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Antitumoral Potential of Carotenoid-Rich Microalgae Strains

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Abstract

This study aims to evaluate the cytotoxic/antiproliferative potential of carotenoid-rich microalgae strains from the class Eustigmatophyceae, and their effectiveness against Glioblastoma (GB), the most aggressive type of primary brain cancer. Recognized for their abundant bioactive compounds, microalgae provide a sustainable source for new anticancer agents. The research focused on the cytotoxic effects of extracts from four eustigmatophycean strains (*Nannochloropsis oculata*, *Munda aquilonaris*, *Vischeria helvetica*, and *Chlorobotrys regularis*) on GB cell lines (U87 and A172) and normal human dermal fibroblast (NHDF) cell. The ethyl acetate (EA) extracts from *N. oculata*, and *V. helvetica* demonstrated significant cytotoxic and/or antiproliferative activity, highly reducing cell viability in GB cells while showing the least toxicity on NHDFs. Analysis of the pigment profile revealed high levels of chlorophyll *a* and carotenoids, known for their antitumor properties. Fatty acid analysis showed a high concentration of eicosapentaenoic acid (EPA) in *V. helvetica* and palmitoleic acid in *N. oculata*, both noted for their antitumor effects. These results highlight the significative potential of carotenoid-rich microalgae extracts in GB therapeutics, after in depth studies. The notable cytotoxic effects on tumoral cells, combined with moderate effects on normal cells, emphasize their selectivity and potential for targeted therapy. This study provides preliminary evidence supporting the potential of microalgae-derived compounds as promising candidates for further research into GB therapeutics. Future research should aim to optimize extraction processes and perform *in vivo* studies to validate these findings and advance the development of microalgae-based therapeutics.

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Abbreviations

ACOI: Coimbra Collection of Algae

BBB: Blood-Brain Barrier

CO_2: Carbon Dioxide

DMSO: Dimethyl Sulfoxide

DMEM: Dulbecco's Modified Eagle Medium

EA: Ethyl Acetate

EPA: Eicosapentaenoic Acid

FBS: Fetal Bovine Serum

GB: Glioblastoma

HPLC: High-Performance Liquid Chromatography

Hx: Hexane

IC50: Inhibitory Concentration 50%

MMPs: Matrix Metalloproteinases

MTBE: Methyl Tert-Butyl Ether

MUFA: Monounsaturated Fatty Acid

NF-κB: Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells

NHDF: Normal Human Dermal Fibroblast

PBS: Phosphate Buffered Saline

PUFA: Polyunsaturated Fatty Acid

RRT: Relative Retention Time

SAG: Culture Collection of Algae at Göttingen University

SFA: Saturated Fatty Acid

TMZ: Temozolomide

TTF: Tumor Treating Fields

DCM:MeOH: Dichloromethane:Methanol

MeOH: Methanol

GC-MS: Gas Chromatography-Mass Spectrometry

1. Introduction

1. Microalgae

1.1.1. Diversity and biotechnological applications

Microalgae encompass a diverse group of eukaryotic microorganisms, primarily photoautotrophic, living either singly or in colonies. These photosynthetic organisms represent one of the most diverse groups on Earth, inhabiting various ecological niches on both marine and freshwater waters (Ávila-Román et al., 2021). Situated at the basis of aquatic food webs, microalgae have short duplication times and adapt to a broad spectrum of environments, from temperate zones to extreme conditions, like cold habitats and hydrothermal vents. They use light energy and fix CO_2, aiding in the reduction of greenhouse gases and the removal of nitrogen and phosphorus compounds that can be pollutants at high levels (Martínez Andrade et al., 2018). Although there are over 200,000 scientifically identified species, only a small part has been exploited for commercial purposes (Yaacob et al., 2022).

The rise of biotechnology in the 1960s introduced new methods for cultivating various microalgae species, leading to a significant increase in global research in this area. Initially, some microalgae were recognized for their potential in the biodiesel and bioethanol industries due to their high lipid and carbohydrate content (Posten & Schaub, 2009; Zhu et al., 2016), and their ecological and socio-economic impact (Popp et al., 2016; Rumin et al., 2020; Smith et al., 2010). Their metabolic flexibility in response to environmental changes also makes them valuable for the food, pharmaceutical, and cosmetic industries (Ávila-Román et al., 2021).

