightly better compared to the solvent control.

The article "*Unlocking the Therapeutic Potential of Freshwater Sapropel Extracts: In Vitro Analysis and Antioxidant Profiling for Skincare Applications*" describes the experimental setup involved diluting the cell culture medium to achieve high extract concentrations up to 40 % dilution for 140 µg/mL FA, that could create double-stress conditions (diluted media, high FA, low pH). Mammalian cells are sensitive to pH changes, and the samples did not have an ideal pH for cell cultures, which could have influenced the results. It is speculated that sapropel's pH buffering capacity might have contributed to cell survival by normalising medium pH, potentially more so than the biological activity of the extracts (Phelan & May, 2016, 2017). The solvent toxicity was particularly visible at high concentrations, and when excluded from the analysis, NRU appeared higher than the S10 control (Jurcsik, 1994).

Cell Growth Assessment (Cell-IQ)

Real-time monitoring using Cell-IQ® was employed to assess changes in the growth of BALB/c 3T3 fibroblasts and human keratinocytes (HaCaT) exposed to sapropel extracts at various concentrations. Low concentrations (3.5 µg/mL and 7 µg/mL) generally had no effect on cell growth, except for inhibitory effects of extracts from the Lakes Mazais Kivdalovas and Dunaklu on HaCaT cells after more than 24 hours. At 17.5 µg/mL, a significant promotion of BALB/c 3T3 cell growth was observed for 12 hours, followed by a significant decrease after 24 hours. HaCaT cell growth decreased after extended incubation (> 18h) with the Mazais Kivdalovas Lake extract. At 35 µg/mL, HaCaT growth was generally unchanged, but the samples from the Lakes Mazais Kivdalovas and Audzelu inhibited growth after more than 24 hours. BALB/c 3T3 cells showed slight initial growth increase (12 h) with Mazais

Kivdalovas Lake extract, followed by inhibition. Audzelu and Dunaklu Lakes extracts also showed an inhibitory effect on 3T3 cells, with less initial stimulation. At 70 µg/mL, a significant inhibitory effect on 3T3 growth occurred after more than 9 hours, appearing sooner for the extracts from the Lakes Audzelu and Dunaklu. The Mazais Kivdalovas Lake extract at this concentration showed initial stimulation (6h) on 3T3 cells, followed by a more profound inhibitory effect. HaCaT growth was not significantly affected for up to 12 hours (Audzelu) or 9 hours (Dunaklu) at 70 µg/mL, with significant inhibition thereafter. The Audzelu Lake extract at 70 µg/mL had a slight stimulating effect on HaCaT growth over a longer period (18h). At 140 µg/mL, the Audzelu Lake extract significantly promoted HaCaT cell growth for up to 18 hours and 3T3 cell growth for up to 3 hours. Mazais Kivdalovas Lake extract showed minimal stimulation on HaCaT cells but significant inhibition after 12 hours. Initial stimulation on BALB/c 3T3 cells from Mazais Kivdalovas and Audzelu Lakes extracts was observed for up to 3 hours, followed by a sharp decrease in growth (Hoang et al., 2021; C. Wang et al., 1996).

Overall, the cell growth findings indicate that biologically active substances (FA and HA) in sapropel extracts can promote cell growth in the short term (up to 3–6 hours) at high concentrations, but higher concentrations over longer durations (e. g. > 12 hours) show cytotoxic effects. HA's ability to generate active oxygen may accelerate wound healing, while its antioxidant activity can compensate and restrict peroxidation, suggesting optimal effects at lower concentrations.

The study successfully demonstrated that sapropel extracts from different lakes in Latvia possess varying levels of HA, FA, and antioxidant activity, correlating strongly with HA content and TPC. These characteristics can potentially be used to identify and characterise sapropel sources for cosmetic and pharmaceutical manufacturing. The high antioxidant activity supports the traditional and potential use of sapropel for skin health and wound care (Y. Ji et al., 2016; Kļaviņa et al., 2024; Mehvari et al., 2024; Sim et al., 2022).

However, the *in vitro* testing revealed complex effects depending on concentration and exposure time. While short-term exposure to higher concentrations showed promising cell growth promotion, longer exposures often resulted in inhibition or cytotoxic effects. The NRU test results were significantly impacted by solvent toxicity and suboptimal cell culture conditions (diluted media, low pH) necessitated by the need to achieve high extract concentrations. This highlights a critical challenge in translating sapropel extract research into practical applications: finding appropriate formulations and concentrations that maintain bioactivity while ensuring biocompatibility and avoiding toxicity to mammalian cells (Phelan & May, 2016, 2017).

The study suggests that sapropel extracts, particularly those with high antioxidant properties, do not cause significant harm in cell cultures under certain conditions and could be tested for human product development. Their antioxidant properties potentially protect skin from environmental stress, offering beneficial properties during short-term use (Hoang et al., 2021).

Overall, FA and HA content vary depending on the lake source, influencing the sapropel extract's potential biological activity. There is a strong correlation between antioxidant activity (TAS, TPC) and HA concentration. Sapropel characteristics can help identify suitable sources for cosmetic and pharmaceutical production. While sapropel extracts did not show significant harm in short-term cell culture tests and exhibited potential beneficial properties, particularly antioxidant effects, higher concentrations over longer durations showed cytotoxic effects. The findings suggest a perspective use for short-term topical therapeutic skin applications (Kļaviņa et al., 2024).

For future studies involving cell cultures, it is essential to conduct detailed analysis of natural sapropel samples before cell testing to better understand their effects. Optimise methods to avoid issues like solvent toxicity and media dilution when testing cell cultures, perhaps by finding ways to better control pH and extract concentration in the cell culture environment. Use non-animal, human-relevant toxicity models. Identify sapropel extracts with optimal composition and beneficial biological effects for skin applications. Explore water-soluble formulations for potentially better skin penetration compared to existing dry sapropel extract products (Kļaviņa et al., 2025). The cytotoxicity assessment was only part of a project aimed at analysing medical sapropel, elaborating extraction methods, and potentially developing products. A patent was filed for a water-soluble sapropel extract hydrogel and its preparation method, that was the innovation of this study (Auce et al., 2022).

Sapropel Extract Hydrogel: Preparation and Stability

The article "*Sapropel-enriched sodium carboxymethyl cellulose gel systems: formulation approaches, stability and bioactive potential*" investigates the development of stable, water-soluble hydrogels containing sapropel extract for potential pharmaceutical and cosmetic uses. Traditional applications like thermal mud baths require stationary places and large amounts of material, making them inconvenient. Water-insoluble creams and patches containing sapropel extract exist (Strus et al., 2018, 2019). In a trial with 23 volunteers who had heightened skin sensitivity to irritants (dermatitis), the cream was applied to the skin every 24 hours, leading to a reduction in inflammation. Patches that incorporate the sapropel extract are also recognised. These patches, which contain the sapropel extract cream, are placed on the skin for 24–96 hours, resulting in decreased inflammation in cases of dermatitis and eczema

(Ozkan et al., 2015; Winkler & Ghosh, 2018), but a stable, water-soluble hydrogel of sapropel extract, preserving bioactive properties and maintaining shape after application, was poorly researched, highlighting the desirability of the current study.

Ointments and gels are popular dosage forms for topical applications and are extensively used in both medicine and cosmetics. Modern skincare products are recognised for their adaptability, delivering diverse and comprehensive effects even with relatively straightforward formulations. A prime example of biological effects observed in commonly used cosmetic products is the application of a hydrolipid occlusion layer or various forms of anti-radical protection to the epidermis. These techniques are employed in medicine, pharmacy, and cosmetics (Barbulova et al., 2015; Bevan et al., 2013; Bom et al., 2019; Costa & Santos, 2017; Ficheux et al., 2019; Gianeti & Maia Campos, 2014).

Researchers have developed a water-soluble hydrogel system incorporating sapropel extract, utilizing sodium carboxymethylcellulose (Na-CMC) as the gelling agent. This hydrogel is designed as a potential delivery system for the bioactive compounds present in sapropel, aiming to address the limitations associated with traditional applications such as mud baths and therapeutic patches.

Sodium carboxymethylcellulose (Na-CMC), a water-soluble cellulose derivative known for its gel-forming capabilities, non-toxicity, biodegradability, and biocompatibility, was used as the gelling agent. Na-CMC is soluble in water, forming a neutral or alkaline transparent viscous liquid that can produce a three-dimensional hydrogel structure. It does not trigger an immune response, unlike some animal-origin polymers, and has shown no signs of irritation or significant adverse effects in toxicity studies (Ghorpade et al., 2018; Mo et al., 2022; Sebert et al., 1994; Wellens et al., 2022).

Eight different hydrogel formulations were prepared and evaluated: four containing sapropel extract and four without, to assess the effect of the extract on the hydrogel. The formulations used Na-CMC (2.5 %), glycerol (8 %), ethanol (8 %), and purified water, with or without sapropel extract (5 % containing 140 µg/g FA). Buffer solutions of sodium chloride, magnesium sulphate, or magnesium chloride were also included in some formulations. The sapropel extract was obtained from mixed sediment layers of the Audzelu Lake in Latvia, identified as promising for medical products. Hydrogel preparation involved mixing ingredients at controlled temperature and speed (Burgardt et al., 2015).

The prepared hydrogels were subjected to various evaluation tests over a 2-year period under different storage conditions (4 °C, 23 °C, 45 °C, dark, UV light) (Kļaviņa et al., 2025). Organoleptic properties, including visual inspection for appearance, colour, odour,

homogeneity, consistency, and signs of instability were determined during each storage period under specified conditions.

Stability was evaluated through physical and chemical tests for each formulation developed in this research (Kļaviņa et al., 2025):

- Centrifugal test: Assessed resistance to centrifugal force.
- Thermal tests: Freeze-thaw and heating-cooling cycles tested thermal stability.
- Contact angle: Measured surface wettability (hydrophilicity/hydrophobicity).
- Water retention: Evaluated moisture retention at 60 °C over time.
- Microscopy (Polarized Optical & SEM): Analysed texture, morphology, and structure.
- pH: Monitored pH changes over time.
- Viscosity: Measured at varying spindle speeds.
- Rheology: Assessed complex viscosity, shear-thinning, yield stress, and viscoelastic moduli (G', G").
- XRD: Identified mineral content in sapropel and hydrogel crystallinity.

All sapropel extract-containing formulations were smooth, homogeneous, and light-yellow with good spreadability and acceptable stability. Hydrogels without extract were transparent. Organoleptic properties scored high initially but decreased over a 2-year period, especially at elevated temperatures and under UV light. Centrifugal and thermal tests showed no phase separation or distress, confirming physical stability. All formulations showed a tendency to wet the surface with contact angles below 90°, decreasing over time, indicating hydrophilic properties. Formulations with buffer salts and sapropel extract showed a shift towards more hydrophobic behaviour but lost water content slower than the formulation without them.

Formulations could hold a considerable amount of water, with water retention between 42 % and 53 % after 16 hours at 60 °C. Formulations with sapropel extract and buffer salts lost water content more slowly. Microscopy and SEM revealed that all formulations had a porous structure, with those containing both buffer salts and sapropel extract being more porous and having a rougher surface.

pH levels ranged from 4.7 to 7.4, within the acceptable range for skin application. Formulations with magnesium chlorate showed greater pH fluctuations, while others, especially those stored at 23 °C in the dark, had stable pH values.

Viscosity analyses showed that sapropel extract decreased viscosity, particularly under fluctuating temperatures. Magnesium sulphate-buffered formulations exhibited the most stable viscosity, while sodium chlorate-buffered formulations also showed stable viscosity over time

with higher values after 2 years. Magnesium chlorate-buffered formulations maintained overall stable viscosity but showed fluctuations or increases over time.

Rheological tests confirmed the shear-thinning nature of the hydrogels, where viscosity decreases with increasing shear rate. They also showed the yield stress required to initiate flow and the thixotropic behaviour (reversible transition between solid-like and liquid-like states under stress). G' and G" values were higher for formulations with magnesium salts.

XRD analysis confirmed the amorphous structure characteristic of hydrogels in all formulations. Analysis of sapropel extracts from different depths revealed variations in mineral composition and crystalline structure of humic substances and fulvic acid.

In the discussion, the feasibility of incorporating sapropel extract into stable, water-soluble hydrogels suitable for therapeutic and cosmetic uses. The hydrogel crosslinking process occurs through two mechanisms. Firsts, ionotropic gelation which is achieved the addition of divalent soluble salts like magnesium chlorate and magnesium sulphate. Magnesium ions (Mg^{2+}) interact with the negatively charged carboxyl groups of Na-CMC, forming a crosslinked network. Magnesium sulphate-buffered formulations showed better stability compared to magnesium chlorate (Fang et al., 2022).

Second hydrogen-bonded hydrogel formation initiated by the addition of acidic sapropel extract containing HA and FA, which lowers the pH level. This process replaces sodium ions (Na^{+}) in CMC with hydrogen ions (H^{+}) promoting hydrogen bonding and decreasing CMC's water solubility, resulting in a flexible hydrogel. HA and FA within the sapropel extract also act as mild crosslinking agents due to their negatively charged functional groups interacting with H^{+} and Mg^{2+} ions, enhancing the crosslinking process. Their amphiphilic nature may contribute to the porous matrix observed in the images. The porosity and water retention capabilities are seen as beneficial for wound healing applications, helping to absorb wound exudation and keep the wound hydrated (Al-Arjan et al., 2022). The slow water loss in formulations with sapropel extract and buffer salts suggests the extract changes the network structure. The presence of Mg^{2+} ions, introduced via the buffer salts, is also discussed for its biological significance, noting its role in various processes and its potential to promote angiogenesis and modulate inflammation, creating a favourable environment for wound healing (Al Alawi et al., 2018). The pH range of the formulations (4.7–7.4) is considered favourable for wound healing, as it aligns with the varying pH of acute and chronic wounds (Sim et al., 2022; Zhang et al., 2022). Sapropel extract's acidity helps in crosslinking and provides extra stability, potentially extending shelf life.

Na-CMC hydrogels are considered good candidates for topical drug delivery systems due to high water content, low irritation, and ability to facilitate deeper skin penetration.

Na-CMC is also a mucoadhesive gel, which could increase contact between the sapropel extract and the wound surface, potentially boosting the effectiveness of HA and FA (Silva da et al., 2022; Pornpitchanarong et al., 2022). Sapropel extract itself is noted for its regenerative abilities on epidermal cells and potential to promote collagen synthesis, eliminate free radicals, and inhibit melanin formation, which could aid in wound healing (Kļaviņa et al., 2024, 2025).

The article presents four possible models for how the hydrogel containing sapropel extract might interact with wounded and undamaged skin. Firstly, the enhancement of transdermal passages due to the permeation enhancers ethanol and glycerol present in the formulation. Secondly, hydrogel and lipid exchange with the stratum corneum, potentially enhancing hydration and permeability, though exact lipid concentration was not determined. Thirdly, free release of HA and FA directly into the wound, leveraging their anti-inflammatory, antimicrobial, antioxidant, and regenerative properties. And finally, intact vesicular skin penetration, if vesicles are formed within the hydrogel, protecting active ingredients and ensuring sustained release (Kļaviņa et al., 2025). Overall, the study suggests the sapropel-enriched Na-CMC hydrogel is a potential pharmaceutical product for preventing, treating, and accelerating wound and scar healing. The use of CMC and sapropel also aligns with the global priority of using natural, biodegradable local resources for environmentally friendly products.

In conclusion, during this study a stable and homogeneous sodium carboxymethylcellulose (Na-CMC) hydrogel system incorporating sapropel extract was successfully developed and optimised for topical application. The resulting formulation was free of crystalline impurities, exhibited high water content, and showed low potential for skin irritation. Enhanced transdermal delivery of bioactive compounds further indicates its potential for use in wound care and drug delivery. The study demonstrated that sapropel extract – rich in humic and fulvic acids – not only contributed to hydrogel stability and crosslinking but may also support wound healing through mechanisms such as promoting collagen synthesis and reducing oxidative stress. The hydrogels maintained favourable pH levels and remained stable under varied environmental conditions, reinforcing their suitability for pharmaceutical development (Kļaviņa et al., 2025).

Preliminary observations in this study suggested possible mechanisms for skin penetration of sapropel compounds, including enhanced permeability due to the hydrogel matrix and the bioactivity of humic substances. However, dedicated studies to experimentally confirm and quantify these mechanisms were not conducted within the scope of this work and remain an important direction for future research.

Despite these promising results, challenges remain. Effective penetration through the *stratum corneum* must be ensured to achieve therapeutic efficacy. Future work should investigate key parameters including hydrogel-skin interactions (e. g. contact angle, surface tension, adhesion energy), fibroblast viability, and the controlled release kinetics of sapropel-derived compounds. This research provides new insight into the role of magnesium salts in hydrogel formulation, offering a potential route to enhance structural integrity and therapeutic function.

Overall, the findings support the potential of sapropel-based hydrogels as an innovative, nature-derived solution for topical therapies. This aligns with broader healthcare goals focused on sustainable, bio-based treatment strategies. Further experimental validation and clinical studies are essential to confirm safety and efficacy in real-world applications.

Conclusions

1 Sapropel is a valuable natural resource found in Latvian lakes and beneath peat layers in bogs. Extraction is governed by a legal framework; guidelines for exploration were developed as part of this research. Its rich bioactive compounds, including humic and fulvic acids, provide important anti-inflammatory, antioxidant, and antimicrobial benefits, making it effective for promoting skin health and treating conditions like acne, rashes, and dermatitis.

2 Tested samples contained heavy metals and pesticide residues; their levels in the tested samples were within acceptable limits for cosmetic use. However, raw sapropel samples exhibited high microbial counts, exceeding limits for topical applications, necessitating mandatory sterilisation or preservation steps for safe pharmaceutical and cosmetic formulation.

3 Bioactive compounds, predominantly humic acids (HA) and fulvic acids (FA), are commonly extracted from sapropel using solid-liquid alkaline extraction methods. The concentration and biological activity of these components vary based on the sapropel source and the specific extraction process employed, highlighting the need for standardised protocols to utilise sapropel extract in pharmaceutical use. Sapropel extracts demonstrated promising antioxidant activity and short-term cell regeneration-promoting effects *in vitro*, although prolonged high concentrations showed cytotoxic effects.

4 Eight formulations of sodium carboxymethylcellulose (Na-CMC) hydrogels were successfully developed, with four incorporating sapropel extract. The presence of HA and FA in the extract facilitates dual crosslinking mechanisms within the Na-CMC polymer network, contributing to the formation of a robust, porous structure. Magnesium salts play a crucial role in enhancing ionic crosslinking and contributing to the hydrogel's porosity, which is beneficial for properties such as water retention and the potential sustained, slower release of therapeutic agents.

5 Sapropel-enriched hydrogels exhibited favourable physical stability, maintained a biocompatible pH range, and retained organoleptic qualities, particularly under all storage conditions, supporting their potential for use in topical products. Formulations buffered with magnesium buffer salts and sapropel extract demonstrated the most consistent stability over time.

6 Physical-chemical and persistence testing confirmed the hydrogels' suitability as a platform for topical application, addressing challenges of traditional sapropel use.

In conclusion, the Thesis supports the hypothesis. Sapropel extract demonstrates effective compatibility with carboxymethyl cellulose gels, contributing positively to their structural stability and enhancing their potential as delivery platforms for therapeutic and cosmetic applications, particularly in areas like skin care and wound healing.

Future Research Proposals

1 Investigate Skin Penetration Mechanisms

While potential pathways for sapropel compound absorption have been hypothesised, targeted experimental studies are needed to confirm and quantify them. Future work should employ *in vitro* and *ex vivo* skin models to better understand diffusion dynamics and optimise therapeutic efficacy.

2 Evaluate Hydrogel–Skin Interface Properties

To enhance product performance and user comfort, further studies should examine physical interactions at the hydrogel–skin interface. Key parameters include contact angle, surface tension, adhesion strength, and moisture retention.

3 Conduct In Vivo and Clinical Trials

Building on promising *in vitro* data, controlled animal studies and clinical trials are essential to evaluate safety, biocompatibility, and therapeutic outcomes in real-world scenarios, particularly for wound care, dermatological, and cosmetic uses.

4 Explore Broader Applications of Sapropel-Based Hydrogels

Beyond wound healing, sapropel-based hydrogels hold potential for treating inflammatory skin conditions and serving as carriers for localised drug delivery. Expanding application fields could enhance the material’s medical relevance.

Publications and reports on topics of Doctoral Thesis

Publications:

1. Vanadzins, I., Martinsone, I., **Klavina, A**., Komarovska, L., Auce, A., Dobkevica, L. and Sprudza, D. 2022. Sapropel – mining characteristics and potential use in medicine. *Proc. Latvian Acad. Sci., Section B,.* Received 29.06.2018. Published 02.06.2022. Vol. 76, No. 2 (737):188–197. doi: 10.2478/prolas-2022-0029.
2. Pavlovska, I., **Klavina, A**., Auce, A., Vanadzins, I., Silova, A., Komarovska, L., Silamikele, B., Dobkevica, L. and Paegle,L. 2020. Assessment of sapropel use for pharmaceutical products according to legislation, pollution parameters, and concentration of biologically active substances. *Sci Rep.* Published 09.12.2020. 10:21527. dio: doi.org/10.1038/s41598-020-78498-6.
3. **Klavina A.,** Auce A., Vanadzins I., Silova A., Dobkevica L. 2019. Extraction of Biologically Active Components from Freshwater Sapropel. *Environmental Technology. Resources. Proceedings of the 12th International Scientific and Practical Conference.,* Published: 20. 06.2019. 3:114–118. doi: 10.17770/etr2019vol3.4135.
4. **Klavina, A.**, Reste, J., Martinsone, I., Vanadzins, I., Lece, A., and Pavlovska, I. 2024. Unlocking the Therapeutic Potential of Freshwater Sapropel Extracts: In Vitro Analysis and Antioxidant Profiling for Skincare Applications. *Medicina*. Published 27.03.2024. 60(4):546. doi: 10.3390/ medicina60040546.
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Guidelines:

1. Scientific workgroup: Vanadziņš, I., Pavlovska, I., Mārtiņsone, I., Sprūdža, D., Silova, A., Dobkeviča, L., Komarovska, L., Auce, A., Paegle, L., Muižnieks, A., **Kļaviņa, A.,** Blāķe, I., Silamiķele, B., Ribinska, L., Čerpakovska, Z. Published on 30.03.2020. The sapropel extraction guidelines. Developed in project No.1.1.1.1/16/A/165. RSU. Riga, Latvia.
2. Scientific workgroup: Vanadziņš, I., Pavlovska, I., Mārtiņsone, I., Sprūdža, D., Silova, A., Dobkeviča, L., Komarovska, L., Auce, A., Paegle, L., Muižnieks, A., **Kļaviņa, A.,** Blāķe, I., Silamiķele, B., Ribinska, L., Čerpakovska, Z. Published on 31.03.2020. Guidelines for stability tests, use and preservation of therapeutic properties of sapropel. Developed in project No.1.1.1.1/16/A/165. RSU. Riga, Latvia.

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Acknowledgments

I extend my heartfelt gratitude to my supervisors, Ilona Pavlovska from the Institute of Occupational Safety and Environmental Health, and Assoc. Prof. Baiba Mauriņa from the Faculty of Pharmacy for their guidance, invaluable advice, and relentless support throughout this study.

Special thanks to Lead researcher Inese Martinsone for introducing me to the fascinating field of sapropel research and its potential medical applications. Their initial guidance and inspiration were fundamental in shaping the direction of this Thesis and initiating the investigation into the valuable properties of sapropel as a raw material for medicinal products.

I also express my sincere gratitude to Sergejs Gaidukovs, Professor of Polymer Chemistry and Technology at Riga Technical University, and his team for their invaluable support and assistance with the rheology testing of the hydrogels.

I am also grateful to the entire project team for their cooperation and assistance during the data collection process for the project "*Analysis of characteristics of medical sapropel and its usage for medical purposes and elaboration of industrial extraction methods*".

Finally, I would like to express my deepest gratitude to my family for their unwavering support and encouragement throughout my academic pursuits.

Annexes

First Publication

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PROCEEDINGS OF THE LATVIAN ACADEMY OF SCIENCES. Section B,
Vol. 76 (2022), No. 2 (737), pp. 188–197.

DOI: 10.2478/prolas-2022-0029

Review

SAPROPEL – MINING CHARACTERISTICS AND POTENTIAL USE IN MEDICINE

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Communicated by Modra Murovska

Found in lakes, freshwater sapropel is a sediment with a fine structure containing more than 10% of organic matter as well as residues of aquatic organisms with a small content of inorganic components of biogenic origin and mixture of mineral ingredients. The mud was first used in medicine by ancient Greeks, and it gained more popularity together with development of balneology in Europe in the 19th century as a remedy for several diseases. The last century also brought wider popularity in its cosmetic use. Despite its wide usage, mechanisms behind its effects are not so clear yet. Broad but fragmented studies on the effects of sapropel are available, but few have used modern research methods. There is evidence suggesting that its positive health effects are linked to its thermal capacity, ability of penetration in tissues and biological activity of its components, e.g., humic substances. Evidence also suggests antimicrobial activity and positive effects on skin regeneration. This review aims at summarising available knowledge on the structure and composition of sapropel and its effects on the human body, as well as its potential for further evidence-based use in medicine and cosmetics.

Keywords: *peloids, general characteristics, mud therapy, health benefits.*

INTRODUCTION

Human beings have always been plagued by the need to protect their health and beauty and defend against various illnesses. This can be seen as far back as the 5th century BC when the ancient Greek scientist Herodotus (484–425 BC) conceived the method on the use of mineral waters. In the works of the founding father of medicine, Hippocrates (460–370 BC) references to the curative effects of salted sea water can also be found (Jackson, 1990). In the 19th century, a new scientific discipline — balneology started to develop thanks to the English doctor J. Currie and the founder of modern hydrotherapy — Austrian doctor V. Priessnitz. Balneology uses mineral and thermal waters as well as different types of mud for the improvement of health and treatment of various maladies (Michler, 2005; Miclaus *et al.*, 2011). During the 17th–19th centuries, mud therapy gained popularity in Europe, where there was a rapid development of balneology, with clinics founded in Germany, France, Italy, Austria, and Romania. Experiments testing the application of mineral waters and mud were carried out, testing for the effects of different variables such as temperature, application periods, and chemical composition such as the level of sulphur and nitrous oxides needed for the treatment of patients suffering from arthritis. Mud remedies also gained popularity in cosmetology and cosmetic surgery of the time, with mud being used to speed up the regeneration and renewal of skin (Routh *et al.*, 1996; Groven, 2013; Correia *et al.*, 2016). Later, during the early 20th century, the use of hydrotherapy was combined with other types of therapies, e.g., peloid therapy (also called mud therapy), massage, iontophoresis, phonophoresis physiotherapy, and physical exercise. These methods were aimed at fortifying the patients' health and were rather effective in many cases (Becker, 1994; Guillemin *et al.*, 1994). These therapeutic methods produced effective outcomes for a variety of health problems, such as rheumatological disorders, osteoarthritis, fibromyalgia, spondylosis, and a number of different musculoskeletal disorders (Falagas *et al.*, 2009). Despite the rather long use of peloids including sapropel, scientifically proven

results that can be called evidence, started to appear only during the last hundred years. With the recent revival and development of non-pharmaceutical methods in medicine, the importance of positive scientific evidence of the health effects of sapropel has increased. Detailed and systematic search of peloids and sapropel is essential to encourage scientifically sound medical and cosmetic use of sapropel based on firm evidence (such as balneology) at the same time facilitating a wider usage of local natural resources in healthcare and medical cosmetics.

