Eng. Sci., 2025, **36**, 1628

Engineered Science
DOI: https://dx.doi.org/10.30919/es1628

Extraction of Lipids from Natural Saline Mud and their Interpretation from Point of Biological Activity in the Context of Salt Lakes of North-East Kazakhstan

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Abstract

Natural muds from saline lakes are attracting growing interest due to their content of biologically active components with proven therapeutic and cosmetic potential, particularly in dermatology. Despite their long-standing use in traditional medicine, the molecular composition of these muds—especially their lipid fractions—remains poorly understood. This study aimed to develop and optimize a protocol for the extraction and identification of lipid classes from saline muds, using samples from lakes in Kazakhstan as a model system. Special emphasis was placed on linking lipid extraction efficiency with antioxidant activity as an indicator of biological relevance. Mud samples were collected from Lakes Moilyd, Tuzkala, Maraldy, Arasan, Shoshkaly, and the eastern shore of Lake Alakol. The study revealed that by varying the eluent system, extraction can be tailored to favor different lipid classes, with specific systems demonstrating optimal selectivity for sphingolipids, glycerophospholipids, glycerolipids, sterols, fatty acyls, and prenol lipids. Extracts obtained with methyl *tert*-butyl ether:methanol and isopropanol:acetonitrile exhibited the highest antioxidant activity, a characteristic found to correlate significantly and that may be attributable to their enrichment in glycerolipids and sterols. Pre-treatment with mineral acid and an increased solvent-to-sample ratio further enhanced lipid yield and diversity. These findings demonstrate the potential of saline lake muds from Kazakhstan as sources of bioactive lipids and provide a practical framework for their application in dermatological, pharmaceutical, and cosmetic formulations.

Keywords: Lipids; Natural mud; Antioxidant properties; Extraction; Antioxidant activity.
Received: 15 May 2025; Revised: 23 June 2025; Accepted: 23 June 2025.
Article type: Original research.

1. Introduction

Natural muds (peloids) exhibit a unique composition that endows them with both medicinal and cosmetic properties. Their utilization is widespread both within formal medical contexts—specifically in peloid therapy—and among the general population, where their application is informed by empirical evidence and traditional knowledge. Research has validated the beneficial effects of peloid-based therapy as a complementary non-pharmacological treatments for rheumatic diseases, as well as their integration into cosmeceutical formulations for the management of scaly dermatological conditions.[1-12] In addition, antimicrobial activity of peloids has been confirmed by microbiological analyses: Ma'or *et al.*[13] reported inhibition of gram-positive and gram-negative bacteria by Dead Sea mud; Al-Karablieh[14] showed effects against *Candida albicans*; and Spilioti *et al.*[15] along with Georgieva *et al.*[16] identified actinobacteria and cyanobacteria in peloids capable of producing antimicrobial compounds such as polyketides and peptides. Natural muds are formed through prolonged and synergistic interactions between physicochemical and biological processes, governed by local geological and climatic conditions from a sedimentological perspective, they are defined as cohesive sediments composed predominantly of silt and clay fractions (<63 μm), exhibiting plasticity in a hydrated state. Medicaly, they are characterized

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Table 1: Objects of study, sampling location data, and coding.

Research object	Code	Sampling Location (Google Maps Coordinates)
Black mud of Arasan Lake	S.1	49° 15′ 51″ N 81° 44′ 28″ E
Black mud of Alakol Lake	S.2	46° 03′ 36″N 82° 02′ 12″E
White mud of Alakol Lake	S.3	46° 03′ 36″N 82° 02′ 12″E
Black mud of Maraldy Lake	S.4	52°16'01"N 77°44'58"E
Black mud of Moiyldy Lake	S.5	52°23'48"N 77°04'03"E
Black mud of Tuz Kala Lake	S.6	51° 52′ 11″ N 77° 28′ 29″ E
Black mud of Shoshkaly Lake	S.7	51°16'57.9"N 78°41'58.7"E

as semi-solid mixture of mineral and organic phases, combined with mineral water in varying proportions.[17] According to the classification proposed by Gomes and Silva[18] liquid phase mass fraction exceeding 90% is indicative of a high organic matter content, whereas values below 30% (*e.g.* peat) denote a predominance of mineral constituents (*e.g.*, fango). Intermediate compositions (*e.g.*, sapropel) represent mixed mineral-organic peloids. A further subclass, designated as sulfopeloids, has been proposed to account for peloids with elevated concentrations of both organic and inorganic sulfur[19]

Although the mineral composition of muds has been extensively studied, the characterization of organic compounds, their extraction methodologies, and the correlation with therapeutic efficacy remain incompletely elucidated, particularly for natural sulfide muds from saline lakes, including those in Kazakhstan[20-21] Consequently, comprehensive investigations have been conducted across various countries over different periods to isolate and qualitatively analyze humic substances from diverse mud origins using sodium hydroxide as an extractant, both with and without pre-treatment, as well as to examine their antioxidant activity and the development of related pharmaceutical preparations.[22-30] The lipid fraction constitutes a significant biologically active component of salt lake sulfide muds, potentially contributing to their physiological effects, particularly on the skin. Cutaneous lipid composition is crucial for the skin's protective functions against environmental stressors, including chemicals, microorganisms, allergens, viruses, and UV radiation, while also maintaining hydration and pH balance. Ceramides, the primary constituents of the skin's lipid layer, consist of a sphingoid base predominantly linked to residues of α-hydroxyl and non-hydroxyl saturated unbranched fatty acids ranging from C16 to C26 in chain length. Ceramides participate in the skin's barrier function, epidermal regeneration, and immune responses.[31] Cholesterol and its esters stabilize the stratum corneum, whereas cholesterol sulfate regulates desquamation.[32] Alterations in the cutaneous lipid profile or microbiome can influence overall skin health and are implicated in various dermatological conditions.[33] Consequently, it is hypothesized that the beneficial effects of topical applications of natural muds on the skin are partially attributable to the inherent lipid composition of the muds, which formed the subject of this investigation.

Studies aimed at elucidating the lipid profiles of muds from diverse origins have employed a variety of extractants, selected based on polarity. For untreated mud samples, solvents such as ethanol combined with non-polar agents (5:1), alcohol-oil mixtures, acetonitrile:chloroform (1:1), and dichloromethane:methanol (1:1) have been reported.[34-37] In cases involving preliminary sample demineralization, petroleum ether and chloroform:ethanol (1:1) have been employed.[38,23] Analysis of these studies revealed a predominant focus on quantitative characterization, with a notable absence of comprehensive qualitative analysis of the lipid fraction composition and its interpretation regarding physiological effects.

