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

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Table of Contents

Table of Contents 3
Abbreviations used in the Thesis 4
Introduction 5
Aim of the Thesis 6
Objectives of the Thesis 7
Hypothesis of the Thesis 7
Novelty of the Thesis 7
Sapropel: A Resource for Health and Beauty 8
Geological and Biological Profile of Sapropel Deposits 10
Extraction and Analysis of Sapropel bioactive components 14
Sapropel Extract Hydrogel: Preparation and Stability 19
Conclusions 22
Proposals 23
List of publications, reports and patents on the topic of the Thesis 24
References 26

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
SCCS	The Scientific Committee on Consumer Safety
SLE	Solid-Liquid Extraction
UAE	Ultrasound-assisted Extraction
SFE	Supercritical Fluid Extraction
HHPE	Hydrostatic pressure extraction
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
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 found 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

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.

Sapropel: A Resource for Health and Beauty

Sapropel is a sediment found in shallow or deep marine basins, lagoons, and lakes, particularly as freshwater sediments in northern and eastern Europe, including Latvia. It is formed from the sedimentation and transformation of organic matter, such as phytoplankton, zooplankton, and macrophytes, along with materials from plants and aquatic animals (Stankevica et al., 2016; Stankevica & Klavins, 2014; Vanadziņš et al., 2022).

Historically, sapropel has been used as a remedy in both veterinary and human medicine, with its therapeutic use dating back to ancient Greece and the development of balneology in the 19th century. It has gained popularity in cosmetology and balneology due to a growing interest in natural remedies (Veniale et al., 2007). Traditionally, sapropel is applied through thermal mud baths or directly to the skin. These methods have demonstrated positive results in treating skin conditions such as eczema and dermatitis, as well as musculoskeletal issues like hand osteoarthritis (Bellometti et al., 1996, 2000). However, traditional application methods require specialised facilities and large quantities of material, limiting accessibility for patients. More user-friendly delivery systems for its bioactive components are needed (Fioravanti et al., 2014; Fortunati et al., 2016; Vanadziņš et al., 2022; Veniale et al., 2007).

The multifunctional effects of sapropel on the body are attributed to its complex chemical and biological structure. Sapropel sediments are rich in humic substances (HSs). These are the primary focus of interest for medicinal use. Formed from the microbiological decomposition of plant and animal waste, HSs are macromolecules with high redox activity and potent antioxidant properties. They are categorised into four fractions based on solubility: humic acid (HA), hymatomelanic acid (HMA), fulvic acid (FA), and humin (Klavina et al., 2019, 2020). Sapropel sediments also have a wide range of vitamins. Sapropel mainly contains water-soluble vitamins such as 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 D and tocopherol (E). Vitamin B12, produced by bacteria and blue-green algae, is vital for blood formation, amino acid metabolism, and nucleic acid synthesis (Kļaviņa et al., 2024; Luhila et al., 2022; Pavlovska et al., 2020; Vanadziņš et al., 2022).

Non-humic substances in sapropel sediments include carbohydrates, amino acids, lipids (such as hydrocarbons, carboxylic acids, esters, steroids, and oxygen-containing compounds), and proteins, all of which exhibit biological activity. Sapropel contains a vast number of microorganisms, some of which produce antibiotics that contribute to its antimicrobial properties. These properties can aid in faster wound healing and reduce inflammation and skin infections (Carretero, 2020a, 2020b; Fernández-González et al., 2017; Gomes et al., 2013).

Sapropel offers various therapeutic properties. If used as a mud application, it has high thermal capacity and low heat transfer. These properties allow sapropel to retain heat for extended periods and transfer it slowly and deeply into tissues, improving blood circulation, relaxing muscles, and reducing pain (Centini et al., 2015; Fioravanti et al., 2014; Gerencser et al., 2010; Glavas et al., 2017; Odabasi et al., 2007). Its fine texture and colloidal structure facilitate the penetration of bioactive components, such as humic substances, vitamins and amino acids, into the skin and deeper tissues, exerting therapeutic effects at the cellular level (Kļaviņa et al., 2024; Vanadziņš et al., 2022; Veniale et al., 2007). Humic and fulvic acids, along with polyphenols, protect cells from free radical damage, thereby preventing chronic diseases, reducing inflammation, and slowing the ageing process. Some research shows that sapropel applications can modulate the immune system, stimulate phagocyte activity, and contribute to tissue regeneration and immune health (Bellometti et al., 1996, 2000, 2002).