Peloids formed during complex biological transformations of Holocene sediment. It consists of mud substance, crystal frames and a colloid fraction. F. Guimaraes and L. Guimaraes were the first researchers who started to use the term "peloids", in 1931 (Gomes *et al.,* 2013). The first chemical analysis of peloids was already performed in 1807 by French chemist Desser (Tserenkhand and Badnainyambuu, 2016). The mud substance is composed of various minerals, while the crystal frame is formed by poorly dissolved plaster, clay, and carbonate particles. The colloid fraction, however, is more complex, consisting from different organic substances such as silicic acid and iron and aluminium hydroxides. The organic substances constitute from 50 to 98% of dry mass in peat mud and from 15 to 95% in sapropel. Organic substances in mud are mainly composed of humic substances, waxes, vitamins, ferments, and amino acids. Peat mud also contains lignin and celluloses. Other various complex substances based on oxygen, carbon, iron, phosphorus, silica, sulphur etc. also possess biologically active characteristics. Hydrogen sulphides found in mud as soluble sulphides, hydrogen sulphide and ferrous-sulphide are believed to have significant medical effects. There are several types of sapropel and the most common classification used is linked to their origin, e.g., seawater sapropel and freshwater sapropel (Segliņš and Brangulis, 1996; Tserenpil *et al*., 2010; Stankeviča and Kļaviņš, 2013).

The structure and content of sapropel depends on the place of extraction and can vary significantly. There have been several attempts to establish a common classification involving the freshwater, seawater, and thermal muds based on their mineralisation level. The most popular classification method used today was elaborated by V. Aleksandrov in 1958 (Dēliņa *et al*., 2016). This classification system defines:

1) inorganic sediment mud — mud from various lagoons, mud from the seabed, rivers and lakes (partially);

2) organic sediment mud, including sapropel — mud from the seabed of various lagoons as well as freshwater and saltwater lakes;

3) peat mud — dominated by a humic organic substance with some inorganic impurities;

4) mixed mud — consisting of inorganic substances and a small amount of plant residues;

5) volcanic mud, including hydrothermal mud;

6) artificial mud with a similar structure to some groups of natural mud (Dēliņa *et al*., 2016; Rautureau *et al*., 2017; Gomes *et al*., 2013).

SAPROPEL

Sapropel is a fine-graded organic sediment found in freshwater lakes that is produced by sedimentation and transformation of residues from aquatic plants and various living organisms together with mineral particles (e.g., sand, clay, calcium carbonate, etc.) (Stankevica *et al*., 2016). Freshwater lakes in Latvia can possibly contain both sapropel and peat mud. The difference between them mostly lays in their finer structure, acidity, content of organic and humic substances, and the types of sedement-forming living organisms. Peat mud was formed in an anaerobic environment while the sapropel mud commonly developed in an environment that contains oxygen. The pH level of peat mud mainly is acidic while sapropel is neutral. The organic part in peat mud is usually around 50% while in sapropel mud it can typically fluctuate between 15 to 85%. Peat mud is mostly formed by various plants that grow in swamps, deciduous and coniferous trees, scrubs, grasses and mosses (Stankeviča and Kļaviņš, 2013), while sapropel is typically formed by aquatic organisms, such as phytoplankton, zooplankton, and water and coastal plants (Korde, 1960; Stankeviča and Kļaviņš, 2013; Leonova *et al*., 2015). The name "sapropel" originated from Greek words: *sapros* — rotten and *pelos* — clay. First the terms "gyttja" un "dy" were used by the Swedish researcher H. von Post in 1862, but German researcher R. Lauterborn proposed the term "sapropel" in 1901, which was widely introduced in the scientific terminology by D. Potonie (Nijenhuis, 1999; Stankeviča and Kļaviņš, 2013).

Sapropel is a renewable natural resource; the average intensity of formation of sediment varies from 0.1 to 6.64 mm per year depending on the type of lake and climate. Sapropel mud develops in lakes and similar water basins where the formation of biomass exceeds mineralisation; such processes are widespread in lakes found in mild climate zones, particularly in forested areas, shallow and overgrown lakes as well as swampy river beds and valleys (Stankeviča and Kļaviņš, 2013).

MINING OF SAPROPEL IN THE WORLD AND LATVIA

The most intensive formation and accumulation of sapropel occurred in the mild climate zones of Europe and Asia, but the deposits of sapropel have not been thoroughly surveyed. They have been found in Belarus, Ukraine, Russia, Mongolia, Germany, Poland, Czech Republic, Romania, Estonia, France, the Scandinavian countries as well as North America (Anderson, 1996; Tserenpil *et al*., 2010; Strakhovenko *et al.*, 2014; Leonova *et al*., 2015; Pleiksnis *et al*., 2015). In some countries the study of sapropel deposits has been mostly focused on saltwater sapropel and freshwater sapro-

pel extraction opportunities have not been properly and systematically addressed (Leonova *et al.*, 2015).

There are more than 2250 lakes in the territory of Latvia, but only 16 of them cover more than 10 km^2. The total lake surface in Latvia is approximately 1000 km^2 or 1.5% of the territory of Latvia. It has been proven that most of the Latvian lakes and some of the swamps can contain sapropel deposits. It has been estimated that the total stock of sapropel in Latvian lakes could be around 700 million m^3 (Nikodemus *et al.*, 2018). Silicate sapropel with ash content of over 65% (as well as limonite sapropel with iron oxide content over 10%) has the potential for further usage (Segliņš and Brangulis, 1996). The largest sapropel deposits with over 300 million m^3 are found in several regions in Latgale: Rēzekne — 94.2 million m^3, Preiļi — 66.1 million m^3 and Daugavpils — 65.3 million m^3. Significant deposits are also found in the regions of Krāslava and Ludza (Lācis, 2003; Noviks *et al.*, 2019, pp. 113–116).

CHARACTERISTICS OF SAPROPEL

As mentioned before, sapropel is a fine-graded organic sediment of freshwater lakes that contains more than 10% of residues made of organic substances and various living organisms with a small content of inorganic biogenic compounds and mineral ingredients (e.g., sand, clay, calcium carbonate etc.). Sapropel is a pasty substance with different colours (light grey, rose, brown, olive or black) with a pH level from 5 to 8 and humidity in the range of 65 to 95% (Korde, 1960; Kurzo *et al.*, 2012; Leonova *et al.*, 2015).

Sapropel sediments in lakes and swamps started to develop after the last ice-age, which took place 12–15 thousand years ago (Stankeviča and Kļaviņš, 2013). However, massive formations of sapropel started to develop during the Holocene period (10 000 years BC) and the oldest know deposits are roughly 11–12 thousand years old (Stankeviča and Kļaviņš, 2013). Sapropel could have an autochthonous origin if the formation had been caused by the sedimentation of the lake biomass, as well as allochthonous origin in cases where the lake biomass deposits were supported by additional biomass and organic compounds (humic substances) from inbound rivers. Higher levels of organic substances are found in sediments of autochthonous origin (Strakhovenko *et al.*, 2014; Yermolaeva *et al.*, 2016).

There are several theories that describe the formation process of sapropel. The most popular theory is that the main mass of sapropel is composed by three main components: mineral substances of allochthonous origin, inorganic compounds with biogenic origin and organic substances (both autochthonous and allochthonous origin) — residues of plants and small aquatic organisms (Schepetkin *et al.*, 2002; Lācis, 2003; Stankeviča and Kļaviņš, 2013; Strakhovenko *et al.*, 2014). A literature review by Stankeviča and Klaviņš (2013), which also covers various classification systems of sapropel, found that there was no agreement on a single theory and that there are various opinions and systems used. The most popular classification system used for freshwater sapropel that can be used for the determination of potential sapropel use is a three-group approach (biogenic, clastic, and mixed sapropel) that is further divided into several classes (organogenic, organogenic-silicates, diatoms, silicates, carbonates, and iron containing) (Stankeviča and Kļaviņš, 2013).

STRUCTURE AND CHARACTERISTICS OF SAPROPEL

Sapropel has a complicated chemical structure that is determined by the biological and biochemical variety of organisms and the compounds forming it from the three main components — organic substances, minerals, and residues of plants and aquatic organisms (Strakhovenko *et at.*, 2014; Leonova *et al.*, 2015). One of the most important characteristics of sapropel is its colloidal structures, as these structures can tie up a significant amount of water that is very slowly released afterwards. After drying, the mud loses its ability to attract more water and thus the specific mass and density are higher.

The potential for the use of sapropel is determined mostly by its composition, physical, and mechanical characteristics. In general, the most important properties of sapropel are the following: high dispersity, good plasticity (colloid characteristics), thermal capacity, low heat transfer, absorption capacity, homogeneity, consistence, biostimulation, antioxidation, and antimicrobial characteristics (Schepetkin *et al.*, 2002; Suárez Muñoz *et al.*, 2015; Dēliņa *et al.*, 2016).

CHARACTERISTICS OF ORGANIC COMPOUNDS

Various authors have slightly different definitions of organic substances that form sapropel. They can be defined as insoluble leftovers of hydrobionts (e.g., fishes) and colloid organic substances that have been brought from inbound waters, including biological and organic components that are mostly biopolymer and adsorption complexes with a low molecular mass (Kurzo *et al.* 2012; Stankeviča and Klaviņš, 2013; Strakhovenko *et al.*, 2014). However, sapropel has a very low content of carbohydrates. Organic and humic substances that can be found in sapropel are shown in the Figure 1 (Stevenson, 1982).

The main substances that are formed in the process of plant humification are fulvic and humic acids; they comprise a general category of naturally occurring, biogenic, heterogeneous, and refractory organic substances of high molecular weight (Stevenson, 1982).

Other important components of sapropel are various waxes (lipids). Their composition is characterised by fatty acids, carotenoids, paraffins, and waxes. The wax components of sapropel have high potential use in medicine as they have

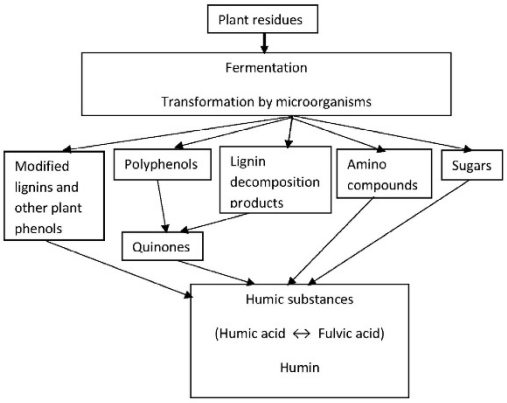

Fig. 1. Formation of organic and humic substances in sapropel (by Stevenson, 1982).

high bactericidal, bacteriostatic and antioxidant activity. There is also a high level of carotenoids in freshwater sapropel (Stankeviča and Kļaviņš, 2013; Kurzo *et al.*, 2017).

CHARACTERISTICS OF HUMIC ACIDS AND FULVIC ACIDS

Humic substances form a significant part of sapropel. Their age can vary from several hundred years up to 10 000 years, depending on their deposition site. Molecular size can vary from 400 up to 1 000 000 daltons. Humic substances in sapropel can form metal complexes, dissolve hydrophobic organic substances and reduce surface tension (Meng *et al.*, 2017).

Depending on their water solubility there are three different fractions:

- humine: fraction that is insoluble in water;
- humic acids: fraction that is soluble in water if the pH is higher than 2;
- fulvic acids: fraction of humic substances that is soluble in water regardless of pH.

Humic substances can form in various environmental settings, usually they have autochthonous origin, or they can be brought from various other environments, e.g., washed out from soil to the surface or from deep-water sediments. Humic substances form as a result from secondary synthesis (humification) during decomposition, when biomolecules are transformed after being produced by various microorganisms that consume residues of plants or aquatic organisms. Humification is an extremely complicated biochemical process — decomposition of residues of plants and aquatic organisms, mineralisation of organic substances, effects of microorganisms to organic resides etc., which leads to further decomposition of organic substances, further transformation and accumulation of different substances (Kireicheva and Khokhlova, 2000). There has been some research on the toxicological properties of humic substances that showed that toxicity of humic substances formed in natural processes is very low (Krūmiņš *et al.*, 2013).

CHARACTERISTICS OF MINERAL SUBSTANCES

The content of mineral substances in sapropel is very different among deposits. Formation of mineral complexes in sediments of freshwater lakes is often linked to inbound river waters in lakes where sedimentation of minerals as well as soluble minerals occurs. The most common sediment materials leached into waters are quartz, dolomite, silicates, and aluminosilicates (feldspar, hydrous mica, chlorite, kaolinite etc.) (Leonova *et al.*, 2015; Glavaš *et al.*, 2017). Biochemical processes lead to deposition of Mg, Ca, Sr, Ba, Fe, Mn, carbonates, plaster, hematite, pyrite, marcasite, and vivianite in sapropel (Tserenkhand and Badnainyambuu, 2016). Iron phosphates as well as iron oxides are also rather common in all types of sapropel and their contents are linked negatively to carbonate content. The content of iron phosphates in carbonated sapropel is 0.4% on average, mixed sapropel — 0.8% and 1.4% in silica sapropel, respectively (Stankeviča and Kļaviņš, 2013).

Depending on the concentration of microelements sapropel can be divided into:

- silica sapropel;
- mixed type sapropel;
- organic sapropel;
- carbonated sapropel.

Sapropel also contains microelements Mn, Co, Mo, Cu, Ni etc. Silica sapropel is particularly rich in microelements, but relatively low concentrations of these microelements can are found in carbonated sapropel. The total content of B, Ni, Cu, Co, V, Mo, Cr can vary from 20 to 150 mg/kg dry mass, and for Ti, Mn, Zn from 200–2000 μg/kg dry mass. Some other elements are also found, e.g., iodine can be deposited in sapropel from plants (Stankeviča and Kļaviņš, 2013). Another research direction is analysis of processes that occur during the maturation of sapropel and other peloids; these are believed to be associated with changes in the redox environment as well as the processes that cause release of ions and other compounds and their incorporation in the sediment. Some of the potentially toxic elements (e.g., Cd, Pb, Hg, Cr, As, Fe, Zn, Mn, Cu) are also of concern to researchers as they can penetrate the skin, especially after contact with sweat (Suárez Muñoz *et al.*, 2015).

Another unresolved issue is the effect of various heavy metals that are found in sapropel as there are risks that, if the sapropel is used for medical or cosmetic purposes, further reactions are possible between these metals and other components of sapropel, especially when they interact with hu-

man biomembranes and can easily resorb through the skin (Mihelčič *et al.*, 2012).

CHARACTERISTICS OF MICROORGANISMS

Freshwater sapropel can contain waste amounts of various microorganisms — 1 gram of sapropel (upper level) can contain up to 1–2 billions of microorganisms (Stankeviča and Kļaviņš, 2013). There are significantly larger bacterial populations in sapropel sediments that are rich with organic substances, compared with mineral sapropels. For example, the bacterial population size can be as high as 7.76 (10^8) cells per cm^3 near surface levels (seawater sapropel) and as low as 1.0 (10^6) per cm^3 below the sea floor (Cragg *et al.*, 1998). Apparently, the available oxygen levels in sediment at different depths influence the diversity of microorganisms and their chemical and biochemical reactions, and also influence the general sedimentation rate of sapropel as the presence of oxygen can both stimulate or inhibit the growth of microorganisms in sapropel (Wurzbacher *et al.*, 2010; Stankevica *et.al.*, 2014).

Fungi and actinomycete are known to participate in synthesis of antibiotics and sulphanilamide, and bacteria and algae produce vitamins that also participate in organic substance decomposition, thus participating in humification (Nikolajev, 2003). Not only the living organisms are responsible for destruction of various organic substances and creation of sediment, but they also can regenerate and preserve some of the organisms. New therapeutically active compounds can be formed during maturation by the action of the growth of living organisms including diatoms, cyanophycean, bacteria, protozoa, and the organic compounds originated by their metabolic activity and degradation (Gomes *et al.*, 2013). Microorganisms are also responsible partly for the release of various gasses (hydrogen sulphide, ammonia, methane etc.). All these processes of biologically active and antibacterial substance sedimentation can lead to potential health effects when sapropel is used in balneology.

However, in the deepest levels of sapropel the microbiological activity is practically nil, thus making the humification processes slow. Studies in Latvia (lakes Kaņieris and Babītes) found that the spread and biochemical activity of microorganisms are directly linked to depth of sediment as well climatic conditions (time of the year) (Sturis, 1965). Similar results were also found in Russia (Lake Ochki) (Leonova *et al.*, 2015). The highest activity of microorganisms was found in the sediment levels ranging from 0.1 to 0.5 meter depth, but activity decreased rapidly from a depth of more than 1 meter (Sturis, 1965). Table 1 lists the groups of microorganisms found in sapropel samples using oat agar. The following groups of bacteria were identified in samples: ammonifying bacteria 1.5 × 105 colony forming units (CFU); *Genus bacillus* 4.7 × 02 CFU; *Oligotrophyc bacteria* 3.8 × 102 CFU. However, the full spectre of bacteria determined using DNS sequencing has not been performed yet.

Table 1. Percentage of microorganisms found in sapropel

Microorganisms	Quantity (%)
Bacteria	92.3
Actinomycetes	5.1
Micromycette	2.6

USE OF SAPROPEL IN MEDICINE

The multidimensional effects of sapropel on the human body can be explained by its complicated chemical composition and biological structure. Its effects are also linked to significant thermal capacity as well as the content of various metals and other chemical elements, hormones, amino acids etc., which have the potential to penetrate the dermal barrier. Biological activity of sapropel is determined by a variety of components that are known to be biologically active, e.g., humic substances, fulvic acid, hemotamalanic acid, water soluble vitamins (ascorbic acid (C), thiamine (B1), riboflavin (B2), pantothenic acid (B5), pyridoxine (B6), folic acid (B9), and cyanocobalamin). A significant number of fat-soluble vitamins like tocopherol (E), vitamins D and P have also been found as well as some microorganisms known to be capable of producing antibiotics that are antagonistic towards several saprophytic pathogens (Kireicheva and Khokhlova, 2000; Szajdak and Maryganova, 2007).

The presence of quartz and calcite in sapropel can also contribute to the beneficial effects of sapropel and its potential application in balneology (including spa and beauty therapy). The small quartz and calcite particles are known to have stimulatory effect on certain mechanisms in the body, for example, circulation (Glavaš *et al.*, 2017).

The acid base balance (pH) of sapropel is rather close to that of a healthy human skin that ensures better penetration into the deeper layers of skin. These characteristics to some extent provide a dual process. During application of sapropel there is penetration from sapropel towards human body and the reverse, as sapropel can absorb some toxins that are being transferred through skin. There is no evidence that sapropel would be responsible for side effects so its use in medicine should be expanded and more research is required to scientifically back the health effects of sapropel. Several sources (Veniale *et al.,* 2007; Gomes *et al.,* 2013; Rautureau *et al.*, 2017) state that peloids can be used for:

- improvement of blood and lymph flow, strengthening of blood vessels, improvement of oxygen exchange;
- improvement of antibacterial therapy due to antibacterial properties;
- enrichment of the body with calcium, magnesium, bromine, iodine, potassium and amino acids;
- reduction of skin aging processes due to its antioxidation properties;

- regeneration of hydro-lipid membranes;
- improvement of skin structure, reduction of subdermatic fat tissue; reduces wrinkles;
- reduction of swellings;
- improvement of nail and hair growth and prevention of hair loss;
- improvement of skin fat gland function;
- improvement of immune system function;
- reduction of symptoms of some skin diseases (psoriasis, seborrhoea, acne etc.).

Currently, sapropel products are mostly used in balneotherapy and cosmetology, especially for chronic conditions. The medical use of peat and sapropel in Europe dates back to the first half on 19th century. Traditional indications in European practice have been various diseases of the musculo-skeletal system, gynaecological, rheumatological, as well as dermatological disorders (Karelina, 1999; Badalov and Krikorova, 2012). A review by Stankeviča and Kļaviņš provided wide information regarding the use of humic substances (especially fulvic acids) for treatment of various diseases: asthma, respiratory diseases, autoimmune arthritis, oncological diseases, virus haemorrhagic fever, ulcerative colitis, diabetes, stomach bleeding, gastritis, duodenal ulcer etc. Several studies have shown that humic substances have anti-inflammatory effects. Some studies also showed that humic substances can protect liver cells from destructive changes caused by external factors and functional overload (Stankeviča and Kļaviņš, 2013). There is also relatively wide information about the effects of humic substances from natural sources on their ability to absorb heavy metals. Humic substances tend to create chelation complexes with toxic heavy metals, which provides an opportunity to use these substances for chelation therapy both for excretion of them and for reduction of toxic side effects. F. N. J. Ridwan observed that even 0.1% application of humic substances was sufficient to demonstrate assimilation of lead and cadmium in test animals (Krūmiņš *et al.*, 2013).

EFFECTS OF SAPROPEL ON MICROCIRCULATION IN SKIN AND MUSCLES (THERMAL CAPACITY)

Several studies have shown that the therapeutic mechanism of peloids in general and sapropel is based on relatively high thermal capacity, poor heat transfer and low convection capacity, which ensure that the accumulated heat is slowly radiated and thus provides slow and deep heat transfer to tissue (Schepetkin *et al.*, 2002; Suárez Muñoz *et al.*, 2015). Already in 1920, a theory was formulated that friction between the skin and peloids creates an electric potential that has a positive effect on microcirculation in skin and muscles. Application of sapropel influences various receptors in skin and mucous membranes, thus initiating reflective stimulation towards the neuro-endocrine system and circulation system, which influences functional changes of microcirculation and metabolism in tissues, organs and organ systems. Warmed sapropel application has been used as a treatment for phlegmons, mastitis, furuncles, chronic gastritis etc. Use of peloids can be viewed as an effective method to induce the whole-body reactivity. Clinical and experimental studies have shown that biological substances of sapropel have a high potential for penetration of skin and tissues and are capable of induction of cell response, thus improving circulation. Sapropel applications are known to improve peripheral blood circulation, oxygen transportation and metabolism. It also improves coronary circulation, changes contractility of myocardial tissue and peripheral resistance. During sapropel application, there is an increase in pulse and breathing rate, elevation of blood pressure, increased sweating, kidney function as well as reduced activity of the gastrointestinal system (Uzbekov, 1958; Kostjakova, 1985; Carabelli *et al.*, 1998; Odabasi, 2008; Espejo-Antúnez, 2012). Several studies showed that changes in microcirculation and small blood vessels cannot be explained solely by vasodilatation (Poensin *et al.*, 2003). Changes in markers responsible for inflammatory mechanisms and articular pain — TNF-alfa, IL-1ß, PGE2 and LTB4 levels were observed after sapropel application. A lower level of reaction is believed to be linked with metabolism stimulation in cartilage. Levels of pituitary hormones were observed to increase due to activation of the hypothalamic-pituitary gland axis as an answer to heat stress induced by the specific thermal capacity of sapropel (Bellometti *et al.*, 2000; 2002). Significant reduction in superoxide dismutase and catalase activity was found after the application of sapropel at 42 °C (Jokić *et al.*, 2010).

EFFECTS OF SAPROPEL ON TISSUE REGENERATION AND IMMUNE SYSTEM

Sapropel has biostimulating effects — it stimulates metabolism and the immune system, encapsulation around foreign bodies in tissue, better healing of wounds and regeneration of tissue and it has desensitisation properties. Sapropel stimulates functioning of phagocytes, resulting in intense tissue regeneration. A 12-day trial showed that after application of hot sapropel (47 °C) there were no significant changes in SP-selectine, IL-1 and TNF-alfa but there was a statistically significant reduction in the IL-6 level, showing that sapropel application could be considered as a safe procedure for patients with atherothrombosis (Basili *et al.*, 2001). Several cytokines and growth factors are responsible for inflammatory processes and degeneration of cartilage, including IL-1 and TNF-alfa that promote reduction of cartilage inflammation, while IGF I is responsible for protection of cartilage structure (Bellometti *et al.*, 1997). A study that involved 37 arthritis patients found that sapropel baths had positive effects both on homeostasis of cartilage and reduction of inflammation, reducing values of NO and myeloperoxidase, while there was no correlation in increase of GSH peroxidase (Bellometti *et al.*, 2000). Another study focused on analysing the fermentative and molecular mechanisms during sapropel application. It was found that TNF-alfa, IL-1ß, PGE2 and LTB4 levels were reduced

(Bellometti *et al.*, 2002), but synthesis of noradrenaline, cortisol, beta endorphins and insulin increased (Bellometti *et al.*, 1996).

ANTIOXIDANT EFFECTS OF SAPROPEL

It is possible to prepare various products from sapropel that have antioxidant characteristics. Research into antioxidant activity of humic substances showed that it had capacity for a direct effect towards neutralisation of various forms of active oxygen as well as several other chemical radicals that are produced as side products in metabolism (Fedko *et al.*, 2005).

Fulvic and humic acids in sapropel are another known antioxidant that reduces the amounts of superoxides and free radicals and thus can play a significant role in inflammatory processes. Fulvic acid is also known for natural chelation with capacity to reduce toxicity of various organic xenobiotics by reducing their transport within the cell. Humic acid in doses from 10 to 100 μg/ml has effect on lipid peroxidation, depending on the dose. Such changes are followed by destruction of glutathione and reduction in activity of catalase, dismutase superoxide, glucose-6-phosphatase and dehydrogenase. Humic acid and fulvic acids increased the amount of informative RNS in cells that is responsible for various biochemical processes. The amount of several enzymes increases, which in turn positively affects the rate of several catalytic reactions (de Melo *et al.*, 2016).

ANTIMICROBIAL AND BACTERIOSTATIC CHARACTERISTICS

Sapropel has antimicrobial and bacteriostatic characteristics — ability to hinder growth of pathogenic microorganisms or to destroy them, thus influencing faster end of inflammatory processes (Strus *et al.*, 2014; Suraganova *et al.*, 2014; Tretjakova *et al.*, 2015). Several microorganisms have been found in sapropel, which are capable of producing antibiotics that are antagonistic to several saprophytic pathogens. This ability has been successfully used in balneology. Antimicrobial activity has been evaluated against *Staphylococcus aureus, Escherichia coli ĄŃŃ 25922, Pseudomonas aeruginosa ĄŃŃ 27853, Bacillus subtilis ĄŃŃ 6633, Proteus vulgaris ATCC 4636, Candida albicans ĄŃŃ 885/653* and it was found out that antimicrobial activity is more evident with application (exposure) time (30, 60, 120, 240 minutes) (Suraganova *et al.*, 2014).

A study of Lake Pribic (Volinska region, Russia) and antimicrobial properties of its sapropel sediment confirmed antimicrobial activity against the following test cultures — *Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853, Basillus subtilis ATCC 6633, Proteus vulgaris ATCC 4636,* and *Candida albicans ATCC 885/653*, while it was not active against *Staphylococcus aureus* (Strus *et al.*, 2014).

Another study analysed the antimicrobial activity of dried sapropel mud (from Lakes Ruson and Ubagova, Latvia). Its antimicrobial activity was tested on the following cultures — *Staphylococcus aureus ATCC 25923, Salmonella enteritidis ATCC 13076, Enterococcus faecalis ATCC 29212, Bacillus cereus ATCC 10876, Escherichia coli ATCC 25922,* and *Candida albicans ATCC 10231* (Tretjakova *et al.*, 2015). It was found that the samples showed activity against a reference culture of *Staphylococcus aureus* (before exposure to direct sunlight) while samples from Ubagova lake did not showed any activity (Tretjakova *et al.*, 2015). It is possible that fresh or correctly stored sapropel has different antimicrobial activity than for dried samples (Tretjakova *et al.*, 2015). A study at Lebazje Lake (Tatarstan) analysed biological activity of sapropel, and found that the biological activity is linked to microorganisms — antagonists: spore forming mould, fungi and actynomicetes. Microorganisms in sapropel (nitrifying, denitrifying, ammonium groups, mycobacteria, funghi etc.) engage in production of nitrogen compounds that have effects on ferment activity of catalase, peroxidase, dehydrogenase. Sapropel is rich with water soluble vitamins and has strong antimicrobial activity against *Staphylococcus aureus*. Antimicrobial activity was also identified against *E. coli, C. perfringens* and *Ps. aeruginosa* (Platonov *et al.*, 2014). It has also been found that sapropel contains large numbers of bacteria and actinomyceta that have antimicrobial activity against pathogens. Antagonism has been identified against typhoid and parathytic pathognes as well some pathogenic fungi (*Achovion Schorleini, Achovion gypseum etc.*) (Marchenko and Gurinovich, 1976).