Lipid extraction and compositional analysis are primarily conducted using the classical chloroform-methanol (2:1) method, originally developed for animal tissue lipid studies and subsequently adapted for the quantitative isolation of phospholipids from thermal muds.[39-41] Salem *et al.*[42] proposed a modification to the classical lipid extraction protocol, substituting chloroform with methyl tert-butyl ether (MTBE) due to its reduced density. This alteration mitigates phase inversion with methanol and inhibits the formation of an amorphous interphase with solid sample particulates. However, comprehensive literature data regarding the complete compositional analysis of lipid fractions from sulfide sapropel extracted using these modified methods are lacking.

This study aims to extract a lipid complex from the sulfide mud of salt lakes in North-Eastern Kazakhstan, renowned for their distinctive therapeutic attributes, and to characterize its

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Table 2: Parameters of extraction systems.

Coding of extraction systems	Description		
	Solvent	Volume of solvent [mL]	Time of vortex stirring, [min]
MTBE:MeOH.	MTBE:MeOH	1 (M1) + 0.65 (M2)	10
MTBE:MeOH. (DM)	M1 - MTBE:MeOH (3:1, v/v) M2 - deionized water:MeOH (3:1, v/v)		10
$CHCl_3$:MeOH.	$CHCl_3$:MeOH (2:1, v/v) followed by 0.2 ml H_2O to induce phase	1.5	15
IPA (1.0-15)	IPA	1.0	15
IPA (1.5-15)		1.5	15
IPA (1.0-45)		1.0	45
IPA (1.5-45)		1.5	45
ACN (1.0-15)	ACN	1.0	15
ACN (1.5-15)		1.5	15
ACN (1.0-45)		1.0	45
ACN (1.5-45)		1.5	45
IPA:ACN(1.0-15)	IPA:ACN (1:1, v/v)	1.0	15
IPA:ACN(1.5-15)		1.5	15
IPA:ACN(1.0-45)		1.0	45
IPA:ACN(1.5-45)		1.5	45

qualitative composition concerning primary classification and antioxidant activity. The novelty of this research resides in the application of the classical Folch *et al.*[39] extraction method alongside a modified protocol proposed by Salem *et al.*[42] adapted specifically for lipid fraction extraction from salt lake sapropels - a methodological approach that has not been previously explored in this context. The significant of this investigation stems from the necessity to augment our understanding of the lipid composition within sulfide sapropels, which exhibit potential therapeutic efficacy for cutaneous and articular conditions. Acetonitrile (ACN) and isopropanol (IPA) were employed as less toxic alternative extraction systems, mirroring the polarity and density of methanol and chloroform/MTBE, respectively. The overarching objective of this study is to procure a lipid complex from North-Eastern Kazakhstan salt lake sulfide muds and to interpret its qualitative composition regarding fundamental classification and antioxidant capabilities. The specific aims of this research are: to compare the efficiency of these novel extraction systems on this matrix in terms of lipid component isolation and potential therapeutic impact; to examine extraction parameters and raw material preparation methodologies; and to conduct an in-depth analysis of lipid compounds, which represent secondary metabolites of microorganisms inhabiting extreme hypersaline and humid environments. The findings of this study are anticipated to unveil the potential utility of sulfide sapropel lipid complexes in the management of dermatological and joint disorders, as well as in pelotherapy.

2. Materials and methods

2.1 Reagents and laboratory equipment

All the solvents used in the study were of LC−MS grade. Methyl tert-butyl ether (MTBE), methanol (MeOH), chloroform ($CHCl_3$), isopropanol (IPA), and acetonitrile (ACN) were purchased from Sigma-Aldrich (Steinheim, Germany). Hydrochloric acid (HCl) of analytical grade was also obtained from Sigma-Aldrich (Steinheim, Germany). Ultra-pure water with a resistivity ≥18 MΩ cm was used in whole analytical process (Milli-Q system, Merck Millipore, Germany). ABTS (>98%), potassium persulfate (>99%), and Trolox (>97%) were supplied by Merck (Germany).

Sample preparation and analysis utilized the following equipment: A Scientz -12N freeze dryer (China) was used for lyophilization. Sample homogenization was performed using standard laboratory porcelain mortars and a Retsch mixer mill (Germany). For mixing steps, an Eppendorf ThermoMixer orbital shaker (Germany) and a Vortex Genie 2 SCIENTIFIC vortex mixer (Scientific Industries, USA) were employed. Sonication was carried out with an ultrasound water bath (VWR, USA). Centrifugation utilized a UNIVERSAL 320R centrifuge (Hettich, Germany). Solvent removal was performed with a Savant SpeedVac rotary evaporator (USA). Spectrophotometric measurements were taken using a Jenway 6400 spectrophotometer (Cole-Parmer, UK). For demineralization procedures, a reflux condenser (Duran, Germany) and a heating plate (IKA, Germany or Heidolph, Germany) were employed. Filtration steps utilized standard laboratory filter paper (413, size: 42.5 mm, VWR, USA) and funnels (Pyrex, USA). Demineralized samples were dried using a Salvis drying oven (Switzerland). Precise mass

Table 3: The number of peaks corresponded with annotated lipids in the different types of muds extracts.

Extraction system	Mud samples							Average ± SE*
	S.1	S.2	S.3	S.4	S.5	S.6	S.7	
MTBE:MeOH	1058	531	328	472	691	610	580	610.00 ± 86.32[a]
MTBE:MeOH (DM)	1039	624	522	625	1197	790	614	773.00 ± 95.52[a]
$CHCl_3$:MeOH	1012	567	491	2411	2242	691	506	1145.71 ± 313.17[b]
IPA (1.0-15)	846	474	385	509	684	644	443	569.29 ± 61.32[a]
IPA (1.5-15)	896	426	405	536	581	593	480	559.57 ± 62.37[a]
IPA (1.0-45)	970	482	361	551	670	679	420	590.43 ± 77.65[a]
IPA (1.5-45)	815	447	404	505	761	651	454	576.71 ± 62.32[a]
ACN (1.0-15)	914	361	382	528	563	568	426	534.57 ± 70.90[a]
ACN (1.5-15)	779	403	357	412	513	540	388	484.57 ± 55.22[a]
ACN (1.0-45)	883	380	340	382	859	562	416	546.00 ± 88.05[a]
ACN (1.5-45)	914	386	353	403	473	554	407	498.57 ± 73.63[a]
IPA:ACN (1.0-15)	989	523	364	608	689	774	565	644.57 ± 75.36[a]
IPA:ACN (1.5-15)	902	566	405	628	630	700	440	610.14 ± 63.05[a]
IPA:ACN (1.0-45)	983	557	366	722	809	1008	495	705.71 ± 92.73[a]
IPA:ACN (1.5-45)	970	459	380	622	738	760	452	625.86 ± 79.81[a]

*Values are presented as Mean ± Standard Error (SE) for N=7 biological replicates (mud samples S.1-S.7).

*Means sharing the same superscript letter are not significantly different ($p>0.05$) based on Tukey's HSD post-hoc test.