Overall, sapropel can improve skin structure, reduce wrinkles, strengthen nails and hair, and normalise sebaceous gland secretion, making it beneficial for conditions like psoriasis, eczema, and acne. Despite its potential, the medical properties of sapropel, particularly those of Latvian sapropel, have not been sufficiently studied using modern scientific methods, leading to a call for more evidence-based research and standardisation (Pavlovska et al., 2020; Vanadziņš et al., 2022). While “raw” sapropel samples generally do not contain active pathogens, their high microbial counts often exceed regulatory limits for topical applications, necessitating sterilisation or the addition of preservatives for safe pharmaceutical and cosmetic formulation. The heterogeneous and polydisperse nature of sapropel, along with variations based on its origin and extraction parameters, complicates the development of universal standard procedures for its extraction and analysis (Pavlovska et al., 2020; Suraganova et al., 2014). To address these challenges, a Doctoral Thesis is exploring the development of stable, water-soluble hydrogels incorporating sapropel extract, which could serve as a promising delivery platform for its bioactive compounds. This approach aims to overcome the limitations of traditional mud therapy by making therapeutic applications easier, more manageable, and more accessible for patients.

Geological and Biological Profile of Sapropel Deposits

Sapropel is a fine-grained, organic-rich sediment primarily found in freshwater lakes. It is often described as a “jelly-like” or “pasty” substance with a colour from light grey, pink, brown, brownish-olive, or greenish-yellow to almost black, depending on its organic substance and mineral additions. Its consistency is typically gel-like. It is considered a partially renewable resource, with formation rates varying from 0.1 to 6.6 mm per year (Stankevica et al., 2016; Stankevica & Klavins, 2014). Latvia’s total sapropel stock is estimated at around 700 million m^3, with significant deposits in the Latgale region, particularly Rēzekne, Preiļi, and Daugavpils municipalities (Obuka et al., 2018).

The primary classification system is based on composition. Sapropel is divided into four main types – organic, silica-containing, carbonate and mixed type sapropel; however, there are several sub-types – peat, carbonated, iron-rich, mixed, silicate with increased ashes contain. The primary type is determined by the biological and mineral content of the sapropel. The organic matter content can vary significantly between lakes, ranging from 15 % to 85 %. Sapropel can also be classified based on its origin – seawater sapropel or freshwater sapropel. The Doctoral Thesis notes that there is no universally accepted classification for all systems of sapropel (Pavlovska et al., 2020; Vanadziņš et al., 2022).

For this study, sapropel sediment samples were extracted from five lakes in eastern Latvia. The lakes were selected by analysing the Latvian lake database (ezeri.lv, accessed in 2018), which contains the official geological survey of Latvian lakes. The lakes were selected based on the depth of the sapropel deposits, the hydrological regime, the agricultural history of the surrounding area, and the potential for exposure to industrial waste. Sapropel was obtained from five lakes: Audzelu Lake, Dunaklu Lake, Ivuskas Lake, Zeilu Lake and Mazais Kivdalovas Lake (Pavlovska et al., 2020).

The extraction of sapropel from the lakes was performed during the wintertime when the surface of the lakes is frozen. Prior to the sample collection by taking probes in several locations, the thickness of the proper sediment layer was determined, and the depth of the sapropel deposit was established to select a well-composed sapropel layer for further analyses. During the sample collection procedure, 21 sediment samples were obtained from each of the lakes in three different depths, resulting in 105 sapropel sediment samples in total (Klavina et al., 2019; Pavlovska et al., 2020; Vanadziņš et al., 2022). The actual thickness of sapropel sediment layers in the studied lakes ranged from 0.9 m to 11.4 m. Optimal sapropel layers were typically found between 2.0 and 9.0 m from the sediment surface; layers less than 1.5 m were considered underdeveloped and were not used (Pavlovska et al., 2020).

All sediment samples were stored in closed opaque plastic containers without oxygen access at 4 °C to prevent oxidation and maintain bioactivity. 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 wintertime. The sediments were refrigerated and kept in these conditions from 4 to 8 months before extraction of biological active substances and analysis (Klavina et al., 2020; Pavlovska et al., 2020; Vanadzins et al., 2020b).