Several publications also note antibacterial anti fungi activity of humic substances (Fedko *et al.*, 2005). Several patents have been registered for sapropel-based products using these characteristics of humic substances (Fedko *et al.*, 2005). Recent studies show mediatory capacity of H_2S (hydrogen sulphide) (gaseous form) and the effects of H_2S on regulation of inflammatory processes in tissues, which can be another mechanism for potential beneficial effects of sapropel towards reduction of inflammatory processes (Brancaleone *et al.*, 2014).

USE OF SAPROPEL IN MEDICINE AND COSMETICS

There is wide potential for use of sapropel in both medicine and cosmetology. Sapropel demonstrates powerful healing and restorative effects on the human body due to its composition and content of variety of chemically active substances — carotenoids, tocopherols, polyphenols, chlorophylls, flavonoids, phospholipids, enzymes as well as humic acids and some other biologically active substances. Traditional indications for balneology in European medical practice are musculo-skeletal disorders, gynaecological, dermatological diseases as well as some diseases in stomatology (Dunaev *et al.*, 1996). Sapropel has also been successfully used for treatment of chronic disorders (Strelis and Zhivotiagina, 1991; Karelina, 1999). Characteristics of fulvic acids and humic acids have been successfully used for treatment of

diabetes, cold stress, rheumatic disorders, immune disorders etc. Fulvic and humic acids chemically link and excrete toxins and heavy metals (Krūmiņš *et al.*, 2013). Sapropel and its extracts are successfully used in cosmetic products due to their ability to prevent and reduce aging effects, improve regeneration of skin and regulate skin moisture levels as well as improve the natural ability of skin to protect the body against ultraviolet light. Sapropel also has antibacterial properties and many cosmetic products using sapropel have been invented and patented as soaps, tonics, cleansing masks, massage oils, shampoos etc.

However, the medical characteristics of Latvian sapropel have been insufficiently studied and further studies are necessary to elaborate evidence-based suggestions for its use in balneology, ensuring development of new medical procedures and services and promoting development of new export products.

ACKNOWLEDGMENT

ERAF Project "Analysis of characteristics of medical sapropel and its usage for medical purposes and elaboration of industrial extraction methods", project No. 1.1.1.1/16/A/165.

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Received 29 June 2018
Accepted in the final form 8 April 2022

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SAPROPELIS — IEGUVES VIETAS, RAKSTUROJUMS UN PIELIETOJUMS MEDICĪNĀ

Saldūdens sapropelis ir nogulumi ar smalku struktūru ezeros, kuri satur vairāk nekā 10% organisko vielu — ūdens organismu atliekas ar nelielu biogēnas izcelsmes neorganisku komponentu saturu, kā arī minerālo ingredientu piejaukumu. Sapropeli lietojuši jau senie grieķi, bet plašāku popularitāti tas ieguva 19. gadsimtā, attīstoties balneoloģijai, kur sapropelis tika izmantots vairāku slimību ārstēšanā. Pēdējā gadsimta laikā tas plaši lietots arī kā kosmētisko līdzekļu sastāvdaļa. Neraugoties uz sapropeļa plašo izmantojumu, zinātniskā izpēte par tā iedarbības mehānismiem nav pietiekami detalizēta. Veikti plaši, taču fragmentēti pētījumi, tomēr tādu, kuros lietotas modernas izpētes metodes, nav daudz. Pētījumi liecina, ka sapropeļa pozitīva ietekme uz organismu saistīta ar tā īpatnējo siltumspēju, kā arī spēju iekļūt audos un tā sastāvā esošo vielu, piemēram, humīnskābju, bioloģisko aktivitāti. Pētījumi liecina arī par sapropeļa antimikrobiālo ietekmi un pozitīvo iespaidu uz ādas reģenerāciju. Šī apskata mērķis ir apkopot pieejamo zinātnisko informāciju par sapropeļa struktūru un sastāvu, tā ietekmi uz cilvēka ķermeni un tā zinātniski pamatotas lietošanas potenciālu medicīnā un kosmetoloģijā.

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Assessment of sapropel use for pharmaceutical products according to legislation, pollution parameters, and concentration of biologically active substances

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Development trends need the necessity for wider use of the local resources and available natural materials are one of the priorities around the world. Freshwater sapropel is a common material in the water basement of the lakes, but still not sufficiently explored. The main goal of the project to start detailed and systematic research on the medical properties of sapropel to be obtained in Latvia, promote its scientifically based use in balneology, develop new medical procedures and services, and promote development of new exportable products. The results include the survey, sampling depths, and processing, evaluation of external signs, physical, chemical, and biochemical parameters, and evaluation of microbiological indicators. Active components from the sapropel samples extracted using the alkaline method. Sapropel extracts were characterized by organic carbon content, humic and fulvic acid concentrations, total phenolic content, trace metal and pesticide concentrations, total antioxidant status, and microbiological flora. Summarizing the article's main findings it was concluded that Latvian freshwater sapropel can be used as raw material for obtaining sapropel extract and use it in the preparation of pharmaceuticals and promote the development of new exportable products and services.

Medical importance. Sapropel might seem something mysterious and incomprehensible that can be found somewhere deep in the water and is sunlight inaccessible. However, an extremely interesting and useful material has long been a major success for health improvement and treatment. Sapropel used in medicine for a long time and is widely used in various health sectors, but still not sufficiently explored. Sapropel is a common material in the water basement of the lakes in Latvia[1–6].

Sapropel is sludge sediment in lakes, with a fine structure that contains incompletely divided organic matter and microscopic aquatic life forms residues with trace of sand, clay, calcium carbonate, and other rock impurities[2,7–10].

Sapropel is a pasty mass of light grey, pink, brown, brownish olive or almost black. Sapropel's deposits in swamps and lakes only occurred on post-ice age, which took place in the Baltic States 12–15 thousand years ago.

Medical mud formed by complex biological transformations of Holocene sediments. The composition of the therapeutic mud depends on the location of the acquisition—freshwater, saltwater or thermal springs. Sapropel sludge is classified as inorganic sediment sludge, river or lake mud, organic sediment sludge, freshwater and saltwater lake mud, peat sludge, mixed sludge, volcanic sludge and artificial sludge[10,11].

In ancient times, people considered that sapropel can cure almost any disorder, even improve the long-term effects on the skin. Even today it is attributed that sapropel is marvellous material for wide range applications.

Sapropel is a multifunctional and widely used medical treatment, and believed to be useful for lymphatic and circulatory enhancement, vascular strengthening, skin structure, cellulite and subcutaneous fat reduction. It has a pronounced antibacterial effect and enriches the body with calcium, magnesium, bromine, iodine, potassium, and amino acids. Sapropel has an antioxidant effect that improves skin structure, smoothens wrinkles and

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prevents new wrinkles, removes swelling, strengthens nails and hair, normalizes sebaceous gland secretion, helps hair loss. The therapeutic effect of sapropel helps to restore immunity, maintain the cellular structure of various skin diseases—dermatitis, seborrhea, acne, and other rashes and other skin diseases. However, today, sapropel preparations are most widely used in balneotherapy and cosmetology, especially in the treatment of chronic or protracted diseases[3,12–20].

Sapropel complex chemical and biological structure explains its multifunctional effect on body. The bioactivity of sapropel determines by its humic acids, fulvic acids and heratomelic acids, various vitamins and microorganisms that release antibiotics. Previously, sapropel commonly used in raw form and there is no standard methods for sapropel extraction, generally. Currently, there are few extraction methods for getting bioactive components from raw sapropel[19]. Latvian freshwater sapropel could be used as raw material for getting sapropel extract and use it as remedy. All mentioned above brings us to the main question for sapropel usage in medicine, balneology and pharmacy "how to develop quality criteria for raw sapropel and its extracts". The quality criteria should include minimum requirements for pollution levels (heavy metals, pesticides), biologically active substance concentration, pH values, antioxidants as well as physical characteristics[21,22].

Sapropel legislation. It is important to monitor and inspect sapropel extraction sites to assess the level of contamination and the environmental impact of anthropogenic activity. Sediment contamination is considered to be a major environmental issue because sediment acts as a reservoir for pollution. Sediments are an integral part of the aquatic ecosystem, which provides food and habitat for various aquatic species.

Production of sapropel in the industrial scale in Latvia is regulated by several Laws and Cabinet Regulations. One of them is the *Environmental Protection Law*[23], which is the main normative act in the field of environmental protection. The purpose of the law is to ensure the preservation of the quality of the environment and the sustainable use of natural resources.

The *Law on Environmental Impact Assessment*[24] defines the activities that require environmental impact assessment. The need for an environmental impact assessment procedure for the extraction of sapropel in lakes is governed by Chapter IV Section I point 1 and point 25 of Annex 1.

Obtaining Sapropel must also comply with the *On Pollution Law*[25]. *Pollution*, purpose is to prevent or reduce damage to human health, property and the environment caused by pollution. The law sets out the procedures and guidelines that must be taken into account when performing polluting activities to minimize the impact on natural resources such as soil, air, and water. The planned extraction of minerals should take into account the emissions of water, and air pollutants.

Law On the Conservation of Species and Biotopes[26] regulates issues related to the protection of protected species and habitats. One of the main aims of the law is to ensure biodiversity by preserving the fauna, flora, and biotopes characteristic of Latvia. Extraction of the sapropel can also pose a threat to the habitat in the lake and affect species diversity.

The acquisition of Sapropel must comply with the *Spatial Development Planning Law*[27]. When planning the extraction of mineral resources, the conformity of the intended land use with the municipal spatial plans shall be taken into account.

It is important to consider the *Protection Zone Law*[28] when planning the acquisition of a sapropel. The main tasks of this Law are to determine the types and functions of protection zones. The task of certain areas shall be to protect different types of objects (natural, as well as artificial) from undesirable external effects, to ensure the exploitation and safety thereof or to protect the environment and people from the harmful effect of an object.

The purpose of the *Natural Resources Tax Law*[29] is to limit the mismanagement of natural resources and environmental pollution, as well as to promote the introduction of new and improved technology that reduces environmental pollution.

When extracting mineral resources, the legal requirements regarding the management of hazardous waste and municipal waste generated by the equipment used in the extraction process *Waste Management Law*[30] must be observed.

When obtaining sapropel on the industrial scale, it is also important to comply with a number of Cabinet Regulations, including the *Regulations on Lists of Specially Protected and Restricted Species*, No. 396[31]; *Regulations on List of Species of Specially Protected Habitats*, No. 350[32]; *Rules on the criteria used to assess the significance of the impact of damage to particularly protected species or habitats*, No. 213[33].

With regard to the extraction of sapropel, it is necessary to assess its impact on lake water quality; to ensure that the environmental quality standards for priority and hazardous substances in surface waters and the priority substances in the lake biota are not exceeded during the extraction process[34].

Regulations on the Discharge of Pollutants into Water, No. 34[35] establishes limit values and a prohibition for the emission of pollutants into water, as well as the procedures by which the operator controls the number of pollutants discharged into water, perform monitoring and provides relevant information.

Before starting the extraction of the sapropel, attention should be paid to the *Law on Subterranean Depths*[36], which is one of the most important normative acts regulating the extraction of natural resources. It defines the procedure for the complex, rational and environmentally friendly use of subterranean depths. Pursuant to section 15 of that law in accordance with the procedures specified by the main requirements for the protection of subterranean depths which may be attributed to the extraction of a sapropel are the rational extraction of minerals and the use of by-products from the field; and use of subterranean depths without adverse effects on mineral resources and subterranean properties.

Figure 1. Sapropel extraction in Latgale District. Workers from geological research company "Geo Consultants, Ltd." extract the samples. All pictures are the property of RSU Laboratory of Hygiene and Environmental Health, prepared in accordance with the *LVS EN 1997-2 AC:2014 L* (*Eurocode 7—Geotechnical design—Part 2: Ground investigation and testing*).

Materials and methods

An important step is to determine the potential sources of sapropel accumulating within the depositional setting. Another consideration for site selection is to ensure that access granted from the relevant landowner and permission sought from the relevant agency if the site is designated as protected. It is vital to ensure that there is no risk of damaging any subsurface utilities (gas, electricity etc.)[19].

In most of the Latvian lakes, there is sapropel, in many swamps and under the peat layers, it also found. Major stocks of this mineral resource are around 300 million cubic meters, mostly located in Latgale districts, eastern Latvia (the light blue to dark blue region on a map, Fig. 1). Samples of sapropel were extracted from five lakes: Audzelu Lake (Istra rural territory), Dunakla Lake (Zvirgzdene rural territory), Ivusku Lake (Cornajas rural territory), Zeilu Lake (Cirmas rural territory) and Mazais Kivdalova Lake (Purenu rural territory). The location of lakes is marked with a circle in Fig. 1.

Official geological survey of Latvia lakes from Latvian lake database of association "Latvijas ezeri" (www.ezeri.lv) used in the selection of the area of the exploration.

The main selection criteria were the sapropel deposits depth, hydrological regime, the history of agriculture next to the lake and the potential exposure to industrial waste. One hundred and five sapropel samples obtained from five lakes (Zielu – Z, Mazais Kivdalova – K, Dunakla – D, Ivusku – I and Audzelu – A) during the wintertime (extraction equipment is installed on a platform over the ice and was therefore more stable).

Since the sapropel accumulates in the lake, there may be differences, depending on inflowing brooks and trenches in the lake, which may bring pollutants that are deposited closer to estuaries and also on the age/depth of the sapropel layer (therefore, there can be differences in the concentration of potential pollutants).

Prior to the sample collection, the thickness of the proper sediment layer was determined and the depth of sapropel deposit established for each of the lakes as well as within each of the lakes by taking probes. Well-composed sapropel layer for further laboratory analyses taken on the three different depths of sapropel sediment at each extraction point through the lake coordinates (Fig. 2). The amount of extraction points through the lake were different from 1 to 11 taking into account the lake dimension (Z 1–9, K 1–10, D 1–11, I 1–7 and A 1–11).

The lakes influenced by several external and internal factors. The soil conditions, climatic conditions, and access facilities to the main road and to the fields applied to mainly natural external factors. Internal factors depend on the type of business enterprises around, farmstead and the relative position of its different buildings. Among general principles that must be taken into account is the availability of transportation between buildings and driveway to the lakes.

To better explore the possibilities of using Latvian sapropel, Riga Stradins university (RSU) researchers have launched a three-year study to test and standardize a composition, properties, storage options and therapeutic effects of the sapropel.

There are main characteristics of the sapropel samples. Organoleptic properties – the color ranges from pale yellow to black, depending on the type of sapropel and the site of exploration. The texture is determined based on the initial description of the site. The smell is neutral (if any changes in the smell observed, the storage conditions of the samples should be checked). Sapropel must be homogeneous inconsistency, with no inclusions or excess water. Another one characteristic of sapropel is dry matter content. The sapropel is dried and weight loss compared to samples of a recognized sapropel site. This is mainly to determine if the series of raw materials are not obtained too shallow at the top of the sapropel layer. Sapropel is divided into four main types – organic, silica-containing, carbonate and mixed type sapropel. There are several kinds (peat, carbonated, iron-rich, mixed, silicate with increased ashes contain, etc.) of each type of sapropel, the main type being determined by the biological and oxide content of the sapropel[37].

Sample collection carried out with a semi-cylindrical chamber with cone cap and longitude closed shutter made of stainless steel used with sample chamber dimensions 1000 × 75 mm. Samples from three different depths at the seven different localizations (21 samples in total) were established for each of the lakes.

The appropriate sapropel layer found from 2.0 to 9.0 m (experimental sapropel layer was from 0.9 to 11.4 m) from the surface of the sediment layer exact depth depending on the lake and the position of the measurement point. Actual thickness and location varied depending on the depth of the lake and degree of the decomposition of organic matter. If the depth is less than 1.5 m from the surface of the sediment layer, sapropel sediments are not fully developed and not used in this study.

Each sample identified by specifying the exact location of the site in the lake and the depth of extraction from the surface of the water and the beginning of the sludge layer. The sediments (Fig. 3a) were removed from plastic containers (Fig. 3b), then refrigerated, and kept at 4 °C (temperature closer to the natural water temperature at the bottom of the lake) and then, stored.

For the extraction of active components from the sapropel samples the alkaline method was selected (Fig. 3c)[38].

Sapropel extracts were characterized by total organic carbon content (TOC), humic acid (HA) and fulvic acid (FA) concentrations by the use of the spectrometric method.

The sample pH level was determined using distilled water (volumetric ration sample: water – 1:2.5).

Sapropel samples were analysed for organic matter and carbonate content using the loss-on-ignition (LOI) method Loss on ignition. The dried sapropel sample was heated for 4 h at 550 °C and 2 h at 900 °C, after each heating the sample weighed and calculated by assuming that all organic matter in the sample is burned at 550 °C, and at the next temperature (900 °C). An important parameter of sapropel is the amount of organic matter. It can be determined whether organic substances mineralize by releasing their nutrients or accumulate in sediment and their mineralization process is slow. The amount of organic matter in the sediments of the lake can vary (from 20 to 90%), depending on the productivity of the lake and the type of land use in the catchment area. The carbonate content (from 1 to 15%), in turn, depends on the amount of carbonate soils in the catchment area, as well as on benthic organisms in molluscs whose shell may contain carbonates[39].

Trace metal concentrations were determined in sapropel samples by electrothermal atomic absorption spectrometry with Zeeman background correction. Before starting the analysis, sapropel samples were dried at 105 °C and finely ground with a mortar and pestle. Sampling was carried out in a closed container with microwaves in the digested system using nitric acid and hydrogen peroxide. The contents of the vessels were quantitatively transferred to 20 mL graduated polypropylene flasks and filled to mark with Milli-Q water[38].

The content of the total phenolic content (TPC) of the extract was expressed as gallic acid (GA) equivalents. The gallic acid was used to set up a standard curve. An aliquot of 500 µl of an extract was mixed with 2.5 ml of Folin-Ciocalteu phenol reagent (10 × dilutions) and allowed to react for 5 min. Then 2 ml of 7.5% Na_2CO_3 solution was added and allowed to stand for 1 h before the absorbance of the reaction mixture was read at 765 nm. All tests were performed six times. The total polyphenol contents of the extract were evaluated from the gallic acid standard curve and expressed as mg of gallic acid per gram of plant material[40,41].

Total antioxidant status (TAS) in samples was measured using Randox Total Antioxidant status kit (Randox Laboratories Ltd.) adapted to the RX Daytona automated chemistry analyzer (Randox Laboratories Ltd)[41].

2,2-diphenyl-1-picrylhydrazyl (DPPH) is a stable organic radical; in a chemical reaction, it functions as a radical and it is a scavenger of antioxidants. DPPH solution is violet with maximum absorption at 515 nm, while its reduced form is yellow. Therefore, the decreased level of absorption at 515 nm adding extracts was proportional to the natural substance antioxidant activity[41].

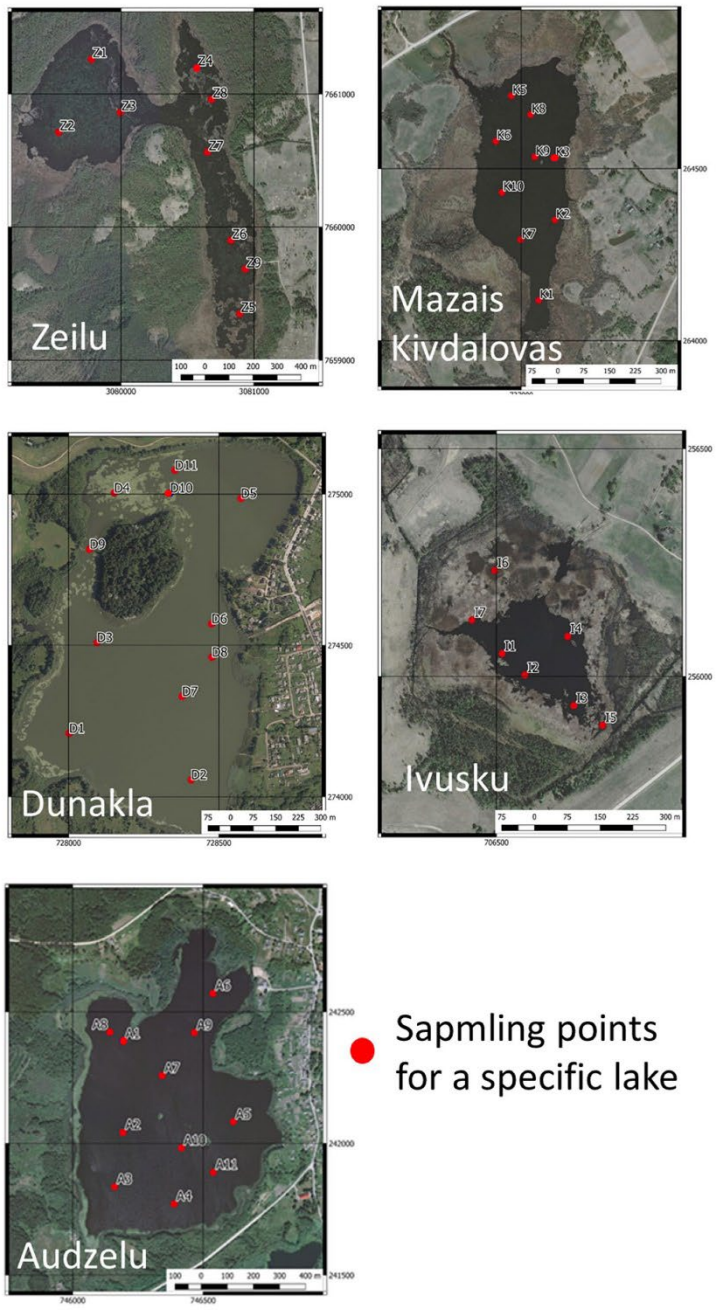

Figure 2. Overview of sapropel sampling points for a specific lake. All pictures were made in cooperation with geological research company "Geo Consultants, Ltd." and are the property of RSU Laboratory of Hygiene and Environmental Health, prepared in accordance with the *LVS EN 1997–2 AC:2014 L* (*Eurocode 7 – Geotechnical design – Part 2: Ground investigation and testing*).

Figure 3. Storage of sapropel in the: (**a**) closed plastic containers, (**b**) removed from plastic containers and (**c**) selected samples for the extraction of active components.

The antiradical activity (six replicates per treatment) was expressed as IC50 (mg·mL ^{1})—the concentration required to cause a 50% DPPH inhibition. The ability to scavenge the DPPH radical was calculated by using the following equation: $\%_{(inhibition)} = 100\cdot(A_0 - A_1)/A_0$, where A_0—average absorption for the "empty" sample (contains solvent), A_1—average absorption for the test sample[41].

The calibration curve was obtained with TROLOX/methanol. The free radical scavenging activity for the sample was calculated after the Trolox equivalent and expressed in millimoles of Trolox equivalent (TE mmol·L-1) of the sample solution[41]. All antioxidant parameters were measured to sapropel extract with fulvic acid concentration 700 mg/L.

Detection and quantitation of dichlorodiphenyldichloroethylene (DDE)/dichlorodiphenyltrichloroethane (DDT) were realized by applying DDE/DDT ELISA kit.

Microbiological measurements were provided by the Institute of Food safety, Animal Health and Environment "BIOR". Test methodology for specific organisms reported the results in CFU (colony-forming unit), the actual count from the surface of a plate, applied the standard ISO 4833-1:2013 Part 1: Colony count at 30 °C by the pour plate technique[41,42]. This standard was last reviewed and confirmed in 2019, therefore the version remains current.

Results and discussion

The main goal of the project is to carry out detailed and systematic research on the origin of sapropel to be obtained in Latvia and its possibility to use it for medical purposes, to promote its scientifically based use in balneology, to develop new medical procedures and services, and to promote the development of new exportable products in the nearest future. So far, studies have been basically focused on sapropel for other purposes, such as agriculture or cosmetology, thus without using sapropel and its acquired mud biomedical and biopharmaceutical potential.

Sapropel is a jelly-like homogeneous mass, its texture in upper layers is close to cream-like, and in the lower layers, it becomes denser. The sediments are odorless except for separate types that smell of hydrogen sulfide (Table 1). Sapropel color depends on organic substance and mineral additions and it refers to caustobiolites[43]. The temperature of 4 °C without exposure to light and oxygen were sufficient for preserving sapropel. Samples from different lakes and depths have different organoleptic characteristic and must be checked on color, texture, visual consistency, impurities and uniformity, as well as smell. Organoleptically sapropel samples found from greenish-yellow to almost black (Table 2).

High silica content usually relates to green and yellow colored sapropel and could be found in moraine lakes. The high organic matter relates to black colored sapropel and could be found in the lakes with low mineral content.

Sapropel consists of a sludge solution, a skeleton, and a colloidal complex. The sludge solution contains water and dissolved substances that mean mineral salts, low molecular weight organic substances, vitamins, and enzymes.

Brown and dark green sapropel affiliate the mixed type of sapropel and its origin comes from lake's plankton, plants and sometimes connected with peat existence. This type of sapropel mostly can be found in Latvian lakes. Sapropel sample pH level is around 7–8 it means that these sapropel sediments have high mineral content (Table 3).

The following factors identified for standardizing and describing sapropel: organoleptic testing (visual look, consistency and smell, coarse composition test), test for heavy metal residue, test for pesticide residue, bacteriological test and pH[44].

Heavy metals are one of the most widespread and significant contaminants in sediment, causing serious environmental effects due to their toxicity, persistence, and bioaccumulation.

Lake sediments can be polluted in many ways, mainly man-made pollution, such as sewage disposal, runoff from agricultural land, lakes and transport from nearby roads. However, the accumulation of heavy metals in lake sediments is not always associated with anthropogenic pollution, and the sediment may be naturally "enriched" with various metals influenced by the local geochemical background. The presence of lead and cadmium in the upper layers may indicate anthropogenic effects. In contrast, metals such as chromium, cobalt, copper and nickel are naturally occurring. Nickel can be found naturally in sediment, erosion and mineral dissolution, as well as in the catchment natural processes, as well as copper, and cobalt. The presence of nickel in the sediment may be elevated if there are municipal waste dumps in the vicinity of the mining site, while the presence of copper may indicate clayey sediment.

	Zeilu	Mazais Kivdalova	Ivusku	Dunakla	Audzelu
Average depth of lake water layer (m)	1.1	1.3	1.0	2.2	2.0
Sapropel layer depth (m)	4.0–9.5	1.7–11.2	2.2–10.4	0.9–9.5	2.65–11.4
Lake surface area (h)	44.8	14.7	1.9	82.7	64.9
Lake bottom structure	Muddy	Muddy	Muddy	Muddy Gravelly	Muddy Sandy Rocky
Proximity of access roads (km)	0.5	0.2	0.2	0.5	0.2
Surroundings	Surrounded by reed beds, marshy forest; rural houses and service buildings; farmland; cemetery	Rural village Nuksi; rural houses with the adjacent area; field; forest	Farmland; cemetery	Close to town Ludza; one island in the lake	Populated area Vecsloboda; forest
Hydrological regime	Streams: 2 inflowing brooks and 6 ditches, 1 outflowing brook	Streams: 1 inflowing river and 3 ditches	Streams	Streams: 1 inflowing brook and 2 ditches, 1 outflowing river	Streams: 1 inflowing river, 1 brook and several ditches, 1 outflowing brook,
Sedimentological description of a section	Brownish grey to dark brown, flowing in upper layers, getting jelly-like in the lower layers, with plant residues, some H_2S odour	Grey to dark brown, flowing, jelly-like with plant residues, top layers have a bit of rough sand, lower layers become denser	Predominantly yellowish-green, sometimes light brown, flowing, jelly-like, contains poorly decomposed peat admixture	Park brown, Flowing in upper layers, getting jelly-like in deeper layers, with plant residues and well-decomposed peat impurities	Dark brown, Moderately flowing in the upper layer, the lower layer becomes extremely dense, mainly jelly-like, in deeper layers with admixture of sand and gravel
Additional data	Water transparency exceeding maximum depth (> 1.6 m); overgrown	Brown water lake	Overgrown	Very intense and regular water blooming; fish thirsting is observed in harsh winters; there was a Ludza bird integrated plant, later a meat processing plant nearby (water intake for economic needs (1000 m^3/day)	Regular water blooming

Table 1. Description of the lakes.