(0.0001 g accuracy, Germany). measurements were made with a Sartorius analytical balance Chromatographic separation was achieved using a Waters Acquity UPLC system (USA) equipped with an ACQUITY UPLC BEH C8 column (2.1 × 150 mm, 1.7 µm; Waters, USA). Mass spectrometry detection was performed on a Q-Exactive Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany). Samples for analysis were stored in 2 mL glass vials (Agilent Technologies, USA).

2.2 Sampling of samples

For the selection of an appropriate extraction methodology and the analysis of biologically active lipid components, the samples detailed in Table 1 were procured. Samples were collected using a point sampling technique, extending to a depth of 20 cm within a single layer at 5-meter intervals across the source radius, and subsequently subjected to quartering according to GOST 17.1.5.01-80. The sampling site locations were determined via the Google Maps application. Sampling was conducted during the summer (June 2023). The resulting composite samples were preserved in sterile polyethylene containers with hermetically sealed lids at 4 °C in darkness.

2.3 Mud samples pretreatment

For the extraction procedure, two distinct groups of samples, each subjected to different pre-treatment protocols, were utilized. The initial group was prepared by lyophilizing the mud samples under a vacuum pressure of 0.038 mbar at -57 °C using a freeze dryer, followed by homogenization in a mortar. These samples underwent extraction with the following solvent systems: methyl tert-butyl ether (MTBE):methanol (MeOH); chloroform ($CHCl_3$):methanol (MeOH); isopropanol (IPA); acetonitrile (ACN); and isopropanol (IPA):acetonitrile (ACN). The second group of samples was subjected to demineralization by the addition of 20 mL of a 2M HCl solution to 1 g (±0.001 g) of dry mud, with a reflux condenser attached to the reaction flask. The resulting mixture was heated at 90 °C for 30 minutes, followed by the addition of 20 mL of distilled water and further heating at 90 °C for another 30 minutes (initially, the temperature was increased to 120 °C for 5 minutes, then reduced to 90 °C for 25 minutes). Subsequently, the mixture was cooled and filtered. The demineralized samples (DM) were then dried at 105 °C for 4 hours and homogenized in a mortar. These samples were extracted using the methyl tert-butyl ether (MTBE):methanol (MeOH) solvent system.

2.4 Mud extracts preparation

Extraction protocol – MTBE:MeOH.

Two extraction solutions were prepared in advance: 100 ml of solvent 1 is prepared by adding 75ml of MTBE and 25 ml of MeOH (3:1, vol/vol); 100 ml of solvent 2 is prepared by adding 75ml of deionized water and 25 ml of MeOH (3:1, vol/vol).[42] Powdered sample (0.0500 – 0.0750g) was weighted into an Eppendorf tube with stainless steel ball, and 1 ml of pre-cooled extraction solvent 1 was added to sample. The mixture was homogenized by 2 min at 25 Hz using mixer mill, incubated on an orbital shaker at 400 rpm for 30 min at 4^0C and subjected to sonication in an ultrasound water bath for 15 min. Then to the mixture was added 0.65 ml of pre-cooled solvent 2, subjected on a vortex mixer at 3000 rpm for 10 min and incubated on ice for 20 min. After that it was centrifugated at a speed 14 000 rpm at 4^0C for 15 min and collected 0.6 ml of upper organic phase extract into new Eppendorf tube. The extracts was evaporated to dryness by rotary evaporator and

stored in a freezer at − 80 °C before analysis.

Extraction protocol – $CHCl_3$:MeOH.

Powdered sample (0.0500 – 0.0750 g) was added into an Eppendorf tube with stainless steel ball, and 1.5 ml of $CHCl_3$: MeOH solution in a ratio of 2:1 was added to sample.[39] The mixture was homogenized by 1 min at 25 Hz using mixer mill, subjected on a vortex mixer at 3000 rpm for 15 min. Subsequently, the homogenate was centrifuged at 14,000 rpm and 4 °C for 15 min, and 1.0 ml of the upper organic phase was carefully collected and transferred into a new 1.5 ml Eppendorf tube, to which 0.2 ml of water was then added to induce phase separation. The water phase was added after the initial extraction to induce phase separation and remove polar impurities. The resulting mixture was subjected on a vortex mixer at 3000 rpm for 5 min and centrifugated at a speed 14 000 rpm at 4^0C for 20 min. The lower organic phase extract was collected for the analysis. The extract was evaporated to dryness by rotary evaporator and stored in a freezer at − 80 °C before analysis.

Extraction protocols using isopropanol (IPA), acetonitrile (ACN), isopropanol (IPA): acetonitrile (ACN).

Solvents IPA, or ACN, or IPA:ACN in a ratio of 1:1 were used for extraction. For this purpose, 0.0500 – 0.0750 g of the powdered sample was added into an Eppendorf tube with stainless steel ball, and added 1.0 ml (1^{st} option) or 1.5 ml (2^{nd} option) of one of these solvent. The mixture was homogenized by 1 min at 25 Hz using mixer mill, for subjected on a vortex mixer at 3000 rpm for 15 min or 45 min (option). After that it was centrifugated at a speed 14 000 rpm at 4^0C for 15 min and collected 0.8 ml of supernatant into new Eppendorf tube. The extract was evaporated to dryness by rotary evaporator and stored in a freezer at − 80 °C before analysis.

Samples reconstruction.

All dry extracts obtained were subjected to the following treatment before UPLC-PDA-HRMS analysis: the sample was supplemented with 0.5 ml of ACN: IPA solution in a ratio of 7:3, subjected on a vortex mixer at 3000 rpm for 5 min and sonicated in an ultrasound water bath for 5 min. After that it was centrifugated at a speed 14 000 rpm at 4^0C for 20 min and transfered into 2 mL glass vials and stored in a freezer at -80 ◦C before analysis. The solvent systems and parameters used, as well as the coding, are presented in Table 2.