Latvian legal framework establishes a robust system of laws, regulations, and permitting procedures that prioritise responsible extraction practices and seek to minimise adverse environmental impacts. Compliance with this framework is essential for any operator wanting to extract sapropel in Latvia. 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 a major environmental concern as sapropel sediment acts as a pollution reservoir and is integral to the aquatic ecosystem (Environmental Protection Law, 2006; Pavlovska et al., 2020). Extraction activities must also consider the quality of surface waters and adhere to the Law on Subterranean Depths, which emphasises the rational and environmentally friendly use of such resources (Environmental Protection Law, 2006; On Environmental Impact Assessment, 1998). However, during the research on the legal framework, the explicit details of mechanisms for enforcing these regulations or the penalties for non-compliance were not found. Further legal research may be necessary to understand the specific enforcement mechanisms and penalties associated with Latvian regulations and freshwater sapropel extraction from lakes (Pavlovska et al., 2020).

Quality criteria for sapropel sediment were established, ensuring the safety, efficacy, and suitability of the sapropel for therapeutic applications. Quality tests include physicochemical testing, pollution parameter assessment of sediments, and microbiological testing. Organoleptic assessment involves evaluating the colour, texture and odour (Pavlovska et al., 2020; Vanadziņš et al., 2020).

The colour of sapropel samples ranged from pale yellow to 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 a mixed type and its origin comes from lakes` plankton, higher plants, and sometimes it is connected with peat layers. The texture of the samples was cream-like to dense, providing insights into the age and formulation speed of the sapropel sediments. The odour was mainly neutral or with the presence of hydrogen sulphide in some exploration points, and in one exploration point in the Audzelu Lake, an oil odour was

determined, which could indicate artificial pollution (Klavina et al., 2019; Pavlovska et al., 2020; Vanadziņš et al., 2020).

The pH level of sapropel is an important indicator of its suitability for medicinal applications. The pH levels of the sapropel samples in the studied lakes were found to be around 7–8. A neutral pH level indicates that these sapropel sediments have a high mineral content. This near-neutral pH is favourable for medicinal applications, particularly those involving topical use (Kļaviņa et al., 2024, 2025; Vanadzins et al., 2020). The organic matter content was determined through methods like loss-on-ignition (LOI). A sediment with an organic matter content of at least 15 % is defined as sapropel. The organic matter content is a fundamental criterion for distinguishing sapropel from other types of sediments. Organic matter content in the tested Latvian sapropel samples varied from 20 % to 90 %, with specific lakes showing 52 % to 54 % organic content and 4.4 % to 5.0 % carbonate. A higher organic matter content generally indicates a greater potential for therapeutic effects (Pavlovska et al., 2020; Vanadziņš et al., 2022).

All 105 sapropel sediment samples were tested for heavy metals lead (Pb), cadmium (Cd), nickel (Ni), cobalt (Co), and chromium (Cr) contamination and compared with standards set by the Scientific Committee on Consumer Safety (SCCS) (Scientific Committee on Consumer Safety (SCCS), 2021). The tested metals were present in all samples, but none of the metals exceeded the maximum acceptable level (Borowska & Brzóska, 2015; The European Pharmacopoeia 11th Edition, 2023; Witkowska et al., 2021). The presence of pesticides, particularly chlororganic pesticides, was a significant concern in the rural regions where these pesticides were used in the past. Sapropel intended for medicinal use should be free from pesticide residues to ensure safety and comply with regulatory requirements. The study found that levels of dichlorodiphenyltrichloroethane (DDT) and its breakdown product were generally low and below the limit of quantification (Pavlovska et al., 2020; Vanadziņš et al., 2020, 2022).

The last important characteristic for sapropel before its use in medicine is microbiological evaluation. Sapropel contains a diverse microbial community, which can impact its safety and therapeutic properties. Sapropel intended for medicinal use must be free from pathogenic microorganisms. Screening for specific pathogens, such as *E. coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, was crucial to ensure the quality (Pavlovska et al., 2020; Vanadzins et al., 2020a, 2020b).

The study identified nine species of bacteria in at various levels across the tested lakes. The Dunakla Lake had the highest value of 2.3×10^7 CFU/g. The Ivusku Lake had the lowest value of 1.1×10^5 CFU/g. The most abundant species were *Paenibacillus amylolyticus* and *Aeromonas bestiarum*, neither of which is known as a pathogen or opportunistic pathogen.