Microalgae are rich in metabolites such as lipids, carbohydrates, proteins, phenolic compounds, vitamins, and carotenoids, which are important for their physiological functions and have practical applications in the pharmaceutical and nutraceutical industries (Gómez-Zorita et al., 2019; González et al., 2015; Lauritano et al., 2020). In biotechnology, microalgae are seen as producers of high-value products with significant market potential (Enzing et al., 2014). However, challenges such as high operational costs, strain selection, dewatering, and large-scale harvesting remain (Alam et al., 2020; Saini et al., 2024). The commercialization of microalgal products is also affected by market and financial dynamics, with the lack of reliable statistical data on the microalgae market impeding comprehensive study (Alam et al., 2020). Current scientific endeavors aim to develop technologies to control abiotic conditions for microalgal biomass production using methods like open water systems, greenhouse ponds, and closed photobioreactors (Alam et al., 2020). Techniques like ultrasonic

sonication and genomic technologies are employed to produce high-value-added products (Ávila-Román et al., 2021).

A promising research area involves developing therapeutics from the marine ecosystems, where the discovery of novel drugs is still minimal compared to its vast potential. The oceans contain diverse organisms with unique metabolic capabilities, leading to numerous secondary metabolites with promising biological activities. Blue Biotechnology, also known as Marine Biotechnology, is a growing field that utilizes marine resources for biotechnological applications. Advances in science and technology over the past decade have greatly improved the prospects for blue biotechnology (De Vera et al., 2018).

A primary goal of marine biotechnology is to develop efficient processes for discovering new and effective drugs. While many key drugs have been derived from terrestrial plants, fungi, and bacteria, microalgae have recently emerged as a sustainable source of bioactive compounds, necessitating the continuous discovery of new chemical structures for therapeutic use (Ávila-Román et al., 2021).

1.1.2. Antitumor properties of microalgae

The growing global population has led to an increased demand for natural bioactive compounds, particularly from sources like microalgae (Gallego et al., 2021; Tabarzad et al., 2020).

The prevalence of diseases, such as cancer and cardiovascular pathologies, has significantly increased (World Health Organization, 2021). Cancer, the second leading cause of death worldwide, caused 9.6 million deaths in 2018. The global cancer burden continues to rise, with 19.3 million cases reported in 2020 and an expected increase to 28.4 million by 2040 (Sung et al., 2021). A major cause of cancer is the high levels of reactive oxygen species (ROS) in cells due to oxidative stress, which overwhelms the endogenous defense system against free radicals and other reactive species, potentially leading to cancer and neurodegenerative diseases (Bibi Sadeer et al., 2020; Pérez-Gálvez et al., 2020).

To support the body's natural antioxidant response, exogenous sources of antioxidants are essential. There is an urgent need for new antioxidant inhibitors of ROS effects and novel antitumoral compounds with higher efficacy, selectivity, and lower toxicity (Pasquet et al., 2011).

Microalgae-derived extracts and bioactive compounds, due to their structural diversity and biological availability, are attractive for drug discovery. Studies have documented the antioxidant, antitumor, antibiofilm, antifungal, antiviral, anti-inflammatory, and antimalarial activities of various microalgae (Fu et al., 2017; Tang et al., 2020). Compounds from microalgae, including pigments, peptides, fatty acids, sterols, polysaccharides, enzymes, vitamins, phycobiliproteins, and polyphenols, have shown promising antioxidant properties and antitumor potential against several types of cancer (Abd El-Hack et al., 2019; Fu et al., 2017). Table 1 provides a summary of microalgal species that have demonstrated antitumor properties, and the specific tumoral cell lines affected.

Table 1. Microalgae and cyanobacteria and active fraction/compounds with antitumor properties in different cancer cell lines.

Microalgae/ Cyanobacteria	**