2.5 UPLC-PDA-HRMS analysis of extracts

For lipid analysis and identification, ultra-high-performance liquid chromatography-high-resolution mass spectrometry (UPLC-HRMS) was used, equipped with a UPLC system including a binary solvent manager, a column manager, a sample manager, a photodiode array detector and coupled to a Q-Exactive Orbitrap mass spectrometer. The injection volume was 2 µL. Compounds were separated on a UPLC BEH C8 column (2.1 × 150 mm, 1.7 µm, Waters) at a temperature of 40 °C. The two mobile phases were of 10 mM ammonium acetate acidified with 0.1% acetic acid in acetonitrile/water (50:50) as the eluent A and acetonitrile/ isopropanol (70:30) as the eluent B in binary gradient at a flow rate of 0.3 mL/min that was applied in the following gradient program: initial–10% B, 1 min–10% B, 9min – 99.0% B, 13 min – 99.0% B, 14 min – 10% B, 17 min 10% B. The PDA detector acquired chromatograms and the UV/Vis spectra in the range 220 – 400 nm with a resolution of 1.2 nm and a frequency of 20 Hz. The mass spectrometer was equipped with a heated electrospray ion source (HESI-II). The HESI-II parameters were set to sheath gas (N2) flow rate = 45 units, auxiliary gas flow rate =15 units, and sweep gas flow rate =3 units. The temperature of the auxiliary gas was 400 ◦C. The other HESI-II parameters were: capillary voltage = 3.5 kV, ion transfer tube temperature = 370 0C, and S-lens RF level =50. Data-dependent scanning was conducted in positive ion mode. The Full-MS spectra were recorded with a resolution of 70000 in the mass range 150 – 2000 Da at 100 ms as the maximum injection time. The following data-dependent MS2 settings were applied: isolation window 1 m/z, 17500 mass resolution, AGC target – 1e5 and 50 ms as maximum injection time for loop count 5. The high energy collision dissociation cell was operated at 35% of the normalized collision energy. Mass accuracy was calibrated using Pierce LTQ Velos ESI positive. The system was run with TUNE 2.8 and XCalibur 4.0 software for instrument control data acquisition, and data analysis. Compounds were manually identified based on their m/z value, retention time, fragmentation spectra. The identification of compounds was confirmed based on their exact masses, and structural formulas with ppm resolution of compound purity in negative ion mode are performed using MSDIAL database (ver.4.92) with accurate mass spectrometry data and molecular characteristics as a reference library to identify lipids. In the initial step, automated peak detection was carried out with the following parameters: MS1 tolerance of 0.01 Da, MS2 tolerance of 0.025 Da, MS1 mass range of 150–2000 m/z, and a retention time window from 1.9 to 13.0 minutes. The mass slice width was set to 0.1 Da. Chromatographic data were smoothed using the Simple Moving Average method with a smoothing level of 3. The minimum peak height was 10,000, and the minimum peak width was 5 scans. Peak merging was allowed with at least one neighboring signal and a maximum of two merged charges. Compound annotation was performed using curated libraries in .msp format, with solvent filtering enabled. The following adducts were considered in positive ion mode: $[M+H]+$, $[M+NH_4]+$, $[M+Na]+$, $[M+K]+$, $[M+ACN+H]+$, $[M+H+H_2O]+$, and $[2M+H]+$. Matching to library entries was based on accurate precursor mass, diagnostic fragment ions, and isotopic pattern similarity. During the alignment step, sample 011–18 was used as a reference. Alignment parameters included a retention time tolerance of 0.2 minutes, MS1 tolerance of 0.015 Da, a retention time factor of 0.4, an MS1 factor of 0.6, and a requirement that a peak be present in at least 3 samples for inclusion. To improve annotation reliability, features with low or borderline similarity scores were filtered and subjected to manual inspection. The corresponding

MS/MS spectra were reviewed to confirm key fragment ions, assess spectral quality, and validate or reject ambiguous identifications. To compare the efficiency of different lipid extraction methods, we did not rely on peak intensities or areas under the curve. Instead, our analysis was based on the number of unique lipid species annotated in each extract. For the quantitative comparison, the total count of distinct annotated lipids was used as an estimator of extraction efficiency. For qualitative assessment, these lipids were categorized into major structural classes — Sphingolipids, Fatty acyls, Glycerolipids, Glycerophospholipids, Prenol lipids, and Sterol lipids. The relative proportion of each class was then calculated as a percentage of all annotated lipids within a given extract. This approach allowed us to determine which extraction methods were most effective in recovering not only a broad diversity of lipids, but also specific lipid classes, without interpreting the data in terms of absolute or relative concentrations.

2.6 Determination of the antioxidant activity in vitro and total phenolic compounds in extracts

The antioxidant potential of obtained extracts was tested using 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$). The following reagents were used in colorimetric assays: potassium persulfate, 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS); the following were used as reference standards: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid ("Trolox"). Used to prepare solutions deionized water was filtered through filter paper. The all determinations were performed on a single-beam visible scanning spectrophotometer. For ABTS measurements, 0.1 mL of the obtained mud extract was diluted tenfold with methanol prior to analysis. Blanks for each determination were performed in parallel to the samples, using pure methanol instead of the plant extract. For colored samples, the background absorbance was measured in a similar manner, replacing the appropriate reagent with water.[43]

2.7 Statistical analysis

All statistical analyses were performed using OriginPro 2018 (OriginLab Corporation, Northampton, MA, USA). A significance level of $p<0.05$ was used for all statistical tests. The relative abundance for each lipid class was calculated as a percentage of the total number of identified lipid peaks, and these values are presented as Mean ± Standard Error of the Mean (SE) based on averaging data across samples. For antioxidant activity data, all analyses were performed in triplicate, and average values and standard deviations (Mean ± SD) were calculated. One-Way ANOVA was employed to determine statistically significant differences among group means in several contexts: to assess the overall effect of different extraction systems on the total number of annotated lipids; to analyze the influence of different extraction systems on the relative abundance of each specific lipid class; to determine overall significant differences in lipid class composition among the three mud samples (S.1, S.4, S.5). Following a significant One-Way ANOVA result, Tukey's Honestly Significant Difference (HSD) post-hoc test was applied for pairwise comparisons between groups. On graphs, statistically similar groups were denoted by a common letter (A, B, AB, etc.) above the corresponding bars; groups not sharing common letters were considered statistically significantly different. For lipid classes where ANOVA did not reveal statistically significant differences ($p\geq0.05$), post-hoc tests were not applied and homogeneous group letters were not assigned. For each solvent type (IPA, ACN, and IPA:ACN), a 2 (solvent volume: 1.0 vs. 1.5 mL) x 2 (shaking time: 15 vs. 45 min) factorial Two-Way ANOVA was conducted to investigate the main effects of extraction parameters (volume and shaking time) and their interaction on the number of annotated lipid peaks. Correlation and Comparison for Antioxidant Activity: Pearson's correlation analysis was conducted to identify relationships between the relative abundance of specific lipid classes and antioxidant activity, by calculating Pearson's correlation coefficients (r). To evaluate the impact of hydrochloric acid pre-treatment on antioxidant activity, paired t-tests were performed for comparing corresponding dry and acid-treated mud samples.