Paenibacillus amylolyticus may even possess antimicrobial properties due to antibiotic production, potentially explaining the lower CFU/g in some samples. However, *Serratia fonticola,* an enterobacteria and opportunistic pathogen, was also found, which could indicate wastewater pollution in lakes (Pavlovska et al., 2020; Suraganova et al., 2014).

Even though no active pathogens were identified in raw sapropel samples, the CFU exceeded the limit allowed in all sapropel samples. For the use of sapropel in cosmetic or medical applications, the sterilisation or the addition of preservatives is necessary to reduce the microbial count before using sapropel in cosmetic or medical applications (Scientific Committee on Consumer Safety (SCCS), 2021; Suraganova et al., 2014; Vanadziņš et al., 2020a). The International Organisation for Standardisation (ISO) standard "ISO 21426:2018 Annexe 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, are also valuable resources for assessing the safety and toxicity of sapropel extracts and their use in medicine. These guidelines can be utilised until standardised extraction and testing protocols are established for sapropel sediments (Kļaviņa et al., 2024; Scientific Committee on Consumer Safety (SCCS), 2021; Technical Committee: ISO/TC 228, 2018).

Extraction and Analysis of Sapropel bioactive components

Although sapropel contains many potentially beneficial compounds, the primary interest lies in its HSs, especially in Humic acid and fulvic acid. HSs are large molecules, known as macromolecules, formed through the breakdown of plant and animal waste by microorganisms (Dolmaa et al., 2011; Tserenpil et al., 2010). This process of formation is a secondary synthesis, meaning HSs are not naturally found in the tissues of living organisms (Aeschbacher et al., 2012).

Humic acid (HA), a main bioactive component of sapropel, is a complex aromatic polymer with diverse functional groups and a molecular weight of ~6500 Daltons. It is water-soluble at pH levels greater than two and precipitates in acidic conditions. HA exhibits strong antioxidant and therapeutic properties, aiding in tissue regeneration and reducing inflammation, and is believed to promote wound healing by generating additional oxygen. Humic acid reacts with cell walls and dermis receptors, penetrates tissues, and produces therapeutic effects. HA facilitates nervous tissue regeneration, stimulates macrophage defence and tissue repair, and has anti-inflammatory effects for tissue burns and cornea diseases. However, it primarily acts on the skin's surface due to its larger molecular size. Though non-toxic, its medical use is limited by structural variability (Almeida et al., 2019; Jacob et al., 2019; Jurcsik, 1994; Mirza et al., 2011; Ozkan et al., 2015; Rensburg van, 2015; Zykova et al., 2018).

Unlike HA, fulvic acid (FA) is a mixture of both weak aliphatic and aromatic acids. This difference in composition contributes to FA's remarkable solubility, allowing it to dissolve in water across the entire pH spectrum. Nevertheless, FA molecules are smaller than HA molecules; their absorption capacity is significantly higher. The enhanced absorption is mainly due to the presence of various functional groups and a smaller molecular size. Its smaller size facilitates deeper penetration into tissues, potentially supporting cell growth and tissue regeneration (Aeschbacher et al., 2012; Beer et al., 2000; Canellas et al., 2015; Chunxia et al., 1996; Lamar et al., 2014; Pena-Mendez et al., 2005; Rensburg van, 2015; Winkler & Ghosh, 2018). As a result, sapropel extract might be beneficial for managing inflammatory skin conditions and complex wound healing. To utilise these biologically active components in medical products, their extraction is necessary (Klavina et al., 2019; Kļaviņa et al., 2024).

Many different techniques can be used to obtain extracts from sapropel, like solid-liquid extraction (SLE) with various solvents, microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), supercritical fluid extraction (SFE), accelerated solvent extraction (ASE) or hydrostatic pressure extraction (HHPE) (Azmir et al., 2013; Hidayah & Abidin, 2017; Klavina et al., 2019, 2020; McDonnell & Tiwari, 2017; Preece et al., 2017; Ryzhova et al., 2013; Vinatoru et al., 2017). All these methods have demonstrated effectiveness in extracting from natural matrices — following principles that enhance extraction yield, can

be modified for industrial use, include steps to eliminate impurities and harmful substances, and ensure that the final product meets pharmaceutical-grade standards (Klavina et al., 2019, 2020). Doctoral Thesis indicates that most extraction methods for sapropel sediments have been developed for the extraction of humic acids and fulvic acids.