The determination of metals, lead (Pb), cadmium (Cd), nickel (Ni), cobalt (Co), copper (Cu), antimony (Sb) and chromium (Cr) in sediments is very important in view of their toxic effects on the environment and their long sustainability in the environment. The analysis of metal contents in the sapropel provides information on the natural and anthropogenic origin of the metal flow in the lake's ecosystem and the influence on sapropel application in medicine. Pb, Cd, Co, Ni and Cu were present in all samples, but none of the metals exceeded maximum acceptable level compared with SCCS's[44] calculated values that tolerated in a different kind of cosmetic (Pb – 20 ppm, Cd – 5 ppm, Ni – 200 ppm, Co – 70 ppm, Cr(III) – 100 ppm, Sb – 100 ppm). The presence of the Pb and Cd in the upper layers of sediments indicates anthropogenic impacts growth on the lake ecosystem. In some samples, the slightly increased Ni concentrations are associated with its natural origin deposited in sediments[45]. Also in very high concentrations, Ni has low potential mobility and low ecological risk. Anthropogenic metals as Co correlated with Ni. The major route of exposure expected to be via the skin, although the potential for absorption of heavy metals through the skin is relatively low.

The use of sapropel in medicine requires that water and sapropel samples are free from pesticide residues and their contents comply with regulatory requirements.

Chlororganic pesticides are among the first to be widely used as effective help to combat unwanted plant pests and pathogens and have bioaccumulation and bioconcentration capabilities. These pesticides include persistent organic pollutants (POPs) which can move very long distances through air and water and accumulate in terrestrial and water ecosystems. The most commonly known and most widely used POP pesticide in the world is DDT.

Some water and sapropel samples at different depth levels showed the presence of DDT (dichlorodiphenyltrichloroethane) pesticide and its decomposition product DDE, DDT was a commonly used pesticide for insect control in the 20rd Century[46]. On 23th June 2015, the International Agency for Research on Cancer (IARC), a part of the World Health Organization specialized agencies of the United Nations, has evaluated the carcinogenicity of the insecticide gamma-hexachlorocyclohexane (lindane) and DDT and the herbicide 2,4-dichlorophenoxyacetic acid[47].

Although DDT has been banned in most countries since 1970, its degradation products are very persistent and can still be found in the environment and in animal and human tissues worldwide.

Limit values for DDT concentrations contained in Legal Acts of the Republic of Latvia Nº 118 Annex 1, Table 2 "*Environmental Quality Standards for Hazardous Substances in Surface Waters*", where the average annual concentration of DDT is 0.025 µg /L or ppb and para-para DDT – 0.01 µg /L or ppb.

Comparing lakes the concentration of DDE/DDT was slightly different. The concentrations of DDE/DDT found in surface water from lakes were in general lower than those found in samples of sapropel. The highest levels of DDE/DDT were found in all depth of Mazais Kivdalova and Zeilu and in the 2nd extraction site of Audzlu lake, but the amount of DDE / DDT was below the limit of quantification, QL.

Table 2. Lithological description of the sapropel exploration points.

		Total concentration, mg/ml				Metal concentration, ppm							Microbiology, uncertainty ± 15,40	
Lake↓	pH	TOC	HA	FA	TPC	Pb	Cd	Ni	Co	Cu	Cr	Sb	CFU/g	Isolated species
Zeilu	7.8	126.4	160.2	74.3	77.2	2.60	0.1	11.8	5.0	9.9	20.1	0.3	$2{,}65 \times 10^6$	Serratia fonticola/Pseudomonas veronii/Pseudomonas chlororaphis
Mazais Kivdalova	7.3	129.1	167.8	72.9	103.6	2.66	0.2	18.4	8.2	12.0	27.2	0.4	$2{,}0 \times 10^5$	Pseudomonas veronii
Ivusku	8.0	106.5	113.1	76.5	70.3	3.10	0.2	3.1	1.7	3.9	9.1	0.3	$1{,}1 \times 10^5$	Paenibacillus amylolyticus/Aeromonas bestiarum
Dunakla	8.0	104.3	138.4	44.5	62.4	5.23	0.2	15.3	5.7	9.4	29.4	0.3	$2{,}3 \times 10^7$	Aeromonas sobria/Pseudomonas marginalis,/Brevundimonas diminuta
Audzelu	7.1	125.4	161.8	70.0	118.5	5.84	0.2	25.2	6.3	13.3	52.4	0.4	$2{,}1 \times 10^5$	Acinetobacter johnsonii

Table 3. Concentration of metals, humic and fulvic acids, total organic carbon and microbiology.

Among the most important groups of organic acids in sapropel are humic acids (HA) and fulvic acids (FA), which are naturally resistant, high-molecular heterogeneous compounds. They consist of both aromatic structures and aliphatic circuits with different functional groups.

Humic substances make up the highest recent amount of carbon on earth that can affect the climate, soil fertility, and degradation[48]. The properties of polyphenols in humus substances in cosmetics and medicine can be used as antioxidants. Antioxidant (AO) activity was determined in fulvic acid with a concentration of carbon fraction (FA-C) till 700 mg/L. AO activity has been measured by a various methods such as Total phenolic concentration (TPC), Total Antioxidant Status (TAS) level, 2,2′-azino-bis(3-ethylbenzothiazoline-6- sulphonic acid (ABTS), and DPPH radical scavenging assays. Results revealed that the AO activity is dependent on the concentration of carbon fraction in FA because lower concentration was not sufficient to scavenge free radicals due to its low TAS level. One tendency is that one of the lakes – Dunaklu – gives considerably lower both AO and humic and fulvic acids levels. However, Ivusku Lake with the lowest AO levels is high at the FA level. The concentration of humic acid and FA and the AO levels varies strongly between different lakes. It was found that AO level is considerably higher in organic sapropel extracts from the lakes Audzelu, Mazais Kivdalovas, and Zeilu. The total AO level is almost threshold between the highest and lowest values. The difference in HA (max. 167.8 mg/ml, min. 113.1 mg/ml) levels between different lakes much or less pronounced than the difference in the FA (max. 76.1 mg/ml, min. 44.5 mg/ml) and AO levels (Table 3).

Therapeutic mud has bactericidal and bacteriostatic (antimicrobial) properties. A special role belongs to the microflora contained in peloids, on the vital activity of which the biological processes occurring in them depend. There is a huge amount of microorganisms in the healing mud – billions per 1 g of peloid. They take part in the breakdown of organic substances, which are closely associated with the formation of mud and the regeneration of spent therapeutic mud.

By oxidizing the organic matter formed at the bottom of the reservoir, with the help of oxygen taken from sulfates – salts of sulfuric acid, seawater, microorganisms get the energy necessary for life.

The high microbiological activity of peloids is their characteristic feature that distinguishes peloids from other similar formations. The active activity of bacteria, fungi, other components contributes to the decomposition of organic and animal residues and enriches therapeutic mud with humic substances, bitumen, produces hydrogen sulfide, ammonia, carbon dioxide, and other gases; only the constant activity of microbes ensures a stable content in the mud of such unstable microcomponents as vitamins, enzymes, and hormones. Due to the microorganisms present in the mud, they are able to self-clean after anthropogenic pollution in the deposits and regenerate after use in the mud baths.

Sapropel is still a living material with its specific biome and microbiological flora that not identified yet in detail. Totally, the nine species of bacteria found in the lakes. The Dunaklu Lake has the highest value of CFU/g – $2.3{\cdot}10^7$, three species of bacteria were most prevalent and one of them – *Aeromonas sobria* – is an opportunistic pathogen[49]. Ivusku Lake has lowest value and two species – *Paenibacillus amyloyticus* and *Aeromonas bestiarum* – were most abundant. Both species not known as pathogens or opportunistic pathogens; however, it reported that *Paenibacillus amylolyticus* might have antimicrobial properties by producing antibiotics[50] that could explain lower value of CFU/g in Ivusku Lake. Sapropel from Zeilu Lake contains $2.65{\cdot}10^6$ CFU/g and three bacterial species are most prevalent, of those two are *Pseudomonas veronii* and *Pseudomonas chlororaphis* that are known as normal soil bacteria, and *Pseudomonas chlororaphis* may have antimicrobial properties due to production of rhamnolipids and some substances with antibiotic characteristics[51]. However, *Serratia fonticola* is an enterobacteria and opportunistic pathogen that could indicate some kind of pollution of wastewaters in this lake. Sapropel form this lake should definitely sterilized as *Serratia fonticola* known to cause skin and soft tissue infections.

Sapropel form Audzelu Lake and Mazais Kivdalova Lake have similar values of CFU/g and have single dominant species – *Acinetobacter johnsonii* (emerging as a fish pathogen) in Audzelu Lake samples and *Pseudomonas veronii* in Mazais Kivdalova Lake.

The Scientific Commission on Consumer Safety has developed guidelines for the use of various mineral, animal and plant-based and biotechnological ingredients in cosmetic products[52]. These guidelines may apply to sapropel as the topical application to the skin or in the form of various gels, creams, shampoos and other products for external use. EU guidelines state that cosmetic products must not contain microbial pathogens and the total aerobic microorganisms must be low. For cosmetic products intended for paediatric or use near to eye zone (Category 1), the CFU or colony-forming units shall according to EU Regulation should not exceed 100 CFU/g (bacteria, yeasts, fungi) and for other cosmetic products (Category 2) should not exceed 1000 CFU/g. Other specific microorganisms such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans*, and others presence should not be observed in 1 g or 1 ml in both Category 1 and 2 end products. Consequently, sapropel, which is intended to be used in various topical applications, also requires routine tests for microbiological and contaminant monitoring. Adaptation of ISO Standards recommended for quality routine tests and use – ISO/TR 19838: 2016, ISO 21148: 2017, ISO 17516: 2014. For the detection of *Aerobic Mesophyll Bacteria* – ISO 21149: 2017, *Staphylococcus aureus* – ISO 22718: 2015, *Pseudomonas aeruginosa* – nd yeast—ISO 16212: 2017 standards needed[53]. It is important to evaluate the utilization of sapropel material after use in therapeutic or cosmetic applications to prevent organic and microbial contamination of the environment. One of the methods, if the material used is of low toxicity or non-toxic and not in high volume, is to dilute it with large amounts of water and then ensure that it is discharged into the sewage system, prevent entry into natural waterways[54]. If preservatives or other compounds are added to the material used before or after application, it should be considered their occurrence in nature, effects on flora, fauna, and potential degradation time.

Conclusions

The use of organic-rich lake sediment like sapropel considered a solution because of the necessity for wider use of the local resources and available natural materials and due to previously insufficient research of sapropels for pharmaceutical needs.

The appropriate sapropel layer found from 2.0 to 9.0 m (actual layer – 0.9–11.4 m) from the surface of the sediment layer. If the depth is less than 1.5 m from the surface of the sediment layer, sapropel sediments are not fully developed.

Sapropel is a jelly-like homogeneous mass, its texture in upper layers is close to cream-like, and in lower layers, it becomes denser. The sediments are odorless except the separate types that smell of H_2S. Organoleptically sapropel found from greenish-yellow (with high silica content) to almost black (with high organic matter and low mineral content). Brown and dark green sapropel, mostly found in Latvian lakes affiliate to the mixed type of sapropel and comes from lake's plankton, plants and sometimes connected with peat existence. Sapropel pH level is around 7–8, it means that sediments have high mineral content.

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The total AO level is almost threshold between the highest and the lowest values. The difference in HA (max. 167.8 mg/ml, min. 113.1 mg/ml) levels between different lakes much or less pronounced than the difference in the FA (max. 76.1 mg/ml, min. 44.5 mg/ml) and AO levels.

Despite the fact that in raw sapropel samples no active pathogens identified, CFU exceeds the limit allowed by tenfold or more in all of sapropel samples. It is necessary to reduce CFU/g in the raw sapropel by sterilization or by adding preservatives before using it in cosmetic or medical applications.

In the framework of this study, the Guidelines for the extraction of sapropel have been developed, thus, practically all the activities that the sapropel industrial miners would have to perform.

Received: 30 April 2020; Accepted: 23 November 2020
Published online: 09 December 2020

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Acknowledgments

The research was co-financed by project "Analysis of characteristics of medical sapropel and its usage for medical purposes and elaboration of industrial extraction methods", No.1.1.1.1/16/A/165.

Author contributions

I.P. Conceptualization, Methodology, Writing—Original Draft; A.K. Conceptualization, Methodology, Writing—Review & Editing; A.A. Writing—Review & Editing, Validation; I.V. Data Curation, Writing—Review & Editing, Project administration, Supervision, Funding acquisition; A.S. Investigation, Validation; L.K. Investigation, Resources, Formal analysis; B.S. Investigation; L.D.Investigation; L.P. Investigation, Resources.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-020-78498-6.

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Third Publication

Environment. Technology. Resources. Rezekne, Latvia
Proceedings of the 12th International Scientific and Practical Conference. Volume III, 114-118

Extraction of Biologically Active Components from Freshwater Sapropel

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***Abstract*—Sapropel has been used for different purposes - in agriculture as fertilizer, in construction as building material, in cosmetic products, in balneology also in medicine and pharmaceuticals as bioactive component. Previously sapropel has been commonly used in raw form and there is no general accepted method or standard method for obtaining sapropel extract. However, most extraction methods follow the same path. Currently, there are few extraction methods using several extractants for obtaining bioactive components from raw sapropel.**

The most commonly used extractant is alkaline solution. When sapropel is subjected to alkaline environment, the humic and fulvic acids, together with some lipids, vitamins and sugar, present in the raw sapropel become soluble, however other organic and mineral content present in the sapropel remain solid. Alkaline extraction is followed by filtration and water present in the aqueous mixture is evaporated off.

Latvian freshwater sapropel can be used as raw material for obtaining sapropel extract and use it as remedy. But the main question for sapropel usage in medicine, balneology and pharmacy is to develop quality criteria for raw sapropel and its extracts. The quality criteria should include minimum requirements for biologically active substance concentration, pH values, antioxidants as well as physical characteristics.

In future studies the differences in extract characteristics of the various deposit sites, as well as the stability of the extracts under different storage conditions should be defined; also, there is need for a common approach to develop method of extraction process for active substances from sapropel and analysis procedures of its extract.

Keywords*—antioxidants, extraction, freshwater sapropel, fulvic acid, humic acid, sapropel

I. Introduction

Sapropel has long been used as a remedy in medicine and veterinary medicine, having a positive effect on the health [1], [2]

Sapropel biological and biochemical structure and composition varies strongly depending on its origin. Its characteristics are determined by organic, mineral and biological compounds that can have a multitude of effects on skin [3]. Sapropel has a high heating capacity that makes it useful for topical applications in medicine and rehabilitation. It is proposed that the medical effects are due to its high heating capacity and a mixture of chemical elements, hormones, various organic acids and vitamins (C, B1, B2, B5, B6, B9, B12, E, D and P) found in sapropel [2], [4]being included in most of ancient Mediterranean/European medical texts and currently used to prepare therapeutic hot-muds (peloids.

Previously sapropel has been used in raw form and there is no general accepted method or standard method for obtaining extracts of its active components. There are number of extraction methods using several extractants for obtaining bioactive components from raw sapropel and most of the extraction methods follow the same path [5].

Extraction is a principal process for recovery and separation of biologically active compounds from nature materials. Extraction converts complex matrix into suitable ingredients for pharmaceuticals, medicine, cosmetics and analytical procedures [6].

Literature suggest different extraction methods for obtaining biological active substances from sapropel. Most popular is solid-liquid extraction (SLE) with alkaline solution [7]. There are recent reports for sapropel extract using microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), supercritical fluid extraction (SFE) and hydrostatic pressure extraction (HHPE) [6]–[8]. All these techniques have proven effectiveness in extraction form natural matrices and could be used as extraction methods for raw sapropel. Also all methods have followed principles of maximizing the yield of extraction, can be adapted for industry and have procedures to avoid impurities [6].

The first step of extraction process is isolation of active components from cells by using physical and chemical processes [9]. Choice for appropriate cell disruption process depends on sapropel sediments, that consist of

Print ISSN 1691-5402
Online ISSN 2256-070X
http://dx.doi.org/10.17770/etr2019vol3.4135

crystalline skeleton like sand and clay, and residues of water organisms – flora, fauna; it all makes colloidal mud solution, which has complex cellular matrix [10]. In case of sapropel extract, cell disruption usually is done by drying samples before solid-liquid extraction process.

II. Materials and methods

A. Sapropel samples

In this work the sapropel samples were extracted from 5 lakes in eastern Latvia. Lakes were selected by analysing the Latvian lake database (*ezeri.lv*) [11], containing official geological survey of Latvia lakes. The sapropel deposits depth, lakes coordinates, history of agriculture next to lake were considered in the selection of the lakes. The sapropel was obtained from 5 lakes: Audzelu lake (*Audzeļu ezers*), Dunaklu lake (*Dūnāķļu ezers*), Ivusku lake (*Ivušku ezers*), Zeilu lake (*Zeiļu ezers*), and Little Kivdalova lake (*Mazais Kivdalovas ezers*) in Latgale region of Latvia.

The extraction of sapropel from the lakes was performed during the winter time when the surface of the lakes is frozen. Prior to the sample collection the thickness of the proper sediment layer was determined and the depth of sapropel deposit was established for each of the lakes as well as within each of the lakes by taking probes in several locations. To select a well-composed sapropel layer for further laboratory analyses the samples were taken from three different depths of sapropel sediment at each extraction point and up to eleven different points through lake coordinates (fig.1). During the sample collection procedure 21 samples were obtained from each for the lakes that resulted in 105 sapropel samples in total.

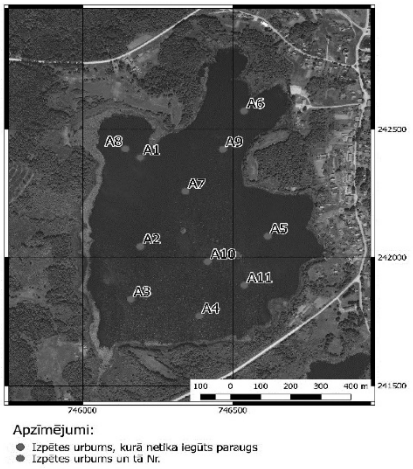

Fig. 1. Sapropel sample coordinates in Audzelu lake (A1 – A11 sample taking points)

Preservation of sapropel samples. All sapropel samples were kept in closed plastic containers without oxygen access to oxygen in order to prevent oxidation of the sapropel and its active components. The sediments were refrigerated and kept at 4°C; in these conditions' samples were stored from 4 to 8 months before extraction process and analysis.

The storage temperature of 4°C was selected as it most closely resembled the natural water temperature at the bottom of the lake during the winter time.

B. Extraction of active componenets from Sapropel

For the extraction of active components from the sapropel samples the alkaline method was selected. Extract was obtained from each of the samples (n=56). Solid-liquid extraction process with 2% NaOH solution was used for the extraction of humic and fulvic acids from the sapropel samples. Sapropel sample with NaOH solution was stirred for 24 h, and then mixture was centrifuged at 5000 rmp for 30 min, and then filtrated. Filtrate was acidified with 5 N H_2SO_4 solution till pH 2 and centrifuged again. Filtrate was separated from solid particles, and both liquid extract and solid extract were stored at 4°C before use.

After sodium hydroxide solution was added pH level rises from neutral to pH 10, all chemical cell disruption processes began, stirring helps with mixing base alkaline solution with sapropel; colloidal mixture is formed. After centrifugation, sand particles and insoluble matter precipitates and is discarded. When acid was added humic acids molecules precipitated from the solution and stay in solid form; fulvic acid remains in the solution. The extraction process results in two forms of extract: solid, crystalline phase; main part of which is humic acids, and the liquid that contains high concentration fulvic acid solution.

C. Characterisation of the sapropel extract

For the characterisation of the sapropel extracts there are no generally accepted guidelines as it is usually the case for many plant extracts. In general the minimal quality indicators for plant extracts are the concentration of active substances, pH values, visual inspection, and raw material quality. The same principles were applied to the characterisation of the sapropel extracts.

Sapropel extracts were characterised by organic carbon content (TOC), humic acid (HA) and Fulvic acid (FA) concentration, pH level, and antioxidant level.

Total organic carbon, HA and FA were determined using spectrometric method.

Sapropel pH level was determined using distillated water (volumetric ration sample: water - 1:2.5).

To determine antioxidants the following methods were used: DPPH radical method, Folin-Ciocalteu method for determination of the total phenolic content and total antioxidants status were calculated.

The total phenolic content in sapropel extract was determined spectrophotometrically according to Folin-Ciocalteu method [12]we will determine the antioxidant properties of methanolic extract of propolis from Ghardaia and Khanchla provinces of Algeria and will correlate the values with total levels of polyphenolic compounds. Methods: The total polyphenol contents of methanolic extract of propolis were measured by using Folin-Ciocalteu spectrophotometric method. Thereafter, the antioxidant properties of these polyphenols were

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determined by using the 2,2-diphenyl-1-picrylhydrazyl (DPPH. This method is based on the reaction of phenol in sapropel extract with Folin-Ciocalteu reagent. The content of phenolic compounds of the extract was expressed as gallic acid equivalents. The gallic acid was used to set up a standard curve. All the samples were analyzed in triplicates.

The total free radical scavenging capacity of sapropel extract was determined using the stable DPPH radical, which has absorption maximum at 515 nm. The radical solution was prepared by dissolving 2.4 mg DPPH in 100 ml methanol, a test sample (5µl) was added to methanolic DPPH. Also, absorption of blank sample (without antioxidant) was measured. A calibration curve was plotted with DPPH scavenged versus concentration of Trolox equivalent (TE mmol/L) [13], [14]. All samples were determined as triplicates.

Total antioxidant status (TAS) in samples was measured using Randox Total Antioxidant status kit (Randox Laboratories Ltd.) adapted to the RX Daytona automated chemistry analyzer (Randox Laboratories Ltd) [15]

ABTS® [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] incubated with H_2O_2 and peroxidase (metmyoglobin), generated the ABTS® radical cation. It has a relatively stable colour of green and blue, which absorbs at 600 nm. The antioxidants present in the sample prevent the formation of the cation; therefore the colour is proportional to its concentration. The result was expressed in milimoles of Trolox equivalent (TE mmol/L) of the sample solution.

III. Results and Discussion

A. Sapropel samples

The location of balneologically usable sapropel layer in the studied lakes was found to be from 2.0 to 9.0 m from the surface of the sediment layer exact depth depending on the lake and the position of the measurement point in the lake. Actual thickness and location of the balneologically usable sapropel layer varied depending on the depth of the lake and the degree of the decomposition of organic matter in the lakes. If the depth is less than 1.5 m from the surface of the sediment layer, sapropel sediments are not fully developed and thus were not used in this study. Organoleptically testing the colour of samples it was found that sapropel colour varies from greenish yellow till black. Green and yellow coloured sapropel usually relates to high silica content and is found in moraine landscape lakes; black coloured sapropel has high organic matter and is found in lakes with low mineral content; brown and dark green sapropel is mixed type and its origin comes from lakes plankton, higher plants and sometimes its connected with peat layers [1]. Sapropel sample pH level is around 7 – 8 it means that these sapropel sediments has high mineral content [16]. The characteristics of the research areas are shown in table I.

TABLE I. THE CHARACTERISTICS OF THE RESEARCH AREAS

Lake name	Characteristics			
	Lake surroundings	Sapropel colour	The depth of the sapropel layer, m	pH
Audzelu	Small village forest	Black	2.65 – 11.4	7.14
Ivusku	Agricultural land	Brown	2.2 – 10.4	7.96
Dunaklu	Towns suburb, has an island	Greenish yellow	0.9 – 9.5	7.56
Zeilu	Forest, agricultural land, cemetry	Dark green	4.0 – 9.5	7.82
Little Kivdalova	Agricultural land, farmstead	Dark brown	1.7 – 11.2	7.27

B. Sapropel extract

All 105 sapropel samples were tested for the presence of heavy metal residues and pesticides. Almost all of the samples tested had the level of heavy metal and pesticides below the level accepted for medical use, 56 samples were selected for the extraction of humic and fulvic acids.

In literature it is reported that the concentration of humic and fulvic acids in the sapropel extract varies due to differences in the chemical structure of humic substances (HS) and physical availability of the organic matter associated with mineral in sapropel [17].

Extraction performed with sodium hydroxide the yielded approximately is 22 -28 g of humic acids and 5 - 9 g fulvic acids from one-kilogram dried sapropel. Outcome of acids is calculated in dried extract form, for fulvic acids excess liquid was evaporated. Humic acids, fulvic acids and total organic carbon, extracted from one g sapropel from each of the lakes are shown in fig. 2; median values of HA, FA and TOC were calculated to show average values of each lake. The highest difference between the sapropel from different lakes is in the yield of the fulvic acids where the highest and lowest values differs by more than 80%, while the total organic carbon is more uniform with the difference between lowest and highest values less being less than 30%. Results show that the highest organic acid concentration is in Audzelu lake and Little Kivdalova lake. The high organic acid concentration in these lakes can be related to the way sapropel forms in these lakes. In both lakes the sapropel sediments are organic sapropel with lower mineral content and with lover pH values.

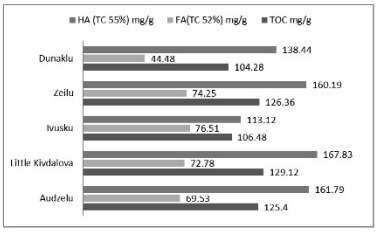

Fig. 2. Concentration of humic acid (HA), fulvic acid (FA) and total organic carbon (TOC) in each lake, mg/g.

It is reported in [18], [19] that not only the color of sapropel sediments can vary, but also the color of extracted HS can be different, indicating the degree of humification and HA and FA concentration in the extracts. Color of extracts was from light yellow to dark brow. Extracts from Dunaklu and Ivusku lake were yellow but extracts from Audzelu and Little Kivdolova lakes were darker almost black and it correlates with HA and FA concentration.

The concentration of humic and fulvic acids in sapropel extract are higher in organic sapropel, it also is related to the age of sapropel layers as the formation of bioactive substances, mineralisation of lakes and degradation of organic matter all influences the concentration of HA and FA in sapropel [3], [20].

In the analysis of the extracts the antioxidant level was measured. Antioxidant levels were calculated for each sapropel layer, there was no significant difference in antioxidant concentration of each sapropel extract from various layer, so median concentration of antioxidants was calculated to show average findings from each lake. It was found that antioxidant level is considerably higher in organic sapropel extracts from the lakes Audzelu, Little Kivdalovu and Zeilu. The difference between the highest and the lowest values is almost threefold for the total antioxidant level. The reason for so drastic differences in the antioxidant levels between different lakes is not fully understood. It seems that the antioxidant level does not correlate with the level of humic and fulvic acids

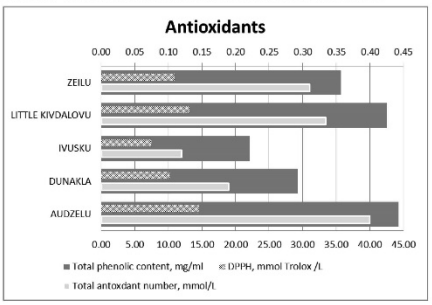

Fig. 3. Antioxidant level in sapropel extract from each lake.

One trend is that one of the lakes – Dunaklu lake gives considerably lower both antioxidant and humic and fulvic acids levels. However, Ivusku lake with the lowest antioxidant levels is high in fulvic acid level. More studies of different samples from the sediments in the same lake might be needed to better understand the variations of these parameters in the sapropel extracts obtained from different sources. The antioxidant measurement results are shown in fig.3 for each lake.