3. Results and discussion

The efficacy of different solvent systems in isolating the lipid fraction from the mud matrix was evaluated based on the average number of annotated lipid peaks across all mud samples (S.1-S.7), as summarized in Table 3. Statistical analysis revealed a significant effect of the extraction system on the number of annotated lipids ($F(14,90)=2.17$, $p=0.015$). The $CHCl_3$:MeOH solvent system consistently demonstrated significantly higher efficacy in isolating the lipid fraction compared to all IPA and ACN based solvent systems tested individually (all $p<0.05$). The $CHCl_3$:MeOH system yielded an average of 1145.71 ± 313.17 annotated peaks, substantially surpassing the average yields of IPA and ACN systems (ranging from 484.57 ± 55.22 to 590.43 ± 77.65 peaks). While the MTBE:MeOH eluent system showed high yields for specific samples (*e.g.*, S.1 and S.7, Table 3), its average performance (610.00 ± 86.32 peaks) was not significantly different from the other solvent systems, including IPA:ACN mixtures, but was significantly lower than $CHCl_3$:MeOH. Similarly, the extractants ACN and IPA individually yielded a lower overall mean quantity of isolated lipid compounds across all samples, relative to other solvents, with ACN exhibiting the lowest isolation efficiency on average; however, these differences were not always statistically significant when compared directly to MTBE:MeOH or IPA:ACN systems.

Conversely, the IPA:ACN solvent systems, particularly IPA:ACN (1.0-45) with an average of 705.71 ± 92.73 peaks, demonstrated a tendency towards superior performance compared to the individual ACN and IPA solvents, although these differences were not statistically significant in all pairwise comparisons.

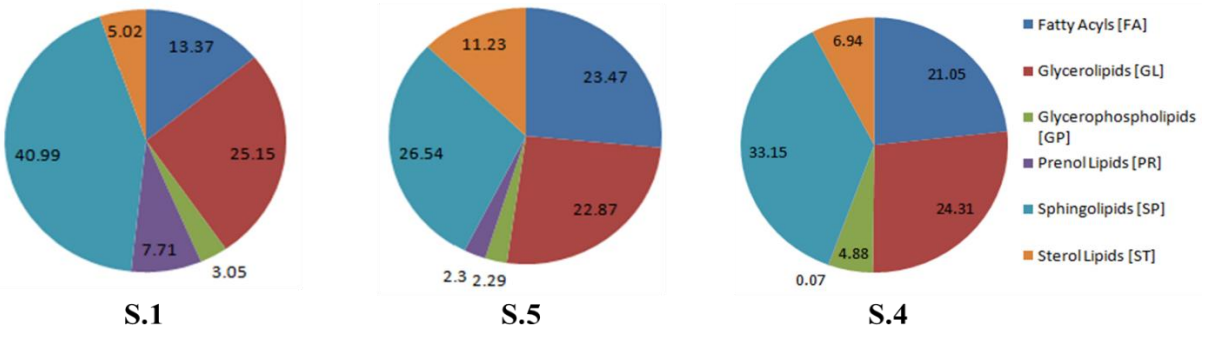

Fig. 1: Average percentage distribution by number of detected lipids for each class in individual mud samples. Percentages represent the mean abundance of each lipid class, averaged across all 15 extraction systems for each sample.

Analysis of the MTBE:MeOH extraction results for both dry and demineralized (DM) samples revealed a consistent increase in the yield of isolated lipid fraction components following mineral acid treatment. Notably, this trend was particularly pronounced for the black mud samples from S.2 (1.6 times increase) and S.5 (1.7 times increase). These findings substantiate that the treatment of mineral fraction carbonates and sulfides with hydrochloric acid facilitates the release of organomineral compounds entrapped within the mineral matrix, thereby suggesting its potential inclusion as a pre-extraction sample preparation step for dry mud.[23-24, 30]

Beyond solvent composition, the influence of extraction parameters, specifically solvent volume and shaking time, was investigated for IPA, ACN, and IPA:ACN systems. Statistical analysis, performed for each solvent type (IPA, ACN, IPA:ACN), indicate that, within the tested ranges, variations in solvent volume (1.0 mL vs. 1.5 mL) and shaking time (15 min vs. 45 min) do not significantly impact the lipid extraction efficiency for IPA, ACN, or IPA:ACN solvent systems. The characterization of the extracted lipids was performed on samples S.1, S.5, and S.4, which, based on our overall analysis of average yields (Table 3), generally corresponded to those with the highest lipid compound quantities.

The former two sources are used in balneological treatments at existing sanatoriums. The mud from S.4 presents a promising resource for pelotherapeutic applications. Fig. 1 illustrates the mean relative abundance of identified lipid classes in these muds, expressed as a percentage of the total annotated lipid peaks. Across all three comparative samples (S.1, S.5, S.4), the sphingolipid class constituted the predominant fraction of isolated lipid compounds, with its mean percentage abundance ranging from 26.54% (S.5) to 40.99% (S.1). Statistical analysis revealed a significant difference in sphingolipid content among the samples ($F(2,42)=8.16$, $p=0.001$). Specifically, S.1 exhibited a significantly higher proportion of sphingolipids compared to S.5, while S.4 displayed an intermediate, non-significantly different profile from both S.1 and S.5. Ceramides constituted the majority of this lipid group (Table 4). Ceramides are essential components of the cutaneous barrier, facilitating moisture retention within the superficial skin layers. The concentration and relative composition of ceramides are subject to alteration in various dermatological conditions.[44] Topical application of ceramide-enriched formulations demonstrates improvement in the cutaneous barrier in senile xerosis and serves as adjunctive maintenance therapy in psoriasis management.[45-47] Sphingolipids (SP) may exhibit antimicrobial properties and provide moisturizing benefits in atopic skin lesions in humans.[48]

Glycerolipids exhibited a relatively uniform mean percentage abundance among the isolated compounds across all samples, spanning from 22.87% (S.5) to 25.15% (S.1). Statistical analysis confirmed no significant differences in glycerolipid content among S.1, S.4, and S.5 ($F(2,42)=0.28$, $p=0.756$). Within this group, diradylglycerols constituted a significant fraction. It is hypothesized that certain diradylglycerols may induce enhanced epidermal pigmentation, manifesting as tanning, through the putative activation of protein kinase C.[49-50] Endogenous pigmentation serves to mitigate both UV-induced damage, yielding a safe photoprotective tan, and the risk of cutaneous malignancies.[51] Glycosyldiradylglycerol subclass lipids, particularly monogalactosyldiacylglycerol (MGDG), which exhibited the highest abundance in the S.4, possess anti-inflammatory properties.[52] The third most prevalent lipid group across all samples analyzed comprised fatty acyls, with mean percentages ranging from 13.37% (S.1) to 23.47% (S.5). Significant differences were observed in fatty acyl content among the samples ($F(2,42)=5.25$, $p=0.009$). Specifically, S.4 (21.05 ± 2.40 %) and S.5 (23.47 ± 2.68 %) showed significantly higher mean percentages of fatty acyls compared to S.1 (13.37 ± 1.57 %), with no significant difference between S.4 and S.5. Fatty acyls were predominantly in the form of fatty amides, which also demonstrate anti-inflammatory

Table 4: Average percentage distribution of the qualitative composition of the lipid fraction of black mud of S.1, S.5 and S.4.