Chemical extraction using the Solid-liquid method is the most common method, which utilises different chemical solutions to extract biologically active components from sapropel sediments. This traditional method involves extracting from fresh, dried, or freeze-dried sapropel with various solvents, solvent mixtures, or aqueous phases with different pH levels. SLE is simple to perform and can achieve high recovery rates; however, it can be time-consuming, uses large quantities of solvents, and can break down humic acid (HA) into lower molecular weight components (Klavina et al., 2019, 2020; Strus et al., 2021). Despite the effectiveness of existing methods, no universally accepted standards exist for biological active substance extraction from sapropel or the analysis of sapropel extracts, which slows down its comprehensive use in pharmaceuticals and cosmetics. The choice of extraction method depends on several factors, including the type of sapropel, the desired components, and cost considerations (Jarukas et al., 2021; Klavina et al., 2020; Strus et al., 2021).

In this study, SLE was performed using sodium hydroxide. After sodium hydroxide solution was added, the pH level rose from neutral to pH 10, and all chemical cell disruption processes began. Stirring helps mix the base alkaline solution with sapropel, forming a colloidal mixture. After centrifugation, the sand particles and insoluble matter precipitate and are discarded. When acid was added to humic acids, the molecules precipitated from the solution and remained in a solid form; fulvic acid, however, remained in the solution. The extraction process yields two forms of extract: a solid, crystalline phase composed of humic acids, and a liquid containing a high concentration of fulvic acid (FA) solution (Klavina et al., 2020).

Extraction performed with sodium hydroxide yielded approximately 22–28 g of humic acids and 5–9 g of fulvic acids from one kilogram of dried sapropel. The highest difference between the sapropel from different lakes is in the yield of fulvic acids, where the highest and lowest values differ by more than 80 %. At the same time, the total organic carbon is more uniform, with the difference between the lowest and highest values being less than 30 %. Results showed that the highest humic acid concentration is in the Audzelu Lake and the Mazais Kivdalovas Lake. The high HA concentration in these lakes can be attributed to the formation of sapropel. In both lakes, the sapropel sediments are characterised as organic sapropel with a lower mineral content and lower pH values (Klavina et al., 2019; Vanadzins et al., 2020a).

For the characterisation of the sapropel extracts, there are no generally accepted guidelines, as is usually the case for many plant extracts (The European Pharmacopoeia 11th

Edition, 2023). 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 in Doctoral Theses to the characterisation of the sapropel extracts (Klavina et al., 2019, 2020; Kļaviņa et al., 2024)

Antioxidant Properties of Sapropel Extract

Sapropel extracts possess significant antioxidant properties, mainly attributed to their high polyphenol content. A high polyphenol content in sapropel extract strongly correlates with their total antioxidant status (TAS) (Kļaviņa et al., 2024). This correlation suggests that polyphenols are significant contributors to the antioxidant activity of sapropel. The antioxidant properties of sapropel may be beneficial in protecting the skin from environmental stressors and in managing skin conditions associated with oxidative stress. They may also benefit individuals with slow-healing wounds, such as those with type II diabetes (Guimarães et al., 2021; Ji et al., 2016; Sim et al., 2022).

In this study, the concentration of humic and fulvic acids in the sapropel extract varies due to differences in the chemical structure of humic substances (HS) and the physical availability of the organic matter associated with minerals in sapropel. It corresponds with the literature. Antioxidant levels showed no significant differences across sapropel layers, leading to the use of median concentrations to represent average findings from each lake. Sapropel samples from five Latvian lakes were analysed, revealing variability in antioxidant activity and humic substance concentrations. The Audzelu Lake had the highest TAS (1.08 ± 0.03 mmol/L) and TPC (146.26 ± 1.16 µg GAL/mL) with a HA concentration of 39.5 mg/g. In contrast, the Ivusku Lake had the lowest TAS (0.31 ± 0.01 mmol/L) and TPC (42.07 ± 0.55 µg GAL/mL), despite having the highest FA concentration (40.8 mg/g). Strong correlations were found between TAS and HA concentrations ($R^2 = 0.90$) and between TPC and TAS ($R^2 = 0.93$), indicating that polyphenols associated with HA are key to antioxidant activity. The differences in antioxidant levels among lakes are not fully understood and do not correlate with humic and fulvic acid concentrations in the same lakes (Klavina et al., 2019; Kļaviņa et al., 2024).