IV. CONCLUSION

Balneologically usable sapropel was found in all studied lakes. The most suitable lake as a source of sapropel was found to be Audzelu lake. It is easy reachable, because of the small village next to it, it has high humic and fulvic acids concentration and it shows the highest antioxidant level.

The concentration of humic and fulvic acids and the antioxidant levels varies strongly between different lakes. In the studied samples the concentration of humic and fulvic acids do not correlate with the antioxidant level.

The difference in humic acid levels between different lakes is much less pronounced than the difference in the fulvic acid and antioxidant levels.

In cases where higher fulvic acids and or antioxidant level are desirable it is important to select correct lake for the raw sapropel extraction since the fulvic acid content and antioxidant level varies strongly between the lakes.

ACKNOWLEDGMENTS

This research was supported by „Analysis of characteristics of medical sapropel and its usage for medical purposes and elaboration of industrial extraction methods", project No. 1.1.1.1/16/A/165.

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Unlocking the Therapeutic Potential of Freshwater Sapropel Extracts: In Vitro Analysis and Antioxidant Profiling for Skincare Applications

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Abstract: *Background and Objective:* Sapropel, a biologically active sedimentary deposit, is high in organic matter and minerals and has been shown to offer health benefits. Its constituents, humic acid (HA) and fulvic acid (FA), have been found to have some therapeutic applications. The aim of this study was to determine the potential therapeutically significant properties of freshwater sapropel extracts: their polyacid content, antioxidative (AO) status, and biological activity in cell culture. *Materials and Methods:* Freshwater lakes from the southeast region of Latvia were investigated layer by layer. The total organic carbon (TOC) was determined through combustion using the catalytic oxidation method, HA and FA were measured via acid perspiration, and the total polyphenol content (TPC) and total antioxidant status (TAS) was analysed spectrophotometrically. Sapropel extracts' regenerative abilities were tested in vitro using a Cell-IQ real-time monitoring system on mouse BALB/c 3T3 fibroblasts and human keratinocyte HaCaT cell lines. Cytotoxicity was measured through neutral red uptake assessment as a concentration-dependent reduction in the uptake of neutral red dye relative to a vehicle control or untreated cells. *Results:* The highest AO activity was observed in sapropel extracts with elevated concentrations of HA and TPC from Audzelu Lake (1.08 ± 0.03 mmol/L), and the lowest activity was found in extracts from Ivusku Lake (0.31 ± 0.01 mmol/L). Correspondingly, the concentrations of HA in Audzelu and Ivusku Lakes were recorded as 45.2 and 27.4 mg/g, respectively. High concentrations of HA promoted in vitro cell growth upon short-term exposure (up to 6 h). *Conclusions:* The results show that high TPC correlates with AO status and sapropel extracts with higher concentrations of HA exhibit greater AO activity and promote in vitro cell growth, suggesting a perspective use for short-term topical therapeutic skin applications. However, higher concentrations over longer durations showed cytotoxic effects, indicating the need for further investigation.

Keywords: antioxidants; biological polyacids; cell growth; humic acid; freshwater sapropel; fulvic acid; in vitro

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Citation: Kļaviņa, A.; Reste, J.; Mārtiņsone, I.; Vanadziņš, I.; Lece, A.; Pavlovska, I. Unlocking the Therapeutic Potential of Freshwater Sapropel Extracts: In Vitro Analysis and Antioxidant Profiling for Skincare Applications. *Medicina* **2024**, *60*, 546. https://doi.org/10.3390/medicina60040546

Received: 20 February 2024
Revised: 19 March 2024
Accepted: 25 March 2024
Published: 27 March 2024

1. Introduction

The increased interest in sapropel use for healthcare, cosmetics, and the pharmaceutical industry means that better knowledge is required regarding sapropel properties and the variations between various sources (the origin lake and depth from which sapropel is extracted).

Sapropel mud is a multi-component system comprising organic mineral complexes and organic matter and is used in healing procedures [1]. Sapropel is a semi-renewable resource from subterranean depths, as it consists of fine-grained and friable continental water sediments with an organic matter content of no less than 15%, mainly consisting of

aquatic animal and plant residues. Sapropel is found in most lakes in Latvia as sediment, and many types of mire also contain sapropel under the peat layer [2,3]. There are several types of sapropel in Latvia—organic sapropel, carbonated sapropel, silica sapropel, and mixed-type sapropel [2]. Sapropel types differ in their concentration of biologically active components and oxide concentration. The sapropel pH level is around 6–8, and the environmental pH reaction is neutral; if pH levels are higher, then sapropel has a high mineral content. The sapropel type can be established based on the ash content [2,3].

Sapropel has been used in resorts with mineral springs, in facilities devoted to relaxation and health (spas), and in aesthetic medicine; its usage has grown due to an increased interest in natural remedies [4]. During sapropel application in balneotherapy, some chemicals penetrate the skin [5]. Sapropel sediments have an antioxidant effect that smooths wrinkles, prevents new wrinkles, and smooths the skin structure, as well as removing swelling and strengthening nails and hair [4,5]. Therefore, the therapeutic effect of sapropel helps to maintain the cellular structure of the skin [6,7] and restore immunity and helps with acne, rashes, and dermatitis [8,9]. In addition, balneotherapy has shown healing capabilities in the treatment of various musculoskeletal and rheumatologic diseases [10].

Sapropel sediments consist mainly of humic substances, which are organic macromolecules [11], major parts of which are humic acid (HA) and fulvic acid (FA) [12,13]. Organic acids are the most important biologically active substances in sapropel sediments. HA and FA participate in healing processes. Organic acids consist of both aliphatic and aromatic structures with different functional groups [8]. The molecular structure of HA is an aromatic polymer containing carboxyl, hydroxyl, methyl, and phenolic groups [14,15]. The average molecular weight is 6500 Da. The molecular weight of FA is from 400 to 2000 Da, depending on the source [16]. The main structural skeleton comprises poly-functional groups with polypeptides and polysaccharides bound to them [14,17]. The HA molecule reacts with the receptors in the dermis due to the aromatic nucleus and functional groups. The anti-inflammatory effect of HA has been supported by its inhibition of the production of inflammatory cytokines. HA produces a therapeutic effect by inhibiting the lipoxygenase pathways of the arachidonic acid cascade [8,14,18]. Arachidonic acid is a component of the cell membrane and a substrate for the synthesis of eicosanoid-based inflammation mediators such as leukotrienes, thromboxane, and prostacyclin. HA, as well as FA, has been found to suppress the heat-induced arachidonic acid release of human promonocytic cells. It is known that wound-healing processes require additional oxygen, and this demand appears in the first minutes after phagocytosis begins. HA has the ability to generate extra oxygen. Based on the molecular size, HA cannot penetrate the deeper layers of the skin, but it can act as a wound-healing agent, while FA, which has a smaller molecular size, can penetrate deeper into the skin and help cell growth by acting on healthy cells. FA's major therapeutic effect relates to the activation of skin metabolism and regeneration processes [6,19,20].

It is important to obtain sapropel extracts to more easily achieve biologically active substance penetration through skin tissues and for therapeutic effects. HA and FA may be extracted for further use from sapropel sediments, and it is necessary to determine their content and full antioxidant properties [21].

This study was established as a part of the European Social Fund project, 'Analysis of characteristics of medical sapropel and its usage for medical purposes and elaboration of industrial extraction methods'. Previously, the average depth of the lake water layer, sapropel layer depth, lake surface area, lake bottom structure, and surrounding and populated areas, as well as the hydrological regime and the sedimentological description of the mud section, have been described [3]. The determination of metals such as lead, cadmium, nickel, cobalt, copper, antimony, and chromium in sediments were measured in all samples, but none of the metals exceeded the maximum acceptable levels given by the Scientific Committee on Consumer Safety (SCCS) [3,22,23].

The use of sapropel in medicine requires sapropel samples to be free from pesticide residues and their contents to follow regulatory requirements. The best-known pesticide in

the world is dichlorodiphenyltrichloroethane (DDT). Some water and sapropel samples at different depth levels showed a minor presence of the DDT pesticide and its decomposition product dichlorodiphenyldichloroethylene (DDE). The concentrations of DDE/DDT found in surface water were, in general, lower than those found in sediments. The highest levels of DDE/DDT were found at all depths in Mazais Kivdalova and Zeilu and in some extraction sites at Audzelu Lake, but the amount of DDE/DDT was below the limit of quantification. The current study continued the research on sapropel properties. Determining the HA and FA content, antioxidants, and biological activity of the sapropel extracts from separate lakes was the main aim [3]. Five lakes located in the eastern part of Latvia in the Latgale Upland were studied.

2. Materials and Methods

2.1. Exploration of Sapropel Samples

Eastern Latvian lakes with the following geographic coordinates were chosen for sapropel extraction: Audzelu Lake (56°15′35.4″ N 27°58′36.2″ E), Dunaklu Lake (56°33′23.1″ N 27°42′49.6″ E), Ivusku Lake (56°24′13.6″ N 27°20′51.4″ E), Zeilu Lake (56°30′40.3″ N 27°40′03.5″ E), and Mazais Kivdalovas Lake (56°32′58.0″ N 27°42′44.6″ E). These lakes have a glacial origin and were formed by a glacier and its melting waters. All the lakes have similar formation conditions (originating in Latgale Upland); however, they are in separate areas of the upland. Mazais Kivdalovas, Zeilu, and Dunaklu Lakes are located in the Rezeknes Lowlands, Ivusku Lake is located in the Raznava Hills, and Audzelu Lake belongs to the Dagda Hills. The freshwater sapropels in these lakes started to form during the Holocene.

During sample exploration, the sapropel sediment layer thickness, type of sapropel, and agricultural history of the territory next to the lake, as well as potential industrial waste exposure, were assessed [3]. Three depths of sediment (upper, middle, and lower layers, based on extraction depth from the top of the mud layer) were considered for exploration. Sediment samples were collected separately with semi-cylindrical chambers and kept in closed neutral plastic containers without oxygen access to prevent oxidation of samples. Sapropel sediments were stored at 4 °C in the dark until later analysis. In this study, 105 freshwater sapropel samples were obtained (each sample was 3 to 5 L). The scheme of the study design is shown in Figure 1.

2.2. Extraction of HA and FA from Sapropel

Sapropel extract was obtained using a solid–liquid extraction process with an alkaline solution [21]. Sapropel sediments, with 2% NaOH (AC, Sigma-Aldrich, Steinheim, Germany) solution, were stirred for 24 h in a closed reactor at room temperature; the mixture was centrifuged at 5000 rpm for 30 min and then filtered. After the addition of the sodium hydroxide solution, the pH rose from neutral to pH 10, and the chemical processes of cell disintegration began; stirring helped to mix the basic alkaline solution with the sediments, and a colloidal solution was formed. After centrifugation, sand particles and insoluble matter precipitates were discarded. Sapropel extract was stored at 4 °C for analysis. Prior to analysis, the HA and FA concentrations were determined for each sample through total organic carbon detection and acid perspiration. For further test purposes, each sample was diluted or concentrated to reach an FA concentration of 700 µg/mL to allow for a better comparison between lakes.

Determination of pH for each extract was performed using digital pH meter (WinLab Data Line pH/mV meter, Clausthal-Zellerfeld, Germany). The electrodes were inserted into 10 mL of sapropel extract for 10 min prior to taking the readings at room temperature, measurements were conducted in triplicate, and average value calculated to detect any pH fluctuation with time.

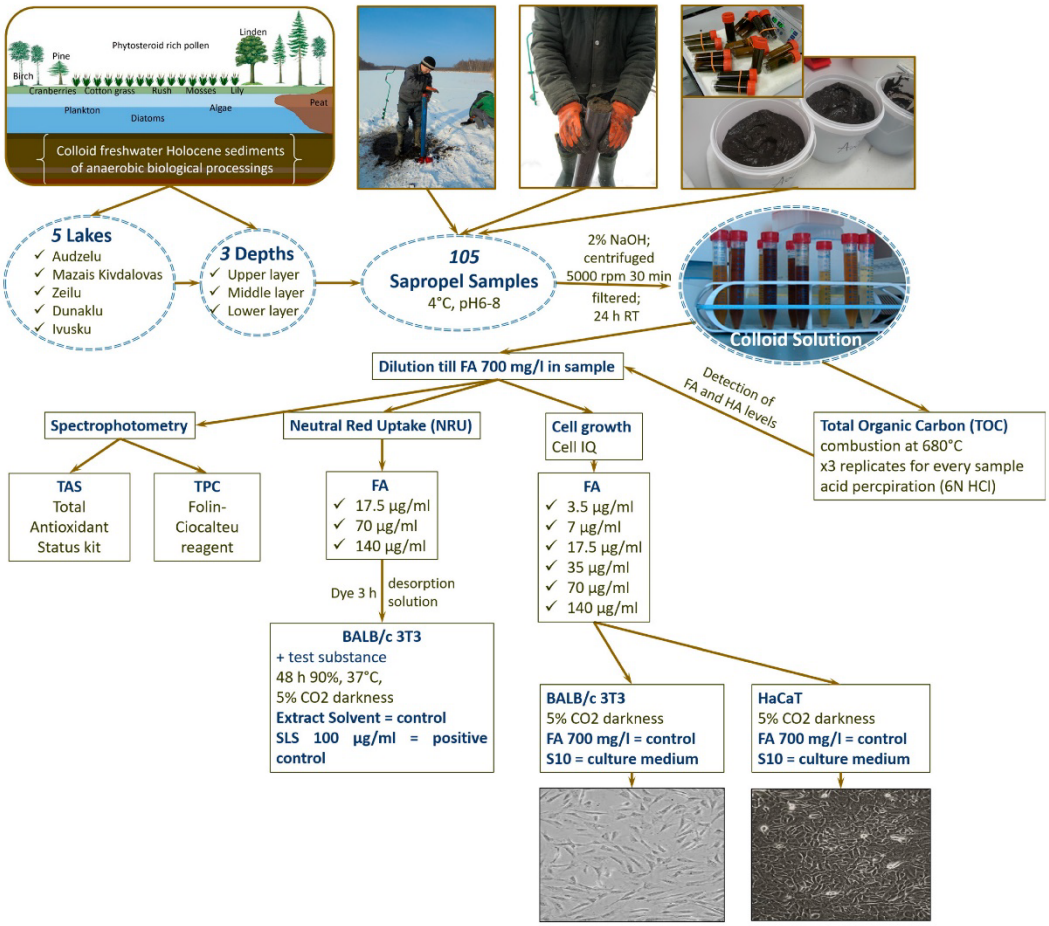

Figure 1. Scheme of the study design (FA, fulvic acid; TAS, total antioxidant status; TPC, total polyphenol content; BALB/c 3T3, cell culture of mouse dermal fibroblasts; HaCaT, aneuploid immortal keratinocyte cell line from adult human skin; SLS, sodium laureth sulphate).

2.3. Determination of Total Organic Carbon (TOC), FA, and HA Concentration

The TOC concentration represents the fraction of organic matter that has escaped mineralisation during sedimentation. The TOC concentration often varies; it indicates changes in an organic deposition under different sedimentary conditions.

The TOC was determined through the combustion of the samples at 680 °C using the catalytic oxidation method (Shimadzu, TOC-V model). All analyses were repeated several times (n = 3) to check the repeatability and validity of the results. Each sapropel sample was measured in three replicates, and an average of three results was obtained.

Acid perspiration was chosen to determine the concentration of HA and FA. Extraction was performed for a dried and crushed sapropel sample (2 g) with 50 mL of 2% NaOH solution, which was stirred for 24 h to achieve perspiration and then centrifuged (30 min) for separation. The test solution containing HA and FA was acidified with 6 N HCl (AR, Sigma-Aldrich, Steinheim, Germany); after 16 h, high-speed centrifugation was used for the final separation. The mixture was filtered through a 0.45 µm membrane filter. The

filtrate was analysed for TOC ($TOC_{filtrate}$). Since the mixture was only composed of HA and FA, after acidification, the HA was precipitated and the solution phase contained only FA ($C_{FA} = TOC_{fitrate}$) based on the definition of the HA and FA. It follows that the difference between the TOC content (TOC_{total}) and $TOC_{filtrate}$ will be equal to the HA concentration ($C_{HA} = TOC_{total} - TOC_{fitrate}$).

2.4. Antioxidant (AO) Activity

The AO activity of sapropel extract with an FA concentration of 700 μg/mL was assessed. AO activity was measured in vitro using spectrophotometric methods to determine total antioxidant status (TAS) and total polyphenol content (TPC) due to polyphenols usually contributing greatly to antioxidant properties of extracts.

2.4.1. Measurement of the TAS

The TAS in the sapropel extract was measured using the Total Antioxidant Status commercial assay kits (Cat. NX2332, Randox Laboratories Ltd., Crumlin, UK) adapted to the RX Daytona™ automated chemistry analyser (Randox Laboratories Ltd., Crumlin, UK) following the manufacturers' instructions. In brief, the assay was based on the formation of ferryl myoglobin radical from metmyoglobin and hydrogen peroxide, which then oxidised ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) to produce the radical cation (ABTS•+), a green soluble chromogen, determined spectrophotometrically. Antioxidant scavenging led to the formation of cation radicals in a concentration-dependent manner, with a proportional decrease in colour intensity. Assay results are expressed as mmol/L. Total antioxidant measurement considers the cumulative effect of all antioxidants present in the extract under investigation.

2.4.2. Total Polyphenol Content (TPC)

The TPC in the sapropel extract was determined spectrophotometrically via UV–Visible spectrophotometer UV-Vis Varian Cary 50 (Varian Australia Pty Ltd., Mulgrave, Australia), applying the widely used Folin–Ciocalteu method [24,25]. The basis of the method is the oxidation of the phenol –OH groups in the reaction with the Folin–Ciocalteu reagent, which is a mixture of phosphomolybdate and phosphotungstate used for the colorimetric in vitro assay of phenolic and polyphenolic antioxidants. It produces a blue colouration with an absorption at 765 nm. The colour of the solution is proportional to phenol concentration. The reducing capacity of the Folin–Ciocalteu reagent depends on the presence of –OH groups in polyphenols. First, 2.5 mL of 10% Folin–Ciocalteu reagent was added to 0.5 mL sapropel extract sample and mixed and incubated at room temperature for 3–8 min, then 2.0 mL of 7.5% sodium bicarbonate was added and mixed and incubated for 30 min at room temperature. The reading was taken at 765 nm against the 'blank' sample. The content of phenolic compounds in the extract was expressed as gallic acid (AR, Sigma-Aldrich, St. Louis, MO, USA) equivalents (μg GAL/mL). The gallic acid was used to set up a standard curve (concentration 100, 50, 25, 12.5, 6.25 μg GAL/mL). Samples were analysed in triplicate.

2.5. Neutral Red Uptake (NRU)

NRU was measured, and the relative comparison of the sapropel extract effect on NRU was assessed. The NRU test was carried out according to the Organisation for Economic Co-operation and Development (OECD) recommendations for NRU protocol [26].

Neutral red (NR) dye captured by a viable cell was released during the desorption step, which resulted in the well staining red, and this colour change was spectrophotometrically quantified in the plate reader (Tecan Infinite F50 with Magellan Tracker software, Tecan, Switzerland, https://lifesciences.tecan.com/products/microplate_readers/infinite_f50 [accessed 26 January 2024]) at 540 nm. Cytotoxicity was expressed as a concentration-dependent reduction in the uptake of NR dye relative to vehicle control or untreated cells, respectively; the more intense the colour of the well, the more viable cells there were.

Routinely, BALB/c 3T3 fibroblasts (Bagg Albino mouse line developed by S.A. Aaronson and G.T. Todaro in 1968) were cultivated in a monolayer to ~80% confluence in cell culture flasks (SARSTEDT, TC Flask T75, Stand., Vent. CAP.) in an incubator with 90% humidity at 37 °C with 5% CO_2 in darkness, with cells visually inspected every day. The growing medium (S10) consisted of 90% of Dulbecco's Modification of Eagle's Medium (DMEM) (Millipore VLE Dulbecco's MEM) accompanied by a 1% mixture of penicillin and streptomycin (Sigma-Aldrich, Penicillin–Streptomycin, Steinheim, Germany) and 10% fetal bovine serum (FBS) (SIGMA, Fetal Bovine Serum, Steinheim, Germany). Subcultivation or recultivation of cells was carried out if the confluence was 50–80%; 1x phosphate-buffered saline (PBS, Sigma-Aldrich, Steinheim, Germany) was used for cell washing, and 0.25% trypsin–EDTA solution (SIGMA, Trypsin–EDTA, Steinheim, Germany) was used to detach cells from the surface. NRU was assessed by using a slightly modified version of the test protocol. It was observed that sometimes, in some samples, a precipitate formed after defrosting; in these cases, they were centrifuged and filtered once again through 0.2 μm filters.

For the NRU test, 100 μL of S10 medium with 3000 cells per well was cultured in the 60 middle wells on the 96-well plate (Microtest Plate 96 Well), and 100 μL of S10 medium without cells were added to the wells along the plate perimeter. The plate was incubated for 24 h in an incubator with 90% humidity at 37 °C and 5% CO_2 in darkness. After 24 h, the medium was gently removed via careful inversion of the plate over the appropriate container (i.e., 'dumped') and blotted on sterile paper towels. Fresh medium containing test substances or controls was added to all wells and incubated for 48 h in an incubator with 90% humidity at 37 °C and 5% CO_2 in darkness. The NRU test was used to check the cytotoxicity of sapropel extracts at three FA concentrations—17.5 μg/mL, 70 μg/mL, and 140 μg/mL; sapropel extract solvent at the proper concentration was used as a control. Sodium laureth sulphate (SLS) (ACS, Sigma-Aldrich, Steinheim, Germany) at 100 μg/mL concentration was used as a positive cytotoxicity control (no uptake of NR dye). Each plate was prepared for chosen extracts and controls at one of the selected concentrations. Then, 25 μg/mL of NR dye was prepared in S5 medium (95% DMEM, 5% FBS). After incubation with test substances, the medium was gently removed and rinsed with 250 μL 1 x PBS, and 250 μL of NR dye was added to each well for 3 h and incubated in an incubator with 90% humidity at 37 °C and 5% CO_2 in darkness. After 3 h, the NR dye mixture was gently removed via inversion of the plate, all wells were rinsed with 250 μL 1 x PBS, and 100 μL of desorption solution was added and incubated for 20–45 min in darkness in a shaker. The desorption solution consisted of a mixture of 1% glacial acetic acid, 50% ethanol, and 49% distilled water (obtained from EMD Millipore SPR00SIA1US SmartPak Direct-Q 3 (Millipore Sigma, St. Louis, MI, USA). After 20–45 min, the plate was removed from the shaker and left in darkness for 5 min. It was measured spectrophotometrically at 540 nm.

2.6. Determination of Changes in Cell Growth Using Cell-IQ®

In vitro assessment of the effect of sapropel extracts on cell growth was performed using a Cell-IQ® method (CM Technologies Oy, Tampere, Finland). Cell-IQ® is an integrated real-time monitoring platform for live cell imaging and analysis. Cell-IQ® was used for single-cell populations to determine basic cell population parameters: cell number, cell proliferation, cell death, shape, size, and rates of growth over time using sapropel extract as growth enhancer. Sapropel extracts were tested on BALB/c 3T3 mouse dermal fibroblasts and HaCaT (human adult low-calcium high-temperature keratinocytes) aneuploid immortal human skin cell line at concentrations of 3.5, 7.0, 17.5, 35.0, 70.0, and 140.0 μg/mL, using FA as the control concentration of 700 μg/mL. HaCaT cells represent a spontaneously transformed human epithelial cell line, developed from a long-term primary culture of human adult skin keratinocytes. As it maintains an epidermal differentiation capacity, it was used to determine sapropel extract effectiveness on human skin. The cells were defrosted from −80 °C, cultured at 37 °C in darkness, and kept in a 5% CO_2 atmosphere for the S10 culture medium. After the defrosting of cells, at least one passage (one reculturing

of cells) was performed. BALB/c 3T3 and HaCaT are adherent cells, so they were separated from the surface of the culture dishes using 0.25% trypsin–EDTA solution.

2.7. Statistical Analysis

Cell growth was monitored using the Cell-IQ real-time cell monitoring system, and relative cell growth was analysed using GraphPad Prism version 5.0. Two-way ANOVA and Bonferroni post-test statistical significance were determined as * if $p < 0.05$, ** if $p < 0.01$, and *** if $p < 0.001$; the measurement count in each test was six for each sample type.

Antioxidant properties and other characteristics were analysed using Microsoft Excel Professional Plus 2010, and IBM SPSS Statistics version 20 was used for calculations. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. General Characteristics

The typical characteristics of the analysed sapropel samples are summarised in Figure 2. The moisture content varied from 80 to 93%. The sapropel samples' pH level was around 7–8, indicating a higher mineral content.

Lake*	pH	LOI (organic matter) [%]	Ash [%]	Total concentration [mg/g] (dry weight)		
				TOC	HA	FA
AUDZELU	7.1	30.9	67.1	60.5	45.2	39.0
DUNAKLU	7.6	37.6	58.8	60.0	34.1	25.6
IVUSKU	7.3	83.2	13.2	68.0	27.4	40.8
MAZAIS KIVDALOVAS	7.5	41.6	56.3	59.6	39.5	22.2
ZEILU	7.7	52.7	42.4	64.2	36.8	28.1

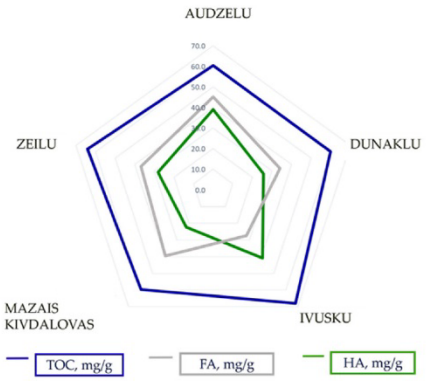

Figure 2. The typical characteristics of sapropel by lake (LOI, loss on ignition; TOC, total organic carbon; HA, humic acid; FA, fulvic acid). * Median values (n = 21 for a lake with three replicates).

The ash determination and loss-on-ignition (LOI) results are between 30.9 and 83.2%, showing that Ivusku Lake has organic sapropel. Dunaklu and Mazais Kivdalovas Lakes have mixed-type sapropel, Audzelu Lake has silica sapropel, and Zeilu Lake has carbonate sapropel.

3.2. TOC, FA, and HA Concentrations

The concentration of FA and HA in sapropel extracts is higher in sapropel with higher TOC. The high organic acid concentration in the lakes can be related to the way in which sapropel forms in lake basins. It is also determined by the formation of organic acids, the degradation of organic matter, and mineralisation processes in the lakes. Figure 2 shows that the highest HA concentrations are in Audzelu Lake (45.2 mg/g), Mazais Kivdalovas Lake (39.5 mg/g), and Zeilu Lake (36.8 mg/g), but the highest FA concentration is in Ivusku Lake (40.8 mg/g). The data show that the ratio between HA and FA is different for separate lakes. As shown in Figure 2, the highest TOC concentration is in Ivusku Lake (68.0 mg/g); the lowest TOC concentration is in Mazais Kivdalovas Lake (59.6 mg/g).

3.3. AO Activity

The concentration of organic acids in sapropel extracts was a key factor in determining whether they possess AO or pro-oxidant activity. All samples showed concentration-dependent AO activity; AO parameters were measured for sapropel extract standardised to an FA concentration of 700 µg/mL.

TAS levels were calculated for each sapropel layer of the lake (n = 21 for each lake with three replicates). No significant differences in the TAS of each sapropel extract from different layers were observed. However, TAS values differ in extracts from separate lakes—the highest values were presented in Audzelu Lake at 1.08 ± 0.03 mmol/L with silica-type sapropel, but the lowest TAS values were found in Ivusku Lake at 0.31 ± 0.01 mmol/L, which has organic-type sapropel. TAS values were found to be higher in sapropel extract samples with higher HA concentrations from Audzelu, Mazais Kivdalovas, and Zeilu Lakes. There is a significant correlation (R^2 = 0.90) between TAS and HA concentrations in sapropel extracts with the same FA concentration. The investigated extracts show a relatively high polyphenol content range: 42.07–146.26 µg GAL/mL of the sample. The highest TPC was found in Audzelu Lake (146.26 ± 1.16 µg GAL/mL), which has high HA content and TAS, but the lowest was in Ivusku Lake (42.07 ± 0.55 µg GAL/mL). Moreover, there is a substantial correlation between TPC and TAS (R^2 = 0.93), as shown in Figure 3. Therefore, there is strong correlation between AO activity assessed as both antioxidant status and polyphenol content and HA concentration, and no correlation between AO level and FA concentration.