	S.1	S.5	S.4
Sphingolipids [SP], %			
Ceramides Cer + CerP	30.40	20.11	25.67
Acidic glycosphingolipids SHexCer	0.00	0.39	0.20
Neutral glycosphingolipids AHexCer + HexCer + Hex2Cer + Hex3Cer	1.82	0.16	0.86
Sphingoid bases SPB + SL	7.90	7.86	11.62
Phosphosphingolipids SM + PE-Cer + PI-Cer	1.43	1.02	0.13
Fatty acyls [FA], %			
Fatty amides NAGly + NAGlySer + NAOrn + NAE + NATau + NAPhe	13.04	22.97	14.98
Fatty esters CAR + FAHFA	0.59	1.66	1.47
Glycerolipids [GL], %			
Diradylglycerols DG + DG O	10.50	10.64	10.70
Glycosyldiradylglycerols DGDG + DGDG O + MGDG + MGDG O + SQDG	0.44	0.40	0.67
Monoradylglycerols MG	0.99	0.69	0.49
Other Glycerolipids ADGGA + DGCC + DGGA + DGTS + LDGCC + LDGTS/A	7.86	5.15	3.80
Triradylglycerols TG + TG O	6.08	7.95	6.93
Glycerophospholipids [GP], %			
Glycerophosphocholines LPC + LPC O + PC + PC O	2.31	2.72	4.64
Glycerophosphoethanolamines LPE + LPE O + PE + PE O + PE P + LPE-N (FA)	0.00	0.12	0.00
Glycerophosphoglycerols BMP + LPG + LPG O + PG + PG O + HBMP	0.30	0.06	0.07
Glycerophosphoinositols PI + PI O + LPI	0.42	0.23	0.13
Glycerophosphoserines PS + PS O + LPS + LPS-N	0.00	0.13	0.22
Prenol Lipids [PR] Vitamin A fatty acid ester VAE	7.18	2.55	0.07
Sterol Lipids [ST] Sterols SE + ST + SG	4.88	10.30	6.20

activity.[53] Sample S.1 exhibited a distinct lipid profile compared to S.4 and S.5, characterized by an elevated mean percentage of prenol lipids. Statistical analysis confirmed this difference ($F(2,42)=10.33$, $p<0.001$), with S.1 (7.71 ± 1.26 %) having a significantly higher proportion of prenol lipids compared to S.4 (0.07 ± 0.07 %) and S.5 (2.30 ± 0.54 %). This group specifically included vitamin A fatty acid esters (Table 4). The S.5, in contrast, displayed a significantly higher mean content of sterol lipids compared to S.1 ($F(2,42)=4.19$, $p=0.022$), with a mean increase of approximately 2.2 times relative to S.1 mud (11.23% vs 5.02%) and 1.6 times relative to S.4 mud (11.23% vs 6.94%). Notably, S.4 did not significantly differ from S.1 or S.5. These lipid classes are implicated in ameliorating age-related and ultraviolet-induced skin aging and associated sequelae.[54-55] Within the glycerophospholipid group, while no overall significant difference was observed between samples, the S.5 sample contained approximately 1.7 times more glycerophosphocholines and glycerophosphoserines compared to the other two samples (Table 4).

This compound group is associated with anti-inflammatory effects and potential benefits in joint pathologies, as well as collagen synthesis enhancement.[56-59]

Qualitative analysis of the lipid complex composition revealed sample-specific variations, reflecting the unique formation conditions of each mud. These findings may inform recommendations for the topical application of these muds for specific dermatological and joint pathologies.

A comparative analysis of lipid class extraction using various eluent systems revealed distinct solvent selectivities for different lipid classes (Fig. 2). Statistical comparisons among extraction systems were performed using one-way ANOVA ($p < 0.05$) followed by Tukey's HSD post-hoc test. Homogeneous groups, where systems did not differ significantly, are denoted by common letters on the figure. For Glycerophospholipids, significant differences were observed ($p=0.001$), with MTBE:MeOH and $CHCl_3$:MeOH systems yielding significantly higher extraction (Group A). Similarly, Prenol Lipids showed significant differences ($p=0.003$), and ACN (1.0-45), IPA:ACN (1.0-45), and IPA:ACN (1.5-45) were identified as the highest-yielding group (Group A). For Sphingolipids, significant variations were found ($p=0.0001$), where ACN (1.5-15) and IPA:ACN (1.5-15) demonstrated significantly superior extraction efficiency (Group A). Lastly, Sterol Lipids also showed highly significant differences ($p=0.0007$), with ACN (1.5-45), IPA (1.0-45), and IPA:ACN (1.5-15) identified as the most effective systems (Group A). Conversely, for Fatty Acyls ($p=0.133$) and Glycerolipids ($p=0.198$), no significant differences were observed among the