The absence of standardised methods for analysing sapropel extracts leads to inconsistent reporting and challenges in comparing results across different studies and sapropel sediments. The complex composition of sapropel extracts, which includes humic substances, non-humic substances, vitamins, peptides, and minerals, complicates analysis and requires the use of a combination of techniques for comprehensive characterization (Azmir et al., 2013; Klavina et al., 2020; Neha et al., 2019; The European Pharmacopoeia 11th Edition, 2023).

Cytotoxicity Assessment

The Neutral Red Uptake test and Real-time cell monitoring were used to check the concentration-dependent cytotoxicity and biological activity of sapropel extracts from various Latvian lakes, focusing on their effects on mammalian cells and potential applications in skincare and wound healing. The NRU test was used to determine cytotoxicity of sapropel extracts on mouse fibroblasts (BALB/c 3T3) at fulvic acid (FA) concentrations of 17.5, 70.0, and 140.0 µg/mL. Results showed a reliable correlation with controls only at the lowest concentration (17.5 µg/mL). At higher concentrations, discrepancies between standard medium and solvent controls suggested solvent toxicity, especially at 140 µg/mL, where NRU decreased significantly compared to the standard control but increased relative to the solvent control. Extracts from the Audzelu Lake consistently showed reduced NRU compared to the standard control across all concentrations. However, they increased NRU compared to the solvent control at higher concentrations, which reinforces concerns about the toxicity of used solvents. Extracts from the Lakes Ivusku and Zeilu at a concentration of 17.5 µg/mL showed no harmful effects and even potential benefits, while the Lakes Dunaklu and Mazais Kivdalovas showed moderate results, again indicating solvent-related stress (Kļaviņa et al., 2024).

The experimental setup required high extract concentrations, which were achieved by diluting the cell culture medium up to 40 %. This process introduced double-stress conditions, low pH and nutrient dilution. Since mammalian cells are sensitive to pH changes, the non-ideal pH of the samples may have influenced results. Sapropel's pH buffering capacity may have helped normalise the medium, contributing to cell survival more than the extracts' biological activity (Phelan & May, 2016, 2017).

Cell Growth Assessment

Real-time cell monitoring using Cell-IQ® provided further insights into cell growth dynamics. At low concentrations (3.5–7 µg/mL), sapropel extracts generally had no adverse effects, except for inhibitory responses in some samples after 24 hours. At 17.5 µg/mL, BALB/c 3T3 cells showed initial growth promotion, followed by inhibition. At 35 µg/mL, HaCaT growth remained stable, though extracts from the Lakes Mazais Kivdalovas and Audzelu inhibited growth after 24 hours. BALB/c 3T3 cells showed similar patterns of initial stimulation followed by inhibition after 24 hours. At 70 µg/mL, significant inhibition of BALB/c 3T3 cell growth occurred after 9 hours. HaCaT cells were unaffected for up to 12 hours, with inhibition thereafter. At 140 µg/mL, extract from the Audzelu Lake promoted HaCaT cell growth for up to 18 hours and BALB/c 3T3 cell growth for up to 3 hours, while other lake extracts showed minimal stimulation and sooner inhibition. Sapropel extracts demonstrated short-term cell growth promotion at high concentrations, but cytotoxic effects with prolonged exposure.

The antioxidant properties of humic and fulvic acids, particularly from lakes with high HA and TPC levels, support their potential for topical therapeutic applications (Kļaviņa et al., 2024). However, solvent toxicity and non-ideal culture conditions complicate interpretation and underscore the need for optimised formulations and standardised testing methods (Phelan & May, 2016, 2017).

Overall, sapropel extracts exhibit dose-dependent effects on cell viability and growth. Lower concentrations tend to support cell proliferation in the short term, while higher concentrations often lead to growth inhibition in long-term applications. Short-term stimulatory effects were observed particularly with sapropel extracts from specific lakes, such as the Audzelu Lake, suggesting certain compositions of sapropel sediments may be more suitable for therapeutic applications than others (Kļaviņa et al., 2024; Vanadziņš et al., 2022). The insights gained from *in vitro* cell culture tests, combined with the analyses of sapropel composition and antioxidant properties, contribute to a broader understanding of the potential therapeutic applications of sapropel extracts, particularly for skincare and wound healing. However, the exact mechanisms by which these extracts interact with cells to exert their antioxidant effects require further investigation, and the whole mechanism was not determined in this study. Future research should focus on human-relevant toxicity models, water-soluble formulations, and controlled pH environments to better assess the therapeutic potential of sapropel (Kļaviņa et al., 2024).