LAKE	Sapropel layer	TPC [µg/ml] *	TAS [mmol/L] *
AUDZELU	Upper layer	133.91 ± 0.99	1.03 ± 0.03
	Mid layer	146.26 ± 1.16	0.99 ± 0.03
	Lower layer	130.78 ± 1.84	1.17 ± 0.03
DUNAKLU	Upper layer	65.34 ± 0.37	0.38 ± 0.01
	Mid layer	67.99 ± 0.46	0.47 ± 0.01
	Lower layer	66.19 ±0.40	0.56 ± 0.02
IVUSKU	Upper layer	42.94 ± 0.50	0.31 ± 0.01
	Mid layer	42.07 ± 0.55	0.32 ± 0.01
	Lower layer	44.28 ± 0.45	0.33 ± 0.01
MAZAIS KIVDALOVAS	Upper layer	90.70 ±0.91	0.83 ± 0.02
	Mid layer	95.59 ±1.31	0.90 ± 0.02
	Lower layer	93.99 ±1.53	0.87 ± 0.02
ZEILU	Upper layer	77.66 ±0.01	0.80 ± 0.02
	Mid layer	74.92 ±0.01	0.72 ± 0.02
	Lower layer	76.45 ±0.02	0.73 ± 0.02

*Median ± SD

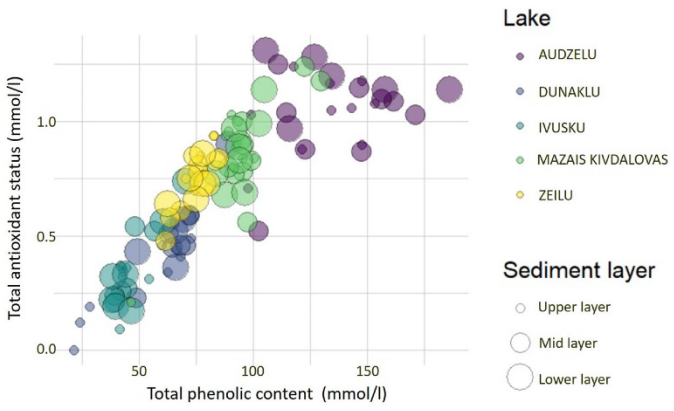

Figure 3. Correlation between total phenolic content and total antioxidant status in different lakes and in different layers.

3.4. Neutral Red Uptake

The concentration-dependent cytotoxicity of sapropel extracts was checked via the NRU method. Knowing that the uptake of neutral red dye by live cells can be influenced not only by the tested sapropel extract but also by other substances present in the medium for cell growth, it is important to consider these factors in the analysis of the data. For this reason, the assessment of the results was conducted in two ways: obtained NRU data were recalculated relative to standard medium (S10) control and solvent control to exclude the effect of the solvent in our experiment. Then, both results were analysed and compared to each other (Figure 4).

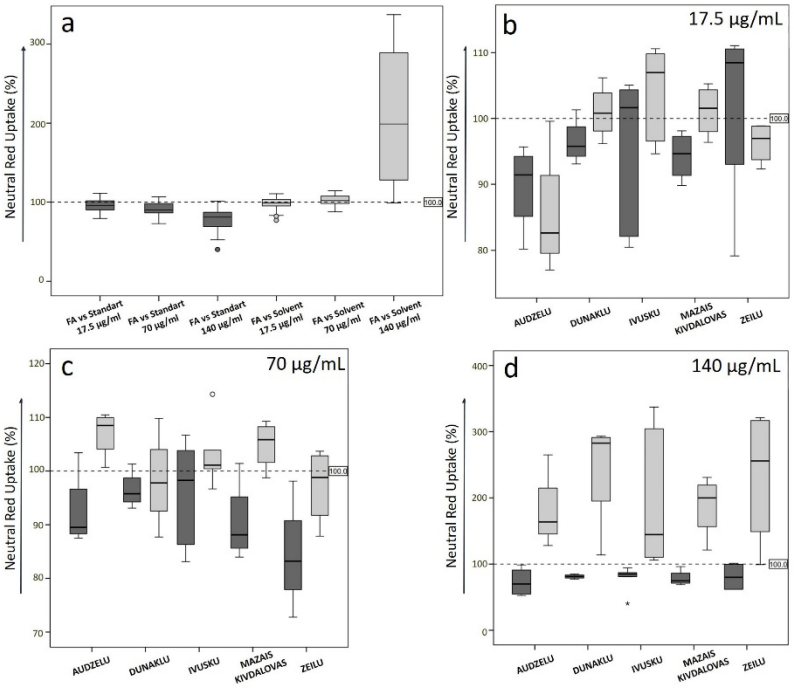

Figure 4. Neutral red uptake by BALB/c 3T3 mouse fibroblasts after exposure to sapropel extract from every lake at 17.5, 70.0, and 140.0 µg/mL concentrations standardised by FA compared to 100% for cells grown in standard medium (S10 control, dark bars) and to solvent control (light bars): (**a**) all lakes together; (**b**) sapropel extract of 17.5 µg/mL concentration by lake; (**c**) 70 µg/mL by lake; and (**d**) 140 µg/mL by lake. Circles indicate outliers, asterisks indicate extreme outliers.

The NRU data in both recalculation scenarios correlated well only at low concentrations of sapropel extract standardised using FA (FA 17.5 µg/mL, Spearman's correlation coefficient $r_s = 0.492$, $p = 0.023$). At higher concentrations, NRU data, compared to S10 and solvent control, did not correlate well, indicating some toxic effects from the solvent (for sapropel extract with FA concentration 70 µg/mL $r_s = -0.010$, $p = 0.964$; for 140 µg/mL $r_s = -0.017$, $p = 0.942$).

Sapropel extracts from different lakes have varying effects on NRU at various FA concentrations. Sapropel extracts from all the lakes at the FA concentration of 140 µg/mL showed a noticeable NRU decrease compared to 100% for cells grown in standard medium (S10 control) (Figure 4a). On the other hand, the comparison to solvent control has shown opposite results, with a significant increase in NRU (Figure 4d). This might indicate the effect of solvent toxicity at high concentrations of sapropel extract.

From further analysis for separate lakes, the data in Figure 4b–d demonstrate that NRU after exposure to sapropel extract from Audzelu Lake at 17.5, 70.0, and 140.0 µg/mL FA concentrations was significantly decreased compared to the S10 control. At the same time, it is important to note that compared to the solvent control, NRU for the sapropel extract from Audzelu Lake was increased at 70.0 and 140.0 µg/mL concentration, probably indicating the toxicity of the solvent more than the toxicity of the sapropel extract itself.

For Ivusku and Zeilu Lake sapropel extracts at a low FA concentration (17.5 µg/mL), no harmful properties were noticed compared to the S10 control and they could potentially have beneficial effects that increase NR accumulation by cells, meaning that the cells in

these samples are more viable than in the control. Compared to the solvent control, the sapropel extract from Ivusku Lake has shown even better NRU results, but the sapropel extract from Zeilu Lake has worse NRU (Figure 4b).

Sapropel extracts from Dunaklu and Mazais Kivdalova Lakes at low concentrations have shown moderate results compared to S10 and solvent controls but still indicate potential solvent toxicity.

Sapropel extracts at a slightly higher FA concentration of 70 μg/mL showed lower NRU in both scenarios compared to S10 and solvent control, regardless of the lake location. Slightly better NRU was seen only for sapropel extracts from Audzelu, Ivusku, and Mazais Kivdalova Lakes compared with the data for the solvent control.

NRU did not differ significantly when analysed by sapropel extraction depth for all FA concentrations tested. The NRU results slightly decreased between the upper level and the deepest layer at the lowest concentration, as well as at the highest 140 μg/mL concentration. Conversely, each lake slightly increased at a 70 μg/mL concentration.

3.5. BALB/c 3T3 and HaCaT Cell Growth

Cell growth changes during exposure to various concentrations of sapropel extracts were observed using the Cell-IQ real-time monitoring system. The cell growth findings demonstrate that at concentrations of 3.5 μg/mL and 7 μg/mL, sapropel extracts do not affect BALB/c 3T3 mouse fibroblasts or HaCaT cell growth during the incubation period; however, extracts from Mazais Kivdalovas Lake and Dunaklu Lake have significant inhibitory effects on HaCaT cell growth if incubated at these low concentrations for longer than 24 h.

Figure 5 shows the results of testing with higher concentrations (17.5, 35.0, 70.0, 140 μg/mL) of sapropel extracts. As can be seen, at 17.5 μg/mL concentration, a significant promotion of BALB/c 3T3 cell growth was observed for 12 h, with a significant decrease in growth after 24 h. A decrease in HaCaT cell growth was also observed after incubation with Mazais Kivdalovas Lake sapropel extract for more than 18 h.

At 35 μg/mL concentration, extracts do not change HaCaT cell growth, though Mazais Kivdalovas Lake and Audzelu Lake samples do inhibit cell growth if co-incubation is longer than 24 h (Figure 5). For BALB/c 3T3 cells, a slight increase in cell growth was observed after the exposure of cells to sapropel extract at 35 μg/mL from Mazais Kivdalovas Lake, but only for the first 12 h, then the later notable inhibitory effect appeared. For Audzelu and Dunaklu Lakes, an inhibitory effect of the extract on BALB/c 3T3 cells was also found, but the initial stimulation was less prominent.

At 70 μg/mL concentration, extracts have a significant inhibitory effect on BALB/C 3T3 cell growth if incubated for more than 9 h, i.e., it appeared sooner, especially for extracts from Audzelu and Dunaklu Lakes. For the Mazais Kivdalovas Lake sapropel extract, an initial notable stimulating effect on BALB/c 3T3 was seen, but only for 6 h; later, a significant and more profound inhibitory effect started. HaCaT cell growth was not significantly affected by 12 h of co-incubation with a 70 μg/mL extract of sapropel from Audzelu Lake and for 9 h with an extract from Dunaklu Lake. After this time, a significant inhibitory effect was observed for extracts from both lakes. Sapropel extract from Audzelu Lake at 70 μg/mL concentration had a slight stimulating effect on HaCaT cell growth over the longer period of 18 h.

Co-incubation with a 140 μg/mL extract from Audzelu Lake significantly promoted cell growth for up to 18 h in HaCaT cells and for up to 3 h in 3T3 cells. An extract from Mazais Kivdalovas Lake had a minimal stimulating effect on HaCaT cells but a significant inhibitory effect after 12 h. In BALB/c 3T3 cells, an initial stimulating effect was seen for extracts from Mazais Kivdalovas and Audzelu Lakes, but only for up to 3 h of co-incubation, with a sharp decrease in cell growth afterwards.

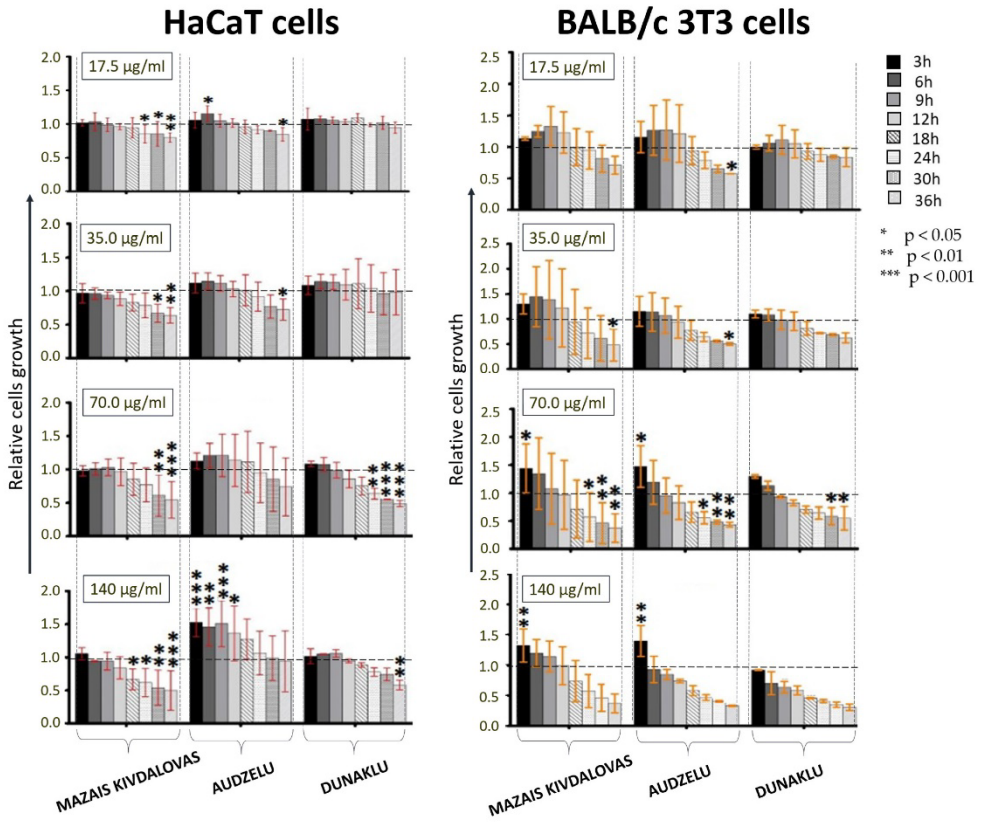

Figure 5. Relative cell growth during 36 h incubation period with sapropel extracts from Mazais Kivdalovas, Audzelu, and Dunaklu Lakes by concentrations of extracts (17.5 µg/mL, 35.0 µg/mL, 70 µg/mL, and 140.0 µg/mL) and cell culture type (HaCaT and BALB/c 3T3 cells).

4. Discussion

In the present study, the sapropel was classified according to the typical characteristics of natural sediments based on the properties (Figure 2) [2]. There are no regulatory acts on quality standards, and there is no international classification for sapropel [3]. However, the international standard "ISO 21426:2018 Annex D: Guidelines for Control Analysis of Peloids and Monitoring" can be used to better understand sapropel sediment evaluation and safety control for use in medical treatments [23]. The investigated sapropel extracts have relatively high AO activity and TPC in various lake samples, which is in accordance with studies reported by Obuka and co-workers [27]. Sapropel extracts show a high polyphenol content, and this has a strong correlation with the TAS, so polyphenols in sapropel extracts are responsible for AO activity [9,28]. The high TPC content may allow sapropel extracts to represent a prospective preparation for the treatment of skin diseases and complex wounds [29,30], as well as for local applications to people suffering from long-healing wounds/injuries (e.g., type II diabetes patients) [8,9], as these wounds do not heal quickly, form scar tissues, and are prone to inflammation.

In order to complete the picture of potential usage in skin applications, the results were analysed from a cosmetic ingredient perspective. The SCCS guidelines indicate that neutral red uptake phototoxicity is a validated in vitro method and its use is mandatory for

testing for phototoxic potential [22]. As sapropel extracts contain fulvic and humic acids and they are known as photosensitisers, this test was used to determine toxicity [6,8]. Also, the SCCS guidelines talk about research focusing on using non-animal, human-relevant models, and, subsequently, more human-relevant testing of HaCaT cell growth was carried out. This testing method allowed us a better understanding of sapropel extracts' effects on human skin and potential wound-healing properties.

The NRU experiment was carried out without the optimisation and adaptation of sapropel extracts for use in mammalian cell cultures, as this was the first phase of assessing whether the selected extracts were harmful (cytotoxic) to cell cultures; the whole purpose was to test sapropel extracts as used in other project activities and to evaluate their potential to reduce cell viability [31,32]. Under the given conditions, 17.5 μg/mL, 70.0 μg/mL, and the highest possible concentration of 140 μg/mL were chosen. However, to acquire this concentration, the medium used for cell culturing was diluted by up to 40% (in the 70.0 μg/mL case, it was 20%), while such a situation provided an opportunity to evaluate NRU under double-stress conditions: diluted media (40% less of the supplements necessary for successful cell growth), a high FA concentration, and a low medium pH. Future experiments should abstain from such dilutions [33,34]. To avoid toxicity in the solvent, finding ways to better control the pH and extract concentration for the cell growth milieu is essential in further studies. It also should be taken into account that while BALB/c 3T3 cells are robust, due to higher extract concentrations, low pH and, perhaps, some other extract/solvent properties, they can lose adherence while performing NRU tests, and, thus, low NRU can be observed in some wells. This was especially relevant (and observed) compared with the solvent control and could be explained by a loss of cell mass due to starvation stress and cell detachment due to high medium dilution and a very low pH [32,35]. It could be hypothesised that under high-starvation/low-pH stress, the high concentration of extract (70 and 140 μg/mL) could help cells maintain viability and adherence to the surface. When interpreting the results, it is important to remember that samples did not have the ideal pH value and consistency for testing in mammalian cell cultures, as these cells are also sensitive to pH change [32]. It could be speculated that perhaps the observed effects of sapropel's ability to influence NRU were not only due to the biological activity of the extracts but also due to their pH buffering capacity; thus, maybe cell survival was more connected to normalising the pH value in the medium than the biologically active substances in the sapropel extract being able to otherwise improve cell viability [33,36,37]. High concentrations of sapropel extracts made the cytotoxic effect of the solvent visible; when it was excluded from analysis, NRU appeared to be even higher than that of the S10 control.

Regarding higher concentrations, it could be that extracts from some lakes might help cells stay viable under stress conditions for a certain time when cell media are diluted, the pH is not optimal, and there is a high FA concentration. However, evaluating the solvent control to see what type of effect the sapropel extract had without the solvent effect for all samples showed that at 140 μg/mL compared to the solvent, it was remarkably higher, so the solvent plays the main role in toxicity. It could be speculated that sapropel extract containing FA and HA has some properties that reduce starvation stress and help cells maintain viability, similar to the S10 control. However, this hypothesis should be fully tested in different sets of experiments [38].

Moreover, the results that we present here on cell growth indicate that biologically active substances (fulvic acid and humic acid) from sapropel in the short-term (up to 3 h) co-incubation of cells with high concentrations of sapropel extract promote cell growth. Consequently, cell growth is not significantly reduced if incubation with an extract at a high concentration is no longer than 12 h. The results suggest that sapropel extracts have beneficial qualities; for short-term applications, they all could potentially be used in cosmetic applications. However, the further determination of other effects on cells is needed [6,28,29]. It is known that HA has the ability to generate active oxygen from the presence of oxygen, water, and radiation. This process can accelerate wound healing. However, oxygen-driven

radicals can cause cell destruction or lipid peroxidation. However, based on HA antioxidant activity, it also has compensation ability; HA solutions produce oxygen only in the amount that is needed, and, at the same time, can restrict peroxidation. This explains why sapropel extracts are effective in lower concentrations, as lower concentrations are optimal for maintaining balance in the system.

Overall, NRU and cell growth data have shown that the biological activity of sapropel extracts can vary a lot depending on the lake location, composition, concentration, and time of exposure. The SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and Their Safety Evaluation recommend general principles for calculating the threshold of toxicology concerns; these calculations also could help determine sapropel extract safety without expensive testing. The calculations represent a pragmatic tool that is based on established principles of systematic adverse event estimation in human health. However, it is not a standalone alternative in cases when the toxicity data availability is limited, so, for future studies, a detailed analysis of natural sapropel samples should be performed before testing on cell cultures to better understand the effects, and non-animal, human-relevant toxicity models are advised. Knowing that sapropel is a natural product with varied contents, sapropel extracts from different locations could have contrasting effects on human cells. For potential use in skin treatment, sapropel extracts with optimal composition and the most beneficial biological effects in cell cultures should be chosen.

Nevertheless, further research must be conducted to find the most promising sapropel extracts for skin applications. Nowadays, there are known compositions with dry sapropel extract in a water-insoluble form for skincare in cases of eczema, dermatitis, and other skin diseases. Sapropel powder extract is added to ointments and other skin care products, but it has limitations when it comes to penetrating the epiderma. Water-soluble skincare products could provide better penetration into the skin and could work on deeper tissues. The current study classifies sapropel extracts based on natural sediment characteristics and explores their potential applications in medical treatments, particularly in skin disease management. Future prospects could involve leveraging international standards to enhance sapropel sediment evaluation and safety control, thereby facilitating their use in medical treatments. Further research could focus on understanding the effects of sapropel extracts on skin cells using non-animal, human-relevant models, and a detailed analysis of natural sapropel samples is recommended before testing on cell cultures to determine the optimal compositions for skin applications. Additionally, exploration into water-soluble sapropel extract formulations may offer improved penetration into the skin for enhanced efficacy in treating skin diseases.

5. Conclusions

The FA and HA contents in the sapropel extracts are comparable in the extracts from separate layers and differ depending on the lake because of its origin; the ratio of the FA and HA acids also varies in the separate lakes. The high concentration of FA and HA in the samples indicates the potentially high biological activity of sapropel extract.

The ratio of HA and FA, along with other characteristics of the sapropel, such as pH, organic matter content, ash content, and polyphenolic content, can be used to characterise the sapropel from a particular lake and used as a tool for the identification of the source of sapropel for pharmaceutical and cosmetic product manufacturing.

A strong correlation was found between AO activity (TAS) and HA concentration, as well as TPC.

According to the results for NRU, the sapropel extract with HA and FA does not cause significant harm in cell cultures of human keratinocytes and mouse dermal fibroblasts and could potentially be tested for the development of products intended for humans. Potentially, sapropel might protect the skin from environmental stress due to its AO properties and exhibit beneficial properties during short-term use. Sapropel extracts from the freshwater lakes of the Latgale Upland are expected to be used in the development of

cosmetic and pharmaceutical products for application to the skin. Furthermore, this could increase the use of locally available natural resources as one of the world's priorities.

6. Patents

We have a patent of the Republic of Latvia LR 15514 A. A61Ql9/00, A61K8/02. A. Auce, A. Klavina, I. Vanadzins, I. Pavlovska, A.Silova, L. Dobkevica, L.Komarovska, B. Silamikele: sapropel extract water soluble gel and the method for its preparation. Pat. Appl. P-19-53, 29 November 2019. Publ. 20 July 2020.

Author Contributions: A.K. Methodology, Conceptualisation, Validation, Visualisation, Writing—original draft; J.R. Conceptualisation, Visualisation; Writing—review and editing; A.L. Writing—Review and editing; I.M. Validation, Resources, Writing—review and editing; I.V. Funding, Resources, Validation; I.P. Data curation, Project administration, Visualisation, Writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This project, "Analysis of characteristics of medical sapropel and its usage for medical purposes and elaboration of industrial extraction methods", was financed by European Regional Development Fund, project no. 1.1.1.1/16/A/165. This publication has been developed with financing from the European Social Fund and Latvian state budget within project no. 8.2.2.0/20/I/004, "Support for involving doctoral students in scientific research and studies", at Rīga Stradiņš University.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Acknowledgments: The authors are thankful to Riga Stradiņš University for supplying necessary facilities to carry out this research work and to Baiba Silamiķele for laboratory work in connection with cell growth.

Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Abbreviations

The following abbreviations are used in this manuscript:

ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AO	antioxidant activity
AR	analytical-reagent (AR)-grade chemicals are chemicals that meet the specifications outlined for analytical applications in laboratories. These chemicals are of high purity, typically exceeding 95 percent, with impurities specified and controlled to ensure accuracy and reliability in analytical procedures.
BALB/c 3T3	Bagg Albino mouse fibroblast cells
DDE	dichlorodiphenyldichloroethylene
DDT	dichlorodiphenyltrichloroethane
DMEM	Dulbecco's Modification of Eagle's Medium
FA	fulvic acid
FBS	foetal bovine serum
HA	humic acid
HaCaT	human adult low-calcium high-temperature keratinocytes (HaCaT cells)
LOI	loss on ignition
NRU	neutral red uptake
OECD	Organisation for Economic Co-operation and Development
PBS	phosphate-buffered saline
SCCS	Scientific Committee on Consumer Safety
TAS	total antioxidant status
TOC	total organic carbon
TPC	total polyphenolic content

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Fifth Publication

Carbohydrate Polymer Technologies and Applications 9 (2025) 100669

Contents lists available at ScienceDirect

Carbohydrate Polymer Technologies and Applications

journal homepage: www.sciencedirect.com/journal/carbohydrate-polymer-technologies-and-applications

Sapropel-enriched sodium carboxymethyl cellulose gel systems: formulation approaches, stability and bioactive potential

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ARTICLE INFO

Keywords:
Fulvic acid
Humic acid
Hydrogels
Pharmaceutics
Sapropel extract
Sodium carboxymethylcellulose (Na-CMC)

ABSTRACT

Sapropel, a sediment rich in organic matter and bioactive compounds, has significant potential for pharmaceutical and cosmetic applications. This study aimed to develop a stable, water-soluble hydrogel containing sapropel extract as a delivery platform for these bioactive compounds. Sodium carboxymethylcellulose (Na-CMC) was used as a gelling agent in eight formulations, with or without sapropel extract, and buffer solutions (NaCl, $MgSO_4$, $MgCl_2$). The gels were evaluated for organoleptic properties, pH, viscosity, stability, and thermal resistance over a 2-year period under different storage conditions.

All sapropel extract containing formulations exhibited a smooth, homogeneous and light-yellow appearance with good stability and spread ability. The pH levels ranged from 4.7 to 7.4, within the acceptable range for skin application, although formulations with $MgCl_2$ showed greater pH fluctuations. Viscosity analyses revealed that sapropel extract decreased viscosity, particularly under fluctuating temperatures. $MgSO_4$-buffered formulations exhibited the most stable viscosity over time. However, some formulations demonstrated decreased stability and viscosity after prolonged exposure to elevated temperatures and UV light. Centrifugal and thermal tests confirmed the physical stability of the gels, with no phase separation observed.

Overall, the study confirms the feasibility of incorporating sapropel extract into stable, water-soluble hydrogels, making them suitable for potential therapeutic and cosmetic uses.

1. Introduction

Sapropel is an organic sediment in fresh and sea water reservoirs with still water and in bogs under the peat layer. Sediments are formed from the remains of aquatic plants and animal organisms, which are mixed with micro and macro minerals. The thickness of the sediment layer can vary from 10 cm to 10 m (Pavlovska et al., 2020; Stankevica & Klavins, 2014; Vanadziņš et al., 2022) and the content of organic substances is up to 95 %. The color is usually dark, olive brown, grey or greenish, but the sapropel itself is gel-like in consistency.

Sapropel's unique structure, characterized by a wide biochemical variety depending on its origin, is influenced by its organic, mineral, and biological components, which impart numerous effects on the skin. With a high heating capacity and mixture of biologically active substances, organic acids, and vitamins, sapropel offers significant therapeutic potential. Freshly obtained sapropel contains a high-water content, but it becomes practically water-resistant after drying. However, treating sapropel with an alkaline solution or organic solvents like propylene glycol or ethanol enables the dissolution of its bioactive components in water, allowing the production of sapropel extract (Klavina et al., 2020; Krivonos & Plaksin, 2010; Sharypov et al., 2015).

Researchers indicate that sapropel is a promising raw material for creating effective remedies in medicine, pharmacy, and veterinary medicine. It has been traditionally used in treatments for skin conditions such as eczema and dermatitis, often in the form of water-insoluble creams or patches (Beer et al., 2003; Kovalenko et al., 2016; Obuka et al., 2018; Rumyantsev et al., 2017; Stankevica & Klavins, 2014; Wollina, 2009).