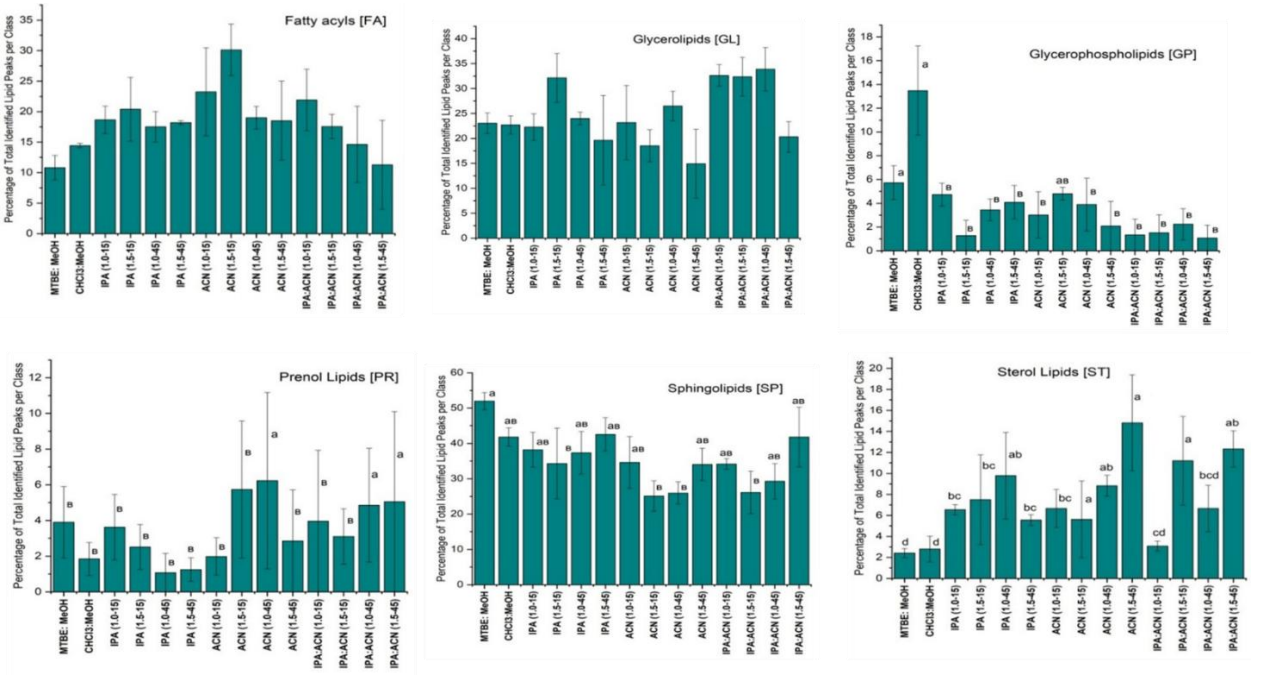

Fig. 2: Mean relative abundance of identified lipid classes across extraction systems, averaged from samples S.1, S.4, S.5. Whiskers represent Standard Error of the Mean (SE). Different letters above bars indicate statistically significant differences ($p < 0.05$) based on Tukey's HSD post-hoc test.

extraction systems, indicating that all tested systems performed similarly for these lipid classes. Furthermore, when assessing the impact of varying solvent volume and shaking time across the tested systems, a statistically significant positive correlation was observed solely for sphingolipids and sterols, contingent on the simultaneous augmentation of both parameters. Conversely, for glycerolipids, fatty acyls, glycerophospholipids, and prenol lipids, no statistically significant effect related to eluent volume and shaking time variation was identified. Consequently, the acquired data may inform the optimization of eluent system selection for the selective isolation of therapeutically relevant bioactive lipid compounds.

The results of the study of antioxidant properties are illustrated in Fig. 3 and 4. The highest abundance of antioxidant compounds was observed in extracts (Fig. 3a) obtained using the IPA:ACN (1.5-45) eluent system for S.1, S.3-4 and S.7 samples. Additionally, extracts from S.1, S.5-6 samples, obtained using the MTBE:MeOH system, also demonstrated high antioxidant activity. Statistical analysis of the data derived from the antioxidant activity and extract qualitative composition studies demonstrated a significant positive correlation between antioxidant activity and sterol lipid content in MTBE:MeOH extracts ($r > 0.95$, $p < 0.02$), and also between antioxidant activity and glycerolipid content in $CHCl_3$:MeOH and ACN (1.5-45) extracts ($r > 0.95$, $p < 0.05$). This correlation may be attributed to the potential UV-protective function of these lipid classes, as described in several studies.[49-51,55] These findings suggest avenues for further investigation into the mechanisms of sterol and glycerolipid antioxidant activity as solar protection factors (SPF). When comparing the antioxidant activity of the

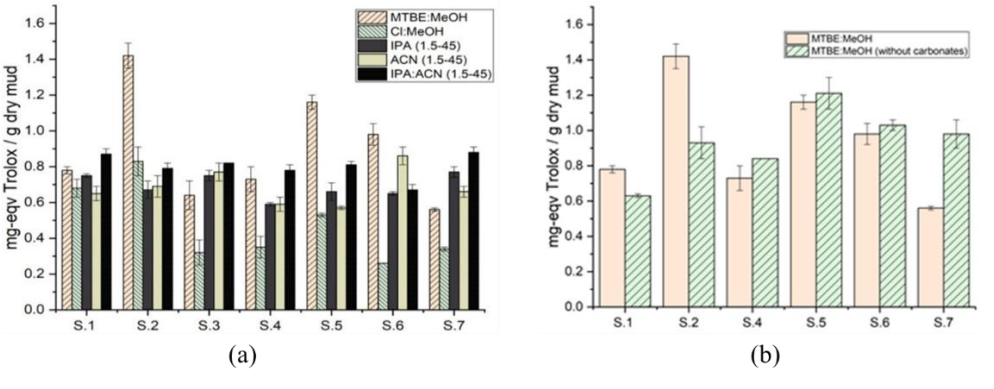

Fig. 3: Antioxidant activity of mud sample extracts, using the ABTS method, converted into milligram equivalents of Trolox per gram: a) across various extraction systems; b) extracts from dry mud samples versus samples pre-treated with hydrochloric acid. Whiskers represent Standard Deviation (SD) of the mean.

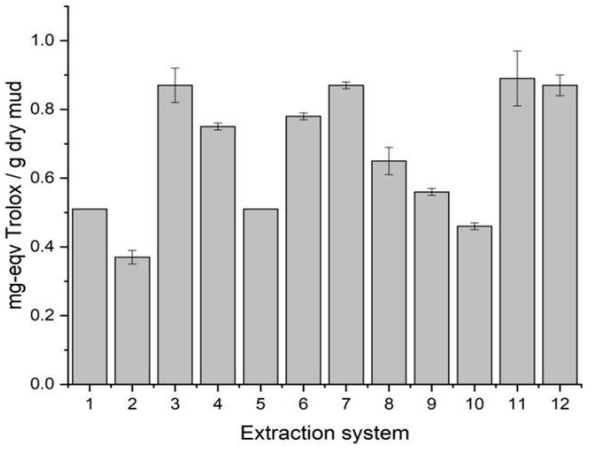

Fig. 4: Results of determination of the content of compounds with antioxidant activity in different extracts from Arasan mud species using the ABTS method, converted into milligram equivalents of Trolox per gram of dry sample (extraction systems: 1 - IPA (1.0-15); 2 - IPA (1.0-45); 3 - IPA (1.5-15); 4 - IPA (1.5-45); 5 - ACN (1.0-15); 6 - ACN (1.0-45); 7 - ACN (1.5-15); 8 - ACN (1.5-45); 9 - IPA:ACN (1.0-15); 10 - IPA:ACN (1.0-45); 11 - IPA:ACN (1.5-15); 12 - IPA:ACN (1.5-45). Whiskers represent Standard Deviation (SD) of the mean.

MTBE:MeOH extracts from dry mud samples and samples pre-treated with hydrochloric acid (Fig. 3b), an increase in activity was observed for the S.4-7 samples after acid treatment. This may indicate the release of substances with antioxidant activity during the decomposition of carbonates. However, for the S.1-3 (Eastern Kazakhstan) samples, this indicator decreased. This may be due to the fact that these samples contain a smaller amount of sterol lipids and glycerolipids in the mineral carbonate matrix, which have potential antioxidant properties.

A comparative analysis of the influence of added solvent volume and shaking time on extract antioxidant activity (Fig. 4) revealed that, for IPA, ACN, and IPA:ACN systems, solvent volume exerted a more substantial effect (1.6-2.0 times) than shaking time.