Sapropel Extract Hydrogel: Preparation and Stability

The Doctoral Theses investigate the development of stable, water-soluble hydrogels containing sapropel extract for potential pharmaceutical and cosmetic uses. The hydrogel was prepared using sodium carboxymethylcellulose (Na-CMC) as the primary gelling agent, combined with glycerol, ethanol, and buffer salts to optimise its physical and chemical properties. The formulation process utilised 2.5 wt. % Na-CMC, a biocompatible polymer known for its gel-forming capabilities and stable viscosity in aqueous environments. Glycerol (8 wt. %) was added as a plasticiser to improve elasticity and spreadability, while ethanol (8 wt. %) served as a preservative, preventing microbial growth and contributing to long-term stability. The active component, sapropel extract (5 wt. %), was standardised to contain 700 µg/g of fulvic acid, ensuring consistent bioactivity across batches (Kļaviņa et al., 2025).

Carboxymethylcellulose is widely used in pharmaceutical and cosmetic formulations due to its water solubility, resistance to organic solvents, and ability to form transparent, stable hydrogels. Its hydroxyl-rich structure facilitates the formation of three-dimensional hydrogel networks under appropriate conditions, with pH stability ranging from 3.5 to 11. Glycerol and ethanol play complementary roles in enhancing transdermal delivery. Glycerol hydrates the skin and increases permeability, while ethanol disrupts the lipid barrier and improves penetration of active ingredients. Together, they create an optimal environment for delivering sapropel-derived antioxidants and humic substances into the skin (Burgardt et al., 2015; Mo et al., 2022; Wellens et al., 2022).

To further refine the hydrogel's stability and rheological behaviour, buffer salts were incorporated. Three different salts – sodium chlorite. magnesium chlorite and magnesium sulphate were prepared as 0.2 M aqueous solutions and added to the formulation. These salts influenced the viscosity, homogeneity, and structural integrity of the hydrogel, with each contributing unique stabilising effects. All formulations were prepared using the same mixing strategy. The solid ingredients (Na-CMC) were mixed with ethanol and glycerol until a thick mixture was achieved. The sapropel extract was added slowly to ensure even distribution. The buffer solution was incorporated as the final step, adjusting the total volume to 100 % by weight percentages (Burgardt et al., 2015; Kļaviņa et al., 2025).

A modular laboratory reactor equipped with an anchor stirrer, a flow breaker, a temperature sensor, and a vacuum system was used to develop the hydrogels. During the process, parameters like mixing speed, temperature, and mixing duration were controlled. Mixing speed started at 50 rpm and then increased to 100 rpm after water addition to prevent polymer aggregation during rapid polymerisation. Temperature maintained at 40 °C, with an optional increase to 60 °C if required. Mixing duration was 3 hours to ensure uniform

polymerisation and dispersion. After mixing, the hydrogel mixture was poured into sterile 100 mL transparent containers. The pouring process was conducted in a sterile environment to prevent microbial contamination, which could compromise the long-term stability of hydrogel. The mixture was allowed to cool to 30 °C before being stored in different storage conditions for stability testing. This cooling step enabled the sol-gel transition, where the liquid (sol) transformed into a gel-like network through hydrolysis and polymerisation reactions. A patent was filed for a water-soluble sapropel hydrogel, marking a leading innovation in this study (Auce et al., 2022; Kļaviņa et al., 2025).

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 lose stability over time. The crosslinking agents used in most cases for the preparation of CMC-based hydrogels are toxic. In this study, the hydrogel crosslinking process occurs through by two mechanisms, which involve changes in physical or chemical conditions (Burgardt et al., 2015; Ghorpade et al., 2018; Mo et al., 2022; Pornpitchanarong et al., 2022; Wui et al., 2014).