This naturally formed sapropel consists mainly of humic substances (HSs) and non-humic substances, all of which have demonstrated biological activity (García-Villén et al., 2018; Winkler & Ghosh, 2018; Wollina, 2009). Humic substances are categorized into four fractions: humic acid (HA), hymatomelanic acid (HMA), fulvic acid (FA), and humin. Most sapropel extraction methods focus on isolating humic and

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https://doi.org/10.1016/j.carpta.2025.100669

Available online 17 January 2025

fulvic acids (Klavina et al., 2020). Sapropel also contains water-soluble vitamins, including ascorbic acid (C), thiamine (B1), riboflavin (B2), pantothenic acid (B5), pyridoxine (B6), folic acid (B9), and cyanocobalamin (B12), as well as fat-soluble vitamins such as vitamin D and tocopherol (E) (Odabasi et al., 2007; Orru et al., 2011). Vitamin B12, produced by bacteria and blue algae, is particularly notable for its role in blood formation, amino acid metabolism, and nucleic acid synthesis (Odabasi et al., 2007).

The primary interest in sapropel lies in its HSs, which are macromolecules formed through the secondary synthesis of microbiological decomposition of plant and animal matter. These substances are known for their high red-ox activity and strong antioxidant properties (Aeschbacher et al., 2012; Dolmaa et al., 2011; Luhila et al., 2022). HA have been utilized in traditional medicine for treating various conditions, including skin diseases, cold stress, rheumatic pain, diabetes, kidney stones, heart ailments, leprosy, and immune system disorders (Jurcsik, 1994; Mirza et al., 2011). Sapropel containing healing mud and peat are mainly used as external remedies (Celik Karakaya et al., 2010; García-Villén et al., 2018; Glavas et al., 2017; Tateo et al., 2009; Tserenpil et al., 2010; Wollina, 2009).

The various anti-inflammatory, immunomodulatory, and radioprotective effects of natural substances seem to be associated with their antioxidant and antiradical effects (Neha et al., 2019). For example, the neuroprotective effect of HA in a focal cerebral ischemia rat model is likely due to the antioxidant properties of HA (Ozkan et al., 2015). The therapeutic effects of HAs were associated with the prevention of oxidative stress as well (Kļaviņa et al., 2024; Zykova et al., 2018).

The traditional method of applying sapropel is thermal mud bath or external sapropel applications. The bath is prepared for 15–20 min, water temperature 37 °C, sapropel concentration – 1 kg per 10 L of water. Very good results were observed when the patients with eczema, dermatitis and hand osteoarthritis were treated (Celik Karakaya et al., 2010; Fioravanti et al., 2014; Veniale et al., 2007). The application of sapropel has limitations, caused by the characteristics of sapropel: its application requires stationary places, e.g. baths or especially suitable rooms or places where sapropel is applied to users. For external sapropel application procedures, a relatively large amount of sapropel is required, which must be stored, applied, and removed afterwards, so it is very difficult to perform these procedures in places convenient for the patient.

A water-insoluble cream containing sapropel extract is known (Bevan et al., 2013). In a study involving 23 volunteers with increased skin sensitivity to irritants (dermatitis), the cream was applied to the skin every 24 h, resulting in a decrease in inflammation. Additionally, patches containing sapropel extract are also known. These patches, containing the sapropel extract cream, are applied to the skin for 24–96 h, leading to a reduction in inflammation in cases of skin dermatitis and eczema (Bevan et al., 2013; Strus et al., 2018). The water-soluble gel of sapropel extract and its production method are poorly researched. It is desirable to develop a stable water-soluble gel of sapropel extract that would be plastic, preserved bioactive properties of sapropel, and would retain its shape for 0.5–3 h after application at 15–35 °C temperature.

Ointments and gels, as forms of preparations for external application, are widely used in medicine and cosmetics. Modern skincare cosmetics are distinguished by their versatility, offering multidirectional and complex effects even in relatively simple formulations. A good example of the biological effects in the most widely used cosmetic applications is the application of a hydrolipid occlusion layer or various forms of antiradical protection to the epidermis. These methods are utilized in medicine, pharmacology, and cosmetics (Barbulova et al., 2015; Ficheux et al., 2019; Hoang et al., 2021).

Hydrogels for medicine, pharmacology, and cosmetics can be prepared from various bases, such as polyethylene glycol (PEG), hydroxyethyl methacrylate (HEMA), acrylic acid (AA), sodium alginate, collagen powder, cellulose derivatives, or Carbopol 940 (Ahmad et al., 2022). Hydrogels can be made from different types of polymers. These polymers may be either water-insoluble, such as polyethylene glycol, or water-soluble, which influences the hydrogel's characteristics, including its biodegradability in the environment (Ahmad et al., 2022; Akhlaq et al., 2023). Hydrogels derived from water-insoluble polymers typically expand when immersed in water. These materials are two-phase systems consisting of a solid phase formed by a water-insoluble three-dimensional polymer network. In contrast, water-soluble hydrogels are formed by cross-linking hydrophilic polymers, such as carboxymethyl cellulose (CMC) and its salts, resulting in a network capable of absorbing and retaining significant amounts of water (Du et al., 2019).

After applying the gel to the skin, the water and alcohol evaporate and the active substances in the gel remain more concentrated, which ensures a faster release of the drug from the dosage form (Mehvari et al., 2024).

Hydrogels based on natural hydrocolloids have great potential to replace synthetic polymers in biomedicine and pharmaceutical technology due to their biological activity, compatibility, and biodegradability advantages (Du et al., 2019).

Carboxymethylcellulose (CMC) and its soluble salts are known to be gel-forming systems. Carboxymethylcellulose salts (CMS) are used as a thickener and binder in food industry, as lubricant for drilling in oil industry and as a stabilizer and binder in cosmetics. CMC is insoluble in organic solvents such as methanol, alcohol, diethyl ether, acetone, chloroform and benzene, but soluble in water. Cellulose and its derivatives have large number of hydroxyl groups due to which they have been used in the preparation of hydrogels.

Its aqueous solution is a neutral or alkaline transparent viscous liquid that can produce a hydrogel three-dimensional structure under appropriate environmental and chemical conditions. CMS forms a transparent gel that is resistant to oils and most organic solvents. It exhibits a stable pH in the range of 3.5 to 11, depending on the forming environment.

The molecular structure of carboxymethylcellulose is derived from the cellulose backbone composed of β-(1→4)-linked D-glucopyranose polymers. The molecular weight of CMC typically ranges from 90,000 to 700,000 g/mol, depending on the degree of substitution and processing conditions (Grießinger et al., 2017; Zare-Akbari et al., 2016). Sodium carboxymethylcellulose (Na-CMC) is methylcellulose replaced by a carboxyl group (–OCH_3, -COOH). The viscosity of Na-CMC is 100–10, 000 mPa·s in concentrations of 1 to 6 %. The viscosity (pseudoplasticity) of the solutions depends on the concentration and viscosity degree of the product (Akhlaq et al., 2023; Grießinger et al., 2017).

Because of its non-toxicity, biodegradability and biocompatibility, CMS solutions have been used as a carrier of water-soluble drugs. Given its plant origin, the main advantage of carboxymethylcellulose over other natural polymers of animal origin, such as collagen and hyaluronic acid, is that CMC does not trigger an immune response. (Final Report on the Safety Assessment of Hydroxyethylcellulose, Hydroxypropylcellulose, Methylcellulose, Hydroxypropyl Methylcellulose, and Cellulose Gum, 1986; Gold et al., 2015).

Based on acute and chronic toxicity studies, CMC shows no signs of irritation or significant adverse effects on mortality, body weight, hematological parameters, urine tests, or microscopic examination. Toxicity was assessed in rabbits by using cosmetic products containing from 0.3 to 3.0 % CMC to the shaved abdominal skin and genital mucosa. The doses administered were 100 times higher than the average estimated daily human consumption (Final Report on the Safety Assessment of Hydroxyethylcellulose, Hydroxypropylcellulose, Methylcellulose, Hydroxypropyl Methylcellulose, and Cellulose Gum, 1986).

Microcrystalline cellulose and Na-CMC are a mixture of two materials considered non-toxic. Microcrystalline cellulose is approved for use as a food additive in Europe, is listed in the U.S. Food and Drug Administration (FDA) ingredient database, the UK-licensed list of non-parenteral drugs, and Canada's list of acceptable non-medical ingredients (Final Report on the Safety Assessment of Hydroxyethylcellulose, Hydroxypropylcellulose, Methylcellulose, Hydroxypropyl Methylcellulose, and Cellulose Gum, 1986; Gold et al.,

2015). It is widely used in oral and topical preparations, primarily to stabilize the product. Additionally, it enhances viscosity and controls the release of active substances from the dosage form (Mo et al., 2022).

Studies suggest the integration of CMC with different polymers and the use of various crosslinking agents. Potential crosslinking agents include calcium chloride, aluminum chloride, disulfides, calcium and sodium nitrates, and organic agents such as glutaraldehyde, monochloroacetic acid, citric acid, carbomer, and formaldehyde. Although these agents are effective crosslinkers, some may be skin irritants or even human carcinogens. The addition of additives such as tamarind gum, xanthan gum, guar gum, chitosan, gelatin, β-cyclodextrin, alginate, poly(vinyl alcohol), and inorganic oxides has been shown to improve the properties, strength, biocompatibility, and drug loading capacity of CMC hydrogels. These hydrogels' ability to encapsulate and release bioactive substances in a controlled manner enhances their therapeutic potential. The development of new crosslinking agents and synthesis techniques could lead to hydrogels with improved or superior drug delivery properties (Akhlaq et al., 2023; Burgardt et al., 2015; A. Das et al., 2015; Ghorpade et al., 2018; Gold et al., 2015; Mo et al., 2022; Nakagawa et al., 2013; Pornpitchanarong et al., 2022; Ramli & Wong, 2011; Wui et al., 2014; Zare-Akbari et al., 2016).

It is difficult to load hydrophobic drug in such hydrogels and control their release due to mutually exclusive nature of hydrophobic and hydrophilic system (Burgardt et al., 2015; A. Das et al., 2015; Ghorpade et al., 2018; Iannuccelli et al., 1993; Mo et al., 2022; Wellens et al., 2022; Wui et al., 2014). Sapropel extract has ability to bind with both hydrophobic and hydrophilic molecules (Klavina et al., 2020).

The hydrogels are better option for topical drug delivery system due to their high-water content and lower epidermal irritation, less mechanical abrasion and more acceptable appearance for using it on wounded skin (Ahmad et al., 2022). Studies suggest that hydrogel systems use all possible action mechanisms of vesicles for drug delivery to deeper layers of the skin. Hydrogels can deliver biologically active substances by diffusion, or the release active component from the hydrogel carrier (Ahmad et al., 2022; Mehvari et al., 2024). This release can be activated with pH, temperature changes or other agents' presence, such as enzymes (da Silva et al., 2022; Pornpitchanarong et al., 2022).

Research suggests that Na-CMC hydrogels can promote faster wound healing on the burn region and regulates transepidermal water loss of wound. Also, Na-CMC gel provides optimum humidity and temperature (Ramli & Wong, 2011). Many medical herbs can aid the healing process by eradication bacteria, improving collagen deposition and enhancing fibroblast growth without second infection or irritation on the wound. But the application of bio-based substances could be limited by their thermal stability, light sensitivity and degradability not only of the biologically active substance itself but also delivery system (Guimarães et al., 2021; Ji et al., 2016; Mehvari et al., 2024; Sim et al., 2022; Yan et al., 2013). Nevertheless, sapropel extract has regenerative abilities on epidermal cells that could help in wound healing as well (Kļaviņa et al., 2024).

It needs to be noted that sapropel characteristic, bacterial composition and pollution levels, as well as Neutral Red Uptake (NRU) tests and antioxidant activity were mentioned in our previous articles and will not be discussed in this article in the broader level (Kļaviņa et al., 2024; Pavlovska et al., 2020; Vanadziņš et al., 2022). In this study, Na-CMC was used to produce a carrier for sapropel extract. This study aimed to develop and evaluate transparent water-soluble gel systems incorporating sapropel extract as a promising delivery platform for various bioactive compounds.

The preparation of the sapropel extract-based gel systems involved the use of water-soluble cellulose derivatives as gelling agents, which provided desirable rheological and physicochemical properties. The sol-gel transition of the formulations (F) was triggered by changes in pH and temperature, allowing for in situ gelation upon administration.

2. Materials and methods

2.1. Components

All reagents used in this study were of pharmaceutical grade. Glycerol, ethanol, magnesium sulphate heptahydrate ($MgSO_4*7H_2O$), magnesium chloride hexahydrate ($MgCl_2*6H_2O$), sodium chloride (NaCl), sodium hydroxide (NaOH) were acquired from Fisher BioReagents (United Kingdom), but carboxymethylcellulose sodium salt (Na-CMC) was acquired from ALFA AESAR (USA).

Previous studies have analyzed sapropel sediments from five different freshwater lakes in the Latgale region of Latvia. These analyses included measurements of microbiological cultures, pollution levels, and concentrations of biologically active substances. Sapropel extracts were obtained from all five lakes. The sapropel extract was found to be non-cytotoxic to human keratinocyte and mouse dermal fibroblast cell cultures and demonstrated high antioxidant potential. Among the studied lakes, Audzelu Lake (56°15′N, 27°58′E) exhibited the most promising characteristics for the development of medical products (Kļaviņa et al., 2024; Pavlovska et al., 2020).

2.2. Extraction

To select a well-composed sapropel extract for development of hydrogel with sapropel extract the sapropel sediments were taken from three different depths – upper layer (2–3 m), middle layer (4–7 m), lower layer (8–11 m). For hydrogel preparation in this study was used mixed sapropel extract from all sediment layers, however for determination of biological active component concentration in each layer and for future guideline development of natural sediment use for medical purposes, extracts form each layer was tested separately. The sapropel extract from all depths was obtained using a solid–liquid extraction process with a 2 % NaOH solution (Klavina et al., 2020). The sapropel sediments were stirred in the alkaline solution for 24 h at room temperature in a closed reactor. This process raised the pH from neutral to 10, initiating cell disintegration and forming a colloidal solution. The mixture was then centrifuged at 5000 rpm for 30 min, followed by filtration to remove sand particles and insoluble matter. The resulting sapropel extract was stored at 4 °C for analysis. Obtained sapropel extract were analyzed to determine biological active substance concentration and antioxidant status (Pavlovska et al., 2020).

2.3. Preparation of hydrogel

The sol-gel transition is used to develop hydrogels by converting small molecules from a precursor solution (sol) into a gel-like network (gel) through hydrolysis and polymerization reactions (Fig. 1). This method allows for the formation of hydrogels with tailored properties, making it suitable for various biomedical and pharmaceutical applications. Hydrogels with sapropel extract were prepared based on previously developed formulations (Auce et al., 2022). The test samples included a blank gel without extract and gels with extract, each incorporating one of three different buffer agents: NaCl, $MgCl_2$, or $MgSO_4$. The formulations contained 2.5 % Na-CMC salt, 8 % glycerol, and 8 % ethanol, the latter of which was used as a preservative. All solid ingredients were initially mixed with ethanol and glycerol until a thick, homogeneous mixture developed. Subsequently, 5 % sapropel extract (containing 140 μg/g of fulvic acid) was added. Buffer salts were dissolved in purified water to create a 0.2 M solution, which was then added to the mixture, bringing the total volume to 100 %. All prepared formulations are shown in the Table 1. The percentages in the table are expressed as weight/weight. The water content was adjusted to achieve a final formulation weight of 100 % by mass.

The mixing process began at 50 rpm, increasing to 100 rpm after the addition of water, with the temperature maintained at 40 °C throughout. Each composition was mixed for 3 h and then poured into transparent

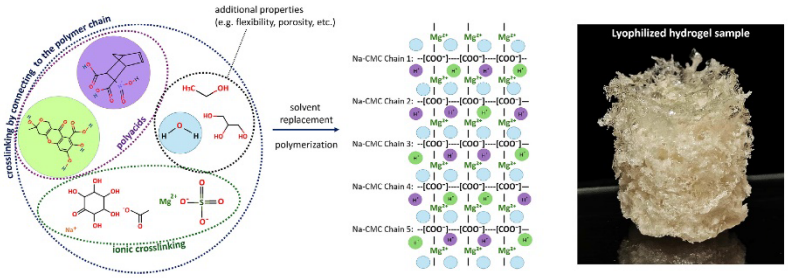


Fig. 1. Schematic representation of Na-CMC-based hydrogel transition from precursors to final hydrogel state.

Table 1
Composition of gel formulations with different buffer solutions.

Ingredients, wt %	Formulation							
	F1	F2	F3	F4	F5	F6	F7	F8
Na-CMC	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
NaCl	–	1.2	–	–	–	1.2	–	–
$MgSO_4$	–	–	2.4	–	–	–	2.4	–
$MgCl_2$	–	–	–	1.9	1.9	–	–	–
Sapropel extract	–	–	–	–	5	5	5	5
Ethanol	8	8	8	8	8	8	8	8
Glycerol	8	8	8	8	8	8	8	8
Water (q.s.)	100	100	100	100	100	100	100	100

100 ml containers at 30 °C to simulate storage conditions. All formulations were mixed using a modular laboratory reactor IKA LR 1000 (Germany), equipped with an anchor stirrer with PEEK scrapers, a flow breaker, a temperature sensor, and a vacuum system. The flow chart of the gel production process is shown in Fig. 2A.

2.4. Organoleptic characteristics

The gel formulations were visually inspected for appearance, color, odor, and signs of instability such as clarity, homogeneity, consistency, particle aggregation, and phase separation. After storage under specific conditions, the gels were examined for appearance and presence of aggregates to verify homogeneity. Consistency was assessed by squeezing a small amount of gel between the thumb and forefinger.

Organoleptic characteristics were evaluated using a three-point scale as follows in Fig. 2B.

- 3 points for samples that were transparent, exhibited excellent homogeneity and a smooth texture, and had a pleasant odor.
- 2 points for samples that were cloudy, displayed good homogeneity and a relatively granular texture, with no changes in odor.
- 1 point for samples that were opaque, showed satisfactory homogeneity, had visible ingredient separation and a granular texture with agglomerates, and exhibited changes in odor.

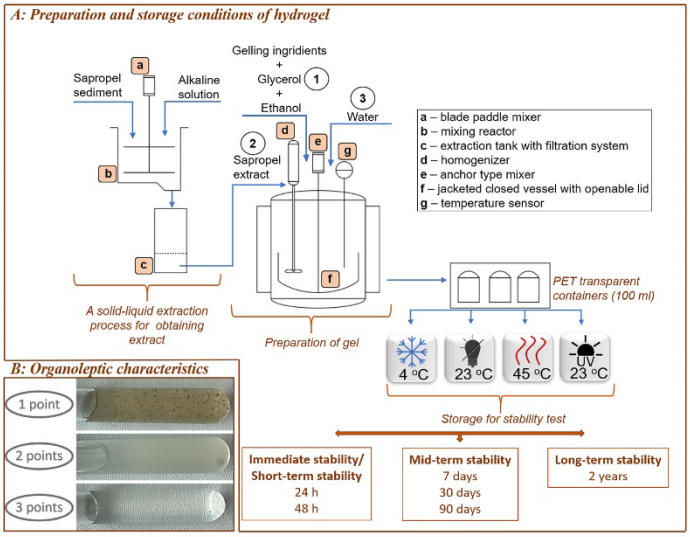


Fig. 2. Design of the study. A - preparation and storage conditions of hydrogel with sapropel extract; B - visual representative of organoleptic characteristics of hydrogels.

2.5. Stability evaluation

Physical stability refers to a product's ability to maintain its original physical properties, such as appearance and texture, over time. Chemical stability ensures that the product's chemical composition and potency remain intact without significant degradation. Carrying out stability testing the shelf-life and expiry date can be calculated. The information about the best storage condition at which drug will contain its characteristic for long time can be obtained. And if there is any specification that can be written on the label (P. Das & Das, 2022).

The determination of the physical and chemical stability of the formulations were conducted according to different cosmetic and pharmaceutical product stability guides (ICH Q1A (R2) Stability Testing of New Drug Substances and Drug Products, 2003). In order to tests the stability of the formulations in different conditions, 24 h after preparation, from each composition 3 samples were placed at 4 °C, 23 °C and 45 °C and held in dark and UV light. Gel formulations were tasted over a period of 24 and 48 hours, 7, 30, 90 days and 2 years (Fig. 2A).

2.6. Centrifugal test

To investigate the gel stability against the centrifugal force, 48 h after preparation, gel samples were transferred into tubes and centrifuged at 3000 rmp for 30 min using centrifugal device (Boeco C 28A, Germany). Gel stability was then evaluated at 5, 15, 30, and 60-minute intervals.

2.7. Thermal tests

To assess the stability of the gel formulations against extreme temperature changes, both freeze-thaw and cooling-heating tests were performed. For each formulation, 25 g of gel was placed in plastic tubes (15 mm diameter, 150 mm height) 48 h after preparation. In the freeze-thaw test, the samples were stored at -18 °C for 48 h, followed by 48 h at 23 °C, and this cycle was repeated for six periods. For the cooling-heating test, each formulation was subjected to 45 °C for 48 h, followed by 48 h at 4 °C. The stability of the formulations was evaluated by checking for the presence of any aqueous phase separation in the tubes; a formulation was considered thermally stable if no aqueous phase separation was observed.

2.8. Surface contact angle

The wetting behavior of the formulations was assessed using surface contact angle measurements to evaluate their hydrophilicity and hydrophobicity. Water contact angle was recorded at 0 min and 2 h to investigate the change in wetting behavior over time. Parallel samples were measured for comparison.

2.9. Water retention

To determine water retention in the prepared formulations, the following procedure was used. First, the formulations were weighed (W_0) in 10 ml glass beakers, which were then placed in an oven set to 60 °C. The weight of the formulations was recorded again (W_t) every hour for 8 h, followed by measurements at 12, 18, 24, 28 and 44 h. The retained water content (M) of the formulations was calculated using the Eq. (1) as follows:

$$\text{Water retention } (\%) - \frac{W_t}{W_0} \times 100 \quad (1)$$

2.10. Polarized optical microscopy

Hydrogel texture and surface morphology of the hydrogel were observed by polarized optical microscope Leica DM EP (Leica Microsystems, Germany) with magnification 100x, using Leica EC3 camera. The sample was placed between the two crossed polarizers. As light passes through the sample, its polarization plane changes because of optical anisotropy (birefringence) of the material, revealing the typical textures of each formulation. Formulation samples were examined both during the gelling phase and after being dried at 60 °C to assess surface morphology and texture.

2.11. Scanning electron microscope (SEM)

Imaging was conducted using the "Hitachi TableTop Microscope TM3000," which provides high-resolution visualization of sample surfaces. Imaging was done at 100x, 200x magnified level. The microscope was operated with a maximum current of 15 A, optimizing electron beam interaction with the sample. The SEM images allowed for detailed observation of the surface structure and provided insights into the physical characteristics and homogeneity of the hydrogels. Prior to analysis, the hydrogel samples were lyophilized to ensure structural stability and to remove moisture, minimizing potential artifacts during imaging. The freeze-drying was used to freeze-dry formulation after they were previously frozen using a laboratory freezer (−25 °C). A high vacuum (63 Pa) and a temperature of − 25 °C were employed during the drying process for 72 h The Christ Alpha 1–2 LDplus freeze dryer (Germany) was used.

2.12. Determination of pH values

The pH of each gel sample was determined in water solutions using a digital pH meter (WinLab Data Line pH/mV meter (Belgium)). Solutions were prepared by dissolving 2.5 g of gel in 25 mL of purified water. Electrodes were inserted 10 min prior to taking readings at room temperature. Each formulation's pH was measured in triplicate, and the average value was calculated to assess any pH fluctuations over time.

2.13. Viscosity

The measurements of viscosity of gels were done with VT550 Thermo Haake Electron Corp. (USA) viscosimeter with sensor MV-DIN. Readings were taken at spindle rotation speeds of 3 and 6 rpm, with the corresponding viscosity values recorded for each speed.

The complex viscosity of the gels was determined by an MCR302 rheometer from Anton Paar (Graz, Austria) in a continuous flow mode at 25 °C. Parallel-plate configuration with PP25 geometry was used. The shear rate was logarithmically increased from 0.01 to 1000 s^{-1}.

2.14. Rheological measurements

Rheological measurements were conducted to determine the sol-gel transition point using an MCR302 rheometer from Anton Paar (Graz, Austria) in oscillatory flow mode at 25 °C. A parallel-plate configuration with PP25 geometry was used. Gel stability was evaluated through a strain sweep test at a fixed frequency of 10 rad/s and strain rates ranging from 0.01 % to 1000 %.

2.15. X-ray diffraction (XRD)

To determine the phase stability of the sapropel extract water-soluble gel formulations and to identify the presence of HA and FA in the sapropel extract from different sediment layers X-ray diffraction was used. Hydrogel samples were dried at 100 °C, thin film was obtained, that was analyzed; sapropel extract from different depths were dried at 100 °C, grounded into powder and analyzed by X-ray analysis (Rigaku Ultima+, Japan) with CuKα radiation and goniometer rotation with 1°/min or 4°/min. The substances were identified using the electronic ICDD database (PDF-5+2024; https://www.icdd.com/pdf-5/) by comparing the obtained data with the corresponding database entry.

3. Results

In this study, an equal amount of sapropel extract was incorporated into a Na-CMC hydrogel system. A total of eight formulations were selected for stability testing: four formulations containing sapropel extract and four without, to assess the effect of sapropel extract on the hydrogel system.

The results indicate that all gel formulations containing sapropel extract exhibited a smooth and homogeneous appearance. Gels without sapropel extract were transparent, while those with sapropel extract were light yellow and clear. All formulations were easily spreadable and demonstrated acceptable stability, with organoleptic properties scoring 3 points for the first 3 months across all temperatures (4 °C, 23 °C and 45 °C). However, after 90 days and after 2 years, some formulations scored 2 points, and after 2 years at 45 °C and under UV light, they scored 1 point. A visual overview of all formulations after 2 years is presented in Table 2. During preparation the inversion test of formulations was performed after formulations were prepared and kept at different conditions to determine gelling time, visual viscosity and formulation stability over time (Fig. 3d).

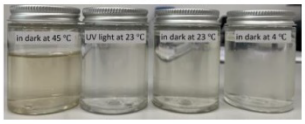
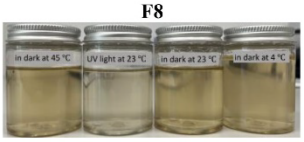
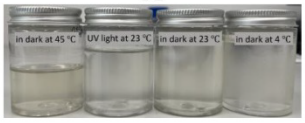
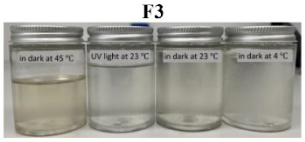
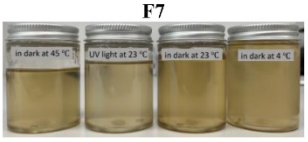
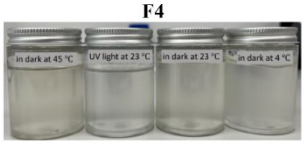
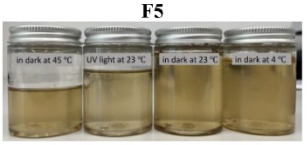
For the evaluating of stability, a centrifugal test was performed. No separation in formulations was determined. All formulations remain stable in all time intervals and samples did not show any distress due to centrifugal force.

During the thermal test – no aqueous phase was observed on the surface of samples, no water crystal formulation in the base under low temperature (-18 °C), no separation noticed during the thermal test. All the samples were stable throughout the cooling and heating periods according to Stability Testing of New Drug Substances and Products (ICH Q1A (R2) Stability Testing of New Drug Substances and Drug Products, 2003).