Consequently, the data analysis indicated that Sterol Lipids and Glycerolipids, which were most efficiently extracted by the IPA:ACN system, exhibited the highest antioxidant activity. Furthermore, increasing both eluent volume and shaking time contributed to an elevated proportion of Sterol Lipids. The study investigating the lipid content of saline muds from lakes in North-East Kazakhstan contributes significantly to the growing field of natural product-based therapeutics, particularly in dermatology and cosmetology. Through an optimized extraction methodology and detailed antioxidant profiling, the research offers a compelling case for the use of bioactive lipids—especially sterol lipids and glycerolipids—as therapeutic agents. When contextualized within existing scientific literature, particularly studies on phytochemicals and natural compounds with antioxidative potential, the biological promise of these mud extracts becomes even more evident.[60,61]

Firstly, the high antioxidant activity demonstrated by glycerolipids and sterol lipids extracted using IPA:ACN and MTBE:MeOH systems aligns with findings from Zahra *et al.* [62] and Zhamanbayeva *et al.*,[63] who emphasized the pharmacological potential of lipid-rich and polyphenol-rich plant species from Kazakhstan and surrounding regions. The positive correlation between antioxidant activity and lipid classes observed in the mud study mirrors observations in plant-based systems, where similar lipid groups contribute to oxidative stress modulation. The lipid classes identified in the mud—particularly sterol lipids—are notable not only for their radical scavenging ability but also for their UV-protective and membrane-stabilizing functions. This is in agreement with the findings of Prasher *et al.*,[64] who reported that lipid metabolites such as spermidine exhibit profound antioxidant and anticancer effects, largely through cellular protection mechanisms that likely involve membrane modulation. This biological mode of action could plausibly be extended to sterol lipids found in the studied muds, suggesting an exciting avenue for natural SPF (sun protection factor) formulation development.

Additionally, the observed release of antioxidant compounds after acid pre-treatment (notably in S.4–7 samples) suggests that bioactive lipids may be trapped within the mineral matrix of saline muds, and their liberation enhances the therapeutic potential of the mud. A similar phenomenon was reported by Rajkovic *et al.*,[65] who explored how calotropin, a bioactive compound with anticancer potential, could be liberated and stabilized through careful manipulation of extraction parameters.

In the broader context of natural therapeutic development, the findings underscore a pressing need to explore underutilized sources of bioactive compounds such as peloid muds. The fact that this is the first detailed lipidomic profiling of such muds from Kazakhstan opens a pathway for regional innovation in cosmeceuticals, similar to how endemic and medicinal plants of Central Asia are being leveraged in multidisciplinary programs.[66,67] Moreover, the antioxidant data from this study may inform the growing interest in natural ingredients for anti-aging dermatological products. The correlation of sterol and glycerolipid content with antioxidant activity strongly suggests that these compounds could serve as active ingredients in next-generation cosmeceuticals targeting skin aging, oxidative damage, and UV-induced stress. Lastly, the ecological dimension of this study should not be overlooked. As Imanaliyeva *et al.*[68] have highlighted in their work on *G. olivieri* populations, sustainable harvesting and ecological monitoring are crucial for natural resource-based pharmacology. Similarly, if Kazakhstan's saline muds are to be developed into dermatological and pharmaceutical products, their extraction must be coupled with environmental of salt

lakes. stewardship, especially considering the sensitive ecosystems.

In summary, the lipid extraction study from Kazakhstani saline muds not only presents a robust methodological foundation but also situates itself within a broader scientific discourse on natural bioactives, plant-derived antioxidants, and sustainable therapeutic development. The observed correlations between lipid classes and antioxidant activity reinforce the potential of glycerolipids and sterol lipids as key ingredients in skincare and anti-aging formulations. Further studies, particularly in vivo efficacy and safety assessments, will be crucial in advancing these findings from laboratory insights to clinical and commercial applications.

4. Conclusion

This investigation successfully developed and optimized aspects of a protocol for the extraction and identification of diverse lipid classes from natural mud matrices, using saline muds from Kazakhstan's lakes as a model system. The study identified optimal solvent systems (MTBE:MeOH, $CHCl_3$:MeOH, IPA:ACN) for maximizing overall lipid yield, and demonstrated that increasing solvent volume-to-mud ratio and mineral acid pre-treatment enhance extraction efficiency by liberating matrix-bound lipids, findings applicable to similar natural matrices. Beyond overall yield, the research precisely characterized the selectivity of various eluent systems for specific lipid classes, providing a versatile framework for targeted extraction: MTBE:MeOH for sphingolipids; $CHCl_3$:MeOH for glycerophospholipids; IPA:ACN for glycerolipids and sterol lipids; and ACN for fatty acyls and prenol lipids. Crucially, linking extraction efficiency with biological relevance, the study found peak antioxidant activity in extracts obtained with IPA:ACN (1.5-45) and MTBE:MeOH, directly correlating this activity with the presence of glycerolipids and sterol lipids, thereby highlighting their significant therapeutic potential for natural muds. These findings provide a scientific foundation for optimizing extraction protocols to selectively obtain therapeutically relevant lipid compounds from peloid muds. The high antioxidant potential of these extracts, particularly may attributed to glycerolipids and sterol lipids, suggests their significant promise in combating skin photoaging and developing novel natural mud-based pharmaceuticals. This first-time comprehensive lipid profile characterization of Salt Lake muds from Northeastern Kazakhstan opens new avenues for the integrated utilization of this natural resource, encouraging further research into its full range of beneficial applications in dermatology and beyond. This study's scope was limited by the range of extraction parameters explored (solvent volumes, shaking times). Further research is needed to fully elucidate the specific mechanisms linking lipid classes to antioxidant activity. Future investigations should broaden extraction condition parameters, conduct in-depth mechanistic studies to confirm the biological activities of isolated lipids, and evaluate the *in vivo* efficacy and safety of mud-based formulations for therapeutic applications.

Acknowledgments

This work was supported by the Science Committee of the Ministry of Science and Higher Education of the Republic of Kazakhstan (grant AP 23488960, titled «Development of technology for producing new cosmeceutical products based on domestic natural raw materials»).

Conflict of Interest

There is no conflict of interest.

Supporting Information

Not applicable.

CRediT Statement

Binur Mussabayeva: data curation, data validation, supervision, writing, review & editing, and funding acquisition. **Khafiza Akimzhanova:** methodology, investigation, writing, original draft**,** and writing, review & editing. **Alfira Sabitova:** methodology, conceptualization, supervision, project administration, and funding acquisition. **Alibek Ydyrys:** visualization, supervision, and data curation. **Dariusz Kruszka:** methodology, formal analysis, resources, and writing, review & editing. **Joanna Cerazy-Waliszewska:** methodology, resources, validation, and writing, review & editing. **Bogusława Łęska:** investigation and data curation. **Łukasz Tabisz:** methodology, visualization, software, and writing, review & editing.

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