The study results indicate that all hydrogel formulations, as explained in the patent, exhibited a smooth and homogeneous appearance. Hydrogels 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 ℃, 23 ℃ and 45 ℃). Nevertheless, 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 (Auce et al., 2022; Fang et al., 2022; Kļaviņa et al., 2025).

Hydrogels with distinct buffer salt compositions (sodium chlorite, magnesium chlorite and magnesium sulphate) demonstrated excellent stability and homogeneity, with the sapropel extract ensuring bioactive functionality. These formulations can be tailored for specific biomedical or environmental applications. Magnesium-containing hydrogels offer unique structural and functional properties that make them highly promising for a wide range of pharmaceutical applications, particularly in wound healing, drug delivery, and other biomedical therapies. The incorporation of magnesium salts, such as magnesium chlorite, enhances the hydrogel's structure, creating a more porous network. This increased porosity is beneficial for sustained drug release and optimised wound hydration, making these hydrogels an excellent choice for topical applications and controlled medication delivery (Al-Arjan et al., 2022; Fang et al., 2022). Hydrogels prepared with Magnesium salt buffer solutions have demonstrated

high stability, as evidenced by supporting scientific literature. Thermal and centrifugal tests further confirm the overall stability of these formulations, ensuring their suitability for long-term use. Compared to traditional hydrogels, magnesium ion-based hydrogels demonstrate superior structural flexibility, porosity, and bioactivity (Kļaviņa et al., 2025).

The addition of sapropel extract, rich in humic acids (HA) and fulvic acids (FA), introduces mild crosslinking and acidification effects, which further enhance the hydrogel's performance. These natural compounds strengthen the hydrogel matrix and help slow water loss, a critical factor in maintaining hydration at the wound site. Nevertheless, HA and FA act as natural polyelectrolytes, enhancing the interaction between the polymer chains and positively charged ions like magnesium ions, further stabilising the hydrogel structure (Ghorpade et al., 2018; Kļaviņa et al., 2025).

One of the most remarkable properties of these hydrogels is their significant ability to retain water, which is essential for wound hydration and healing. Additionally, these hydrogels exhibit a responsive behaviour to changes in environmental pH, allowing them to swell and shrink reversibly (Al-Arjan et al., 2022; Wach et al., 2003). Rheological and spectroscopic analyses confirmed the hydrogel's structural consistency and stability over time, and that the developed system can be classified as a hydrogel. The sol-gel transition method enabled precise control over the hydrogel's properties, making it suitable for biomedical applications (Burgardt et al., 2015; Kļaviņa et al., 2025; Stojkov et al., 2021). The results demonstrated that sapropel-enriched hydrogels are feasible as delivery systems for bioactive compounds, with promising implications for wound healing and skin treatments. This formulation approach highlights the potential of integrating natural antioxidant-rich extracts into modern hydrogel systems, offering a biocompatible and effective platform for topical therapeutic use.

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 hydrogels, 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.

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.

List of publications, reports and patents on the topic of the 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.
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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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2. **Klavina, A**., Auce, A., Silova, A., Dobkevica, L., Vanadzins, I. 2019. Freshwater sapropel extract for use in medicine and pharmaceutics. 3rd Global Conference on Pharmaceutics and Drug Delivery Systems Abstracts: 24.–26.06.2019., 84. Paris, France.
3. Auce, A., **Klavina, A**., Komarovska, L., Silova, A., Pavlovska, I., Vanadzins, I. 2019. Freshwater Sapropel as raw material in medicine and pharmaceutical production. 3rd Global Conference on Pharmaceutics and Drug Delivery Systems Abstracts: 24.–26.06.2019., 109. Paris, France.
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3. **Klavina, A.,** Vanadzins, I., Dobkevica, L., Auce, A., Komarovska, L. 2019. Extraction of Active Ingredients for Pharmaceutical Use from Freshwater Sapropel in Latvia. Rīga Stradiņš University International Research Conference on Medical and Health Care Sciences "Knowledge for Use in Practice": Abstracts, 01.–03.04.2019, 401. Riga, Latvia.

Patent:

1. Auce, A., Dobkevica, L., Komarovska, L., **Klavina, A.**, Silova, A., Silamikele, B., Pavlovska, I. and Vanadzins I. 2022. Water-soluble gel system with sapropel extract and the method of its preparation. P-19-63. LV15514, 20.05.2022. 5/2022. [Available https://databases.lrpv.gov.lv/patents/P-19-63].

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