The ability of a hydrogel to wet a surface reflects its hydrophilic or hydrophobic properties, which can help in understanding the behavior of the formulations on the skin's surface. To determine wetting ability, the surface contact angle was measured. All formulation samples exhibited a tendency to wet the surface, with surface contact angles lower than 90°. The contact angle ranged from 50° to 78° in 0 minutes and from 20° to 52° at 120 min. Over time, the surface contact angle decreased, indicating that the formulations spread out on the solid surface. These results suggest that all formulations possess hydrophilic properties. The shift from more hydrophilic to more hydrophobic behavior correlated with changes in buffer solutions and the addition of Sapropel extract. Formulation F1 had the lowest surface contact angle both immediately after preparation and after prolonged contact. Formulation F7 exhibited a higher surface contact angle and a slower decrease in contact angle over time. Fig. 3c shows the surface contact angle measurements for formulations F1, F7, and F8 as examples of surface wetting behavior. Formulations prepared with buffer salts and Sapropel extract showed lower water loss at 60 °C over time. Water retention for all formulations was between 42 % and 53 % after 16 h, when equilibrium was reached. Formulation F1 lost approximately 60 % of its water content in the first 16 h, while formulations containing Sapropel extract, and buffer salts lost water more slowly. Water retention curves for formulations F1, F7, and F8 are shown in Fig. 4b.

To determine the structure of the formulations, polarized optical microscopy and SEM imaging were performed. The images revealed that all formulations exhibited a porous structure; however, formulations containing both buffer salts and Sapropel extract were more porous than those containing either buffer salts or Sapropel extract alone, or the formulation without both. SEM micrographs also showed that the formulation with both buffer salts and Sapropel extract had a rougher surface compared to formulations where the ingredients were added separately. Fig. 3d illustrates the difference between formulations F3 and F7, both of which contain $MgSO_4$ as the buffer salt. The formulation

Table 2
Chart of formulations: visual overview after 2 years of storage under various conditions.

Buffer solution	Without Sapropel extract	With Sapropel extract
H_2O	F1 	F8 
NaCl	F2 	F6 
$MgSO_4$	F3 	F7 
$MgCl_2$	F4 	F5 

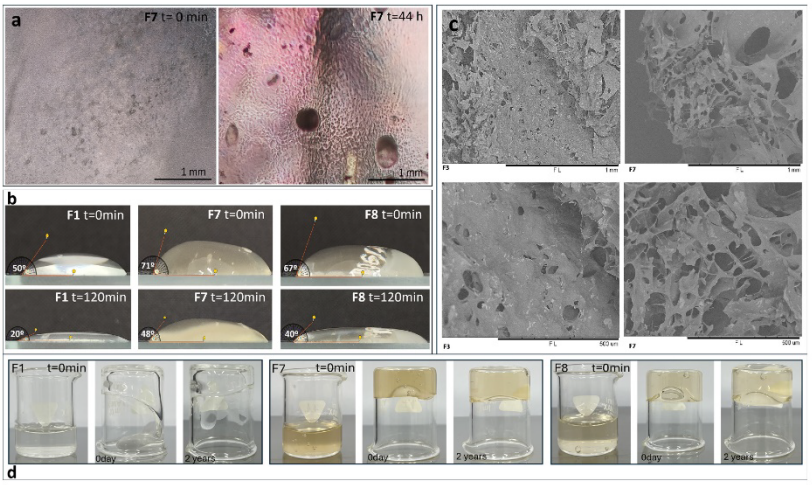


Fig. 3. The surface morphology of formulations (a) – Optical micrograph of formulation F7 at magnification 100x after drying at 60 °C for 44 h and before drying; (b) – The surface contact angle of formulation F1, F7, and F8 at 0 and 120 min; (c) – SEM micrographs of formulation F3 and F7 after lyophilization at 100x and 200x magnification; (d) – the inversion test of formulations F1, F7, and F8 after preparation in the first day and after 2 years samples hold at 23 °C.

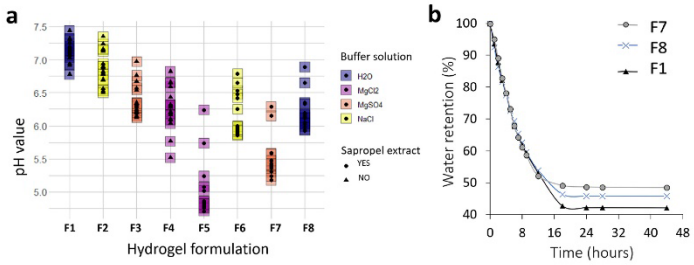


Fig. 4. (a) - The pH values of formulations (F1-F8) over time, stored in the dark at 23 °C with different buffer solutions. Each point represents the median result of the pH measurements at specific time intervals, including parallel samples of each formulation. The time intervals are immediately after preparation, 24 h, 48 h, 7 days, 30 days, 90 days, and 2 years; (b) – Water retention diagram of formulation F1, F7, and F8.

without Sapropel extract was less porous and had a smoother surface than F7, where Sapropel extract was included during hydrogel preparation. The rough surface of F7 is shown in Fig. 3a, captured with a polarized optical microscope. Optical microscopy images of the hydrogel reveal a porous and uniform structure, highlighting the material's consistent morphology and network formation (Fig. 3a).

All formulations maintained an optimal pH level for skin application and appropriate viscosity for a gel. Fig. 4 displays the pH values of the formulations over time. F1 and F2 (without sapropel extract) and F6 and F8 (with sapropel extract) exhibited stable pH values when stored at 23 °C in the dark. In contrast, F4 and F5, which contained a buffer solution of $MgCl_2$, showed greater fluctuations in pH compared to the other formulations. The pH values ranged from 4.7 ± 0.1 to 7.4 ± 0.2, which is considered acceptable; however, formulations with lower pH values may pose a risk of irritation upon skin application.

Viscosity is one of the most important properties of topical formulations, as it affects drug release and provides insights into formulation stability. Formulations using water as a solvent showed minor changes in viscosity over time, particularly in those without sapropel extract. Lower viscosity was observed in samples stored at 45 °C for 90 days and those kept at 23 °C for 2 years. However, the addition of sapropel extract caused a fivefold decrease in viscosity when storage temperatures fluctuated. Formulations containing NaCl as a buffer solution exhibited more stable viscosity over time, with higher viscosity observed in samples stored for 2 years. In contrast, formulations with $MgCl_2$ as a buffer solution maintained overall stable viscosity both with and without sapropel extract, with the 2-year formulation without sapropel extract showing a twofold increase in viscosity. The formulation with $MgSO_4$ as a buffer showed the least variation in viscosity, with samples containing sapropel extract and those without exhibiting similar viscosity values under identical conditions. Fig. 5a shows the viscosity values at a spindle rotation speed of 6 rpm for different formulations, with similar trends observed at a spindle rotation speed of 3 rpm.

Storage modulus G′ and loss modulus G″ versus shear stress at a fixed oscillation frequency (Fig. 5b) is usually used to determine the yield stress, that indicate the flow (typically in the extrusion process, e.g. hand creams, shampoo etc.) (Stojkov et al., 2021). The steep decrease in the loss and storage modulus at shear stress >50, indicates the start of the flow. In general, the crosspoint between the loss and storage modulus indicates the moment when the liquid component (loss modulus) dominates, and the material is in a liquid state. This behavior also confirms the thixotropic nature of the gel (formulation), as it highlights the

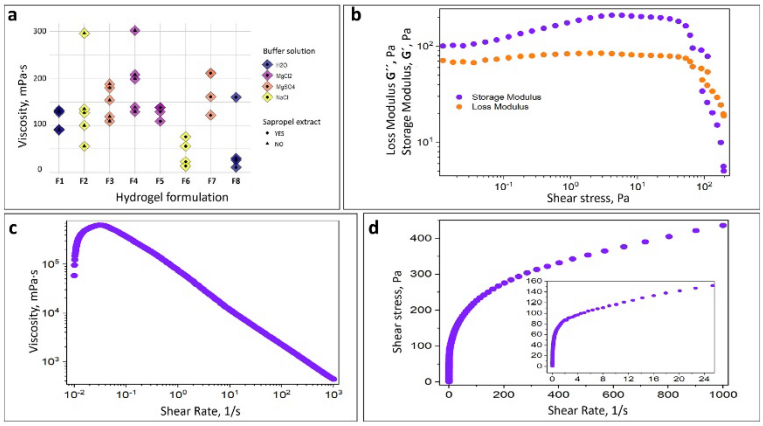


Fig. 5. The rheological properties of formulations: (a) - The viscosity values of formulations F1-F8 measured at a spindle rotation speed of 6 rpm, expressed in mPa·s. Each point represents the median result of measurements from parallel samples taken at two time points: after 90 days and after 2 years; (b) – storage modulus G′ and loss modulus G″ versus shear stress at a fixed oscillation frequency of 1 Hz of hydrogel F7; (c) – viscosity curve - shear rate curves of hydrogel F7; and (d) – shear stress - shear rate curves of hydrogel F7.

reversible transition between solid-like and liquid-like states under applied stress. The G′ and G″ values were higher for formulation with Magnesium salts as buffer salts.

The viscosity of the developed formulations (Fig. 5c) exhibits typical shear-thinning behavior, where viscosity decreases as the shear rate increases. At low shear rates, a slight viscosity increase is observed, attributed to the need to overcome the yield stress required to break the gel structure. Prior to this, the formulation undergoes elastic deformation, with the viscosity peak indicating the transition from elastic behavior to flow as the gel structure breaks down (Stojkov et al., 2021).

The shear stress versus shear rate curve in Fig. 5d demonstrates that the formulation follows a Herschel-Bulkley fluid flow model with shear-thinning behavior (Stojkov et al., 2021). This graph is commonly used to determine the static yield stress (τy), which represents the stress required to initiate flow of the gel. Fig. 5b shows that the curve starts to deflect from steep vertical line at around 52 Pa, after which the slope starts to form, indicating breakage of the formulation structure and flow.

XRD analysis allowed the detailed identification of mineralogical components in sapropel extracts (Fig. 6a). By examining sapropel extracts from different depths, XRD can reveal variations in mineral composition and crystalline structure, which can impact the distribution and effectiveness of biologically active substances. This analysis helped in understanding how depth-related changes in mineral content affect the potential pharmaceutical and cosmetic applications of sapropel.

Overall, XRD was employed to analyze sapropel from different sediment layers, specifically to identify humic and fulvic acids at varying depths. The 2θ angle is a critical factor in understanding the crystallographic structure of materials. It reflects how the (hkl) planes of minerals and organic substances within the sapropel extract are arranged and how they change across different depths. This angle represents the position of diffracted rays detected after interaction with the crystal planes of the sample.

For the lower sediment layer (E3), the absence of crystalline development in humic substances suggests a lack of well-defined crystal planes. Consequently, no sharp peaks were observed in the XRD pattern (Fig. 6a). This absence of specific (hkl) planes indicates the presence of unformed organic matter, which does not produce distinct diffraction peaks at particular 2θ values. In the mid-layer extract (E2), peaks associated with humic substances were evident, suggesting improved crystallinity and structural order. The (hkl) planes are more defined, resulting in sharp and more intense 2θ angles.

In the upper layer extract (E1), sharp diffraction peaks aligning partially with the presence of fulvic acid. However, shifts in the peaks suggest impurity, likely due to variations in the molecular arrangement of FA. These shifts in the 2θ angle reflect changes in the interplanar spacing (d-spacing) and the crystallographic arrangement of the sample.

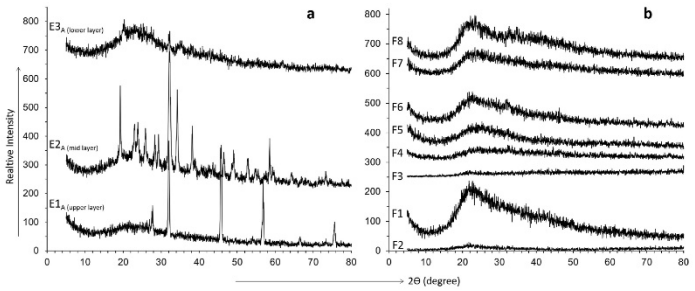


Fig. 6. X-ray diffractograms of: a - sapropel extracts from Audzelu Lake at different depths (lower, mid-, and upper layer), and b - hydrogels with sapropel extract (F1-F8).

For HA and FA, shifts in the diffraction peaks (2θ) and the appearance of new peaks indicate changes in their crystal structure and the level of crystallinity or ordering at different sediment depths.

Hydrogels (F1-F8, Fig. 6b), composed of cross-linked polymer networks, typically exhibit an amorphous structure in X-ray diffraction analysis due to their highly disordered molecular arrangement. The presence of water within the hydrogel matrix disrupts any potential crystallinity, as the water molecules interact with the polymer chains and increase their mobility. Cross-linking further contributes to the amorphous nature by preventing the polymers from aligning into a regular, repeating structure. Thus, all formulations (F1–F8) display this amorphous behavior, which is essential to the nature of hydrogels.

4. Discussion

In this research, the goal was to develop a homogeneous and time-stable hydrogel system with sapropel extract, free of any crystalline impurities and ready for use.

Studies suggest that CMC based hydrogels are prepared using physical or chemical crosslinking. Nevertheless, the hydrogels obtained by physical crosslinking are weak in nature and loses stability over time (Ahmad et al., 2022; Mehvari et al., 2024). The crosslinking agents used in most cases for the preparation of CMC-based hydrogels are toxic (Ghorpade et al., 2018; Mehvari et al., 2024; Mo et al., 2022). In this study the hydrogel crosslinking process happens by two mechanisms changing physical or chemical conditions. First - ionotropic gelation by cross-linking in the hydrogel initiated by the addition of divalent soluble salts. The $CaCl_2$ is the most popular salt used for crosslinking agent as review study shows (Ahmad et al., 2022), only few studies mention magnesium salts as hydrogel forming agents (Fang et al., 2022), however magnesium and calcium has similar chemical properties as they have the same number of electrons in their valence shells. Hydrogels prepared whit magnesium salts show more porous structures when dried and this could help in wound healing and drug release from hydrogel (Fang et al., 2022), due to this reason in this study $MgCl_2$ and $MgSO_4$ buffer solutions were used. The ionotropic method uses the gelling principle of polyelectrolyte solution $Na^+CMC(COO^-)$ with oppositely charged Mg^{2+} and $2Cl^-$ or Mg^{2+} and SO_4^{2-} multivalent ions. More stable formulations were with $MgSO_4$ buffer solution that corelates with Fang et al. (2022) study (Fang et al., 2022). Formulation whit $MgCl_2$ buffer solution overall showed good stability in the 2-year period but there were no studies that used $MgCl_2$ as crosslinking agent for hydrogels to compare our results. Na-CMC is one of the most important polysaccharides and can easily form complexes through ion interactions. Due to its high content of carboxyl groups, it readily binds to positively charged cations in an aqueous solution. As a result, the gelling process with $MgCl_2$ and $MgSO_4$ occurs more rapidly, and the gel formed is slightly thicker.

The second process – an H-bonded hydrogel is obtained by lowering the pH of an aqueous solution. The mechanism involves replacing sodium (Na^+) in CMC with hydrogen (H^+) in the acid solution to promote H-bonding. Sapropel extract is an acid solution containing HA and FA in soluble form, after adding sapropel extract second process starts. The H-bonds cause the water solubility of CMC to decrease and result in the formation of a flexible hydrogel. Hydrogels were put under thermal and centrifugal tests and did not show any instability in formulations. This formulation shows advantages over traditional CMC hydrogels preparation with $CaCl_2$ or organic crosslinkers like citric acid. To continue, the FA and HA act as natural polyelectrolytes. Their negatively charged functional groups (carboxyl- and hydroxyl-) enhance the crosslinking process by providing sites for interaction with positively charged species (e.g., H^+ and metal ions – Mg^{2+}) (Fang et al., 2022).

These acids also influence the network structure, introducing irregularities that contribute to porosity. Their amphiphilic nature may promote phase separation during gelation, forming a porous matrix as SEM and polarized optical microscope micrographs showed.

This can also be seen in the results of surface contact angle. As results show by adding Sapropel extract the contact angle is higher that means that formulation is more hydrophobic and has lower wetting ability. Nevertheless, sapropel extract adding also keeps network structure more stable, as the surface contact angle in prolonged time has the percentual lower angle value loss that it was in formulation with only water. In general, the surface contact angle shows that the preparations are more hydrophilic, which will help absorb more wound exudation and keep the burn or chronic wound hydrated (Al-Arjan et al., 2022; Grießinger et al., 2017).

Nevertheless, the results of water retention show that formulations have the ability to hold a considerable amount of water inside of their structures. The quantity of water in the formulation, which is typically in an amorphous, swollen state, is determined by the nature of the system itself, it could mean that it will respond to changes in environmental pH, hydrogels can swell and shrink in a reversible manner, and it will keep the wound hydrated (Ahmad et al., 2022; Ramli & Wong, 2011; Zare-Akbari et al., 2016). However, this needs to be tested in future experiments. Therefore, the formulations with sapropel extract and buffer salts lose water content more slowly, that could mean that sapropel extract has ability to change network of the system itself (Fang et al., 2022; Pornpitchanarong et al., 2022).

It means that HA and FA serve as a mild crosslinking agent and acidifying component, increasing the concentration of H^+ ions in the system. H^+ ions facilitate hydrogen bonding and ionic interactions between polymer chains, reinforcing the gel network.

To continue, the formation of porous, water-soluble hydrogels can be attributed to the chemical composition and interactions among the components in formulations and with the skin surface.

The buffer salts, like $MgSO_4$ provides Mg^{2+} ions, which interact strongly with negatively charged functional groups (e.g., carboxyl groups of sodium carboxymethyl cellulose). This enhances the ionic crosslinking efficiency, promoting a robust and interconnected matrix (Fang et al., 2022).

The simultaneous presence of Mg^{2+} and H^+ ions allow for dual crosslinking mechanisms: covalent-like ionic interactions and hydrogen bonding. This synergy contributes to the formation of a stable, porous, hydrogel network.

The porosity arises during the gelation process, as the interplay between rapid ionic crosslinking and slower polymer interconnection may create microphase separation. The release of water during gelation (syneresis) contributes to the development of pores (Al-Arjan et al., 2022).

Mg^{2+} ions are essential cofactors in various biological processes, such as DNA replication, protein synthesis, and energy metabolism. Mg^{2+} ions are known to promote angiogenesis (formation of new blood vessels), a critical process in wound healing. They also modulate inflammation by reducing the levels of pro-inflammatory cytokines, creating a more favorable healing environment (Al Alawi et al., 2018; Pan et al., 2024).

This results in a more flexible and bioactive gel structure, enhancing the hydrogel's performance in wound healing. pH of the delivery system should be close to the pH of sink local area to decrease potential local irritation. The healthy skin surface pH range is from 4.5 to 5.3 with an increasing pH gradient over the time to 6.8. The microenvironment of the wound pH can vary, for acute wounds it was reported around pH of 7.4 and for chronic wounds ranges from 7.4 to 8.9; alkaline environment can increase bacterial growth (Sim et al., 2022). The pH values for selected formulation ranged from 4.7 ± 0.1 to 7.4 ± 0.2 which can give favorable environment for wound healing.

As the sapropel extract contains high levels of HA and FA, adding it to the formulations affects the pH values (sapropel extract pH levels were from two to four), helps with crosslinking and gives extra stability to the hydrogel system, that gives extra shelf time (Ghorpade et al., 2018). Hydrophobicity and weak gel forming capacity at neutral and alkaline pH were reported in different studies (Erceg et al., 2021; Mo et al., 2022; Nakagawa et al., 2013). In general, in aqueous dispersion of

Na-CMC with increase of concentration increases viscosity and a stronger gel then can be formed; in this study, Na-CMC was standardized to 2.5 % (w/w), as the maximum mucoadhesive strength could be reached at concentration from 2–3 %. With increase in viscosity that could decrease release rate of biological active substances (da Silva et al., 2022). This formulation of anionic Na-CMC, compared to other non-ionic cellulose derivatives, may exhibit better adhesion to certain biological surfaces, making it potentially suitable for transdermal applications. However, adhesion experiments are necessary to confirm its strength. A transdermal delivery system based on a CMC nanocomposite hydrogel incorporating silver particles has demonstrated significant potential in wound healing (A. Das et al., 2015).

While stable hydrogel formulations were successfully created, the discussion now focuses on their potential applications in the medical field. The main problem of the topical applications of biologically active substances are the penetration ability through the stratum corneum that is outermost layer of the epidermis; in order to have medical effect the biological active substance needs to cross epidermal barrier and reach deeper layers of the skin (Costa & Santos, 2017; Ficheux et al., 2019).

The study highlights hydrogels as effective topical drug delivery systems due to their high-water content, low irritation, and ability to facilitate deeper penetration of bioactive substances into the skin (Ahmad et al., 2022; Mehvari et al., 2024). Na-CMC hydrogels, in particular, show promise in promoting wound healing and managing transepidermal water loss (Ramli & Wong, 2011). While bio-based substances like sapropel extract offer regenerative benefits, their application may be limited by factors such as thermal stability and light sensitivity (Guimarães et al., 2021; Ji et al., 2016; Mehvari et al., 2024; Sim et al., 2022; Yan et al., 2013).

CMC hydrogels is mucoadhesive gel which increases contact between the wound surface and the sapropel extract and boosts effectiveness of HA and FA (Pornpitchanarong et al., 2022). The application of the sapropel sediments to the sustained release of functional substances for skin treatments was explored in many studies (Fernández-González et al., 2017; Gerencser et al., 2010; Glavas et al., 2017; Gomes et al., 2013; Stankevica & Klavins, 2014; Vanadziņš et al., 2022) however hydrogels or other materials containing sapropel extract are less explored (Bevan et al., 2013; Strus et al., 2018). Sapropel extract can promote collagen synthesis, eliminate free radicals and inhibit melanin formation (Vanadziņš et al., 2022); enclosed in the Na-CMC gel it receives the swelling properties of the hydrogel and regulated biologically active substance release (Mehvari et al., 2024).

The Fig. 7 schematically shows hydrogel containing sapropel extract interaction with wounded skin and fibroblasts. There are 4 possible models how hydrogel can interact with skin (Costa & Santos, 2017; da Silva et al., 2022):

1. Enhancement of the transdermal passages due to ethanol and glycerol in the formulation. Ethanol acts as a permeation enhancer by disrupting the lipid structure of the stratum corneum, increasing fluidity and making the skin more permeable. Glycerol hydrates the skin by drawing moisture into the stratum corneum, which can further increase permeability. It can also soften the skin, making it more elastic and allowing easier diffusion of active ingredients. When combined, ethanol and glycerol create an optimal environment for enhanced transdermal delivery by increasing hydration and disrupting the natural lipid barrier, allowing more efficient drug penetration through the skin.
2. Hydrogel and lipid exchange with stratum corneum. The hydrogel typically interacts with the stratum corneum by releasing or facilitating the penetration of water and active ingredients. Lipid from the hydrogel may replace or integrate with the natural lipids of the stratum corneum, enhancing hydration, permeability, or therapeutic effects. Exact lipid concentration in hydrogel containing sapropel extract was not determined; this is protentional mechanism that could happen if lipid concertation was in high levels.
3. Free release of HA and FA directly in the wound. The free release of HA and FA directly into the wound enhances the healing process: HA aids in wound healing through its anti-inflammatory and antimicrobial properties, helping to prevent infections and promoting tissue regeneration. FA contributes to wound repair by acting as an antioxidant, reducing inflammation, and supporting cellular repair and nutrient transport to the damaged tissue. The combination of these acids creates a beneficial environment for rapid wound healing, protection against infections, and reduced inflammation.
4. Intact vesicular skin penetration. Intact vesicular skin penetration refers to the ability of vesicles, such as liposomes or other nanoparticle carriers, to remain structurally intact while penetrating the

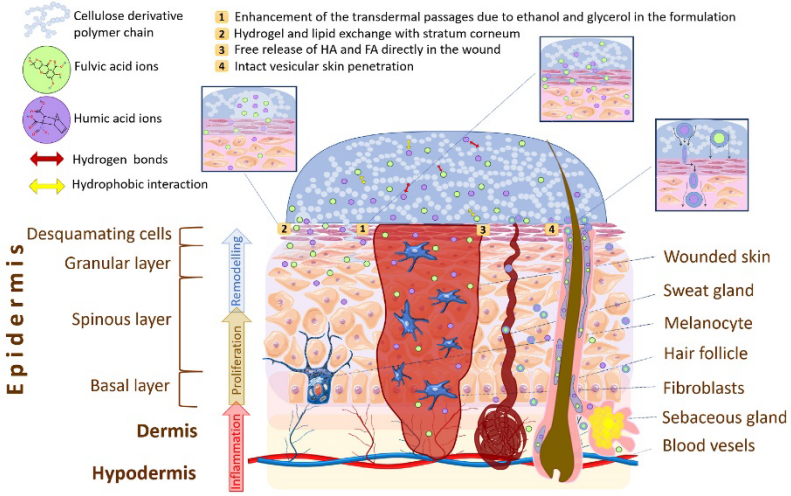


Fig. 7. Possible models of hydrogel interaction with wounded and undamaged skin.

skin layers. This process enhances the delivery of active ingredients deep into the skin, allowing for more effective therapeutic action. By maintaining their structure, these vesicles can protect sensitive ingredients (like drugs or nutrients) from degradation before they reach their target, ensuring sustained and controlled release at the site of action, leading to improved efficacy in treatments such as transdermal drug delivery.

A hydrogel containing sapropel extract is proposed as a potential product for the pharmaceutical market due to its promising applications in preventing, treating, and accelerating wound and scar healing. However, further experiments and clinical trials are essential to validate its efficacy and safety before it can be confidently recommended for widespread use. The potential of this sapropel gel in wound care emphasizes the need for continued research and development. Carboxymethylcellulose (CMC) and CMC-based hydrogels offer significant advantages in the development of environmentally friendly cosmetic and therapeutic products due to their low environmental impact. CMC is biodegradable, meaning it breaks down naturally without contributing to harmful waste. Additionally, CMC and hydrogels are derived from renewable resources like cellulose, reducing dependence on fossil fuels. Their non-toxicity and biocompatibility ensure safety for both consumers and the environment, minimizing pollution and ecological harm. These characteristics align with the global priority of utilizing natural, biodegradable local resources.

5. Conclusion

The homogeneous, time-stable Na-CMC hydrogel system incorporating sapropel extract was successfully developed, free of crystalline impurities and optimized for potential topical applications. The hydrogels exhibited high water content, low epidermal irritation potential, and enhanced transdermal delivery of bioactive compounds, offering a promising platform for wound care and drug delivery. Sapropel extract, rich in humic and fulvic acids, contributed to crosslinking and gel stabilization, potentially promoting wound healing by enhancing collagen synthesis and mitigating oxidative stress. Favourable pH values and stability under various conditions further support their suitability for pharmaceutical use.

However, challenges remain in ensuring sufficient stratum corneum penetration for therapeutic efficacy. Future research should address key gaps, including the hydrogel's interaction with skin surfaces (e.g., contact angle, surface tension, and adhesion energy), fibroblast viability, and release kinetics of sapropel extract. Experimental validation and clinical trials are crucial to comprehensively establish safety and therapeutic performance.

This study highlights the potential of sapropel-based hydrogels as innovative therapeutic solutions, aligning with global priorities in leveraging natural resources for sustainable healthcare advancements.

CRediT authorship contribution statement

Aneka Kļaviņa: Writing – original draft, Methodology, Investigation, Formal analysis. **Jeļena Reste:** Writing – review & editing, Methodology. **Inese Martiņsone:** Resources, Funding acquisition. **Ivars Vanadziņš:** Project administration. **Inna Juhņeviča:** Investigation. **Ilona Pavlovska:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

This project "Analysis of characteristics of medical sapropel and its usage for medical purposes and elaboration of industrial extraction methods" was financed by European Regional Development Fund, project no. 1.1.1.1/16/A/165. This publication has been developed with financing from the European Social Fund and Latvian state budget within project no. 8.2.2.0/20/I/004 "Support for involving doctoral students in scientific research and studies", at Rīga Stradiņš University.

We would like to express our sincere gratitude to professor Sergejs Gaidukovs, professor of polymer chemistry and technology at Riga Technical University, and his team for their invaluable support and assistance with the rheology testing.

Data availability

Data will be made available on request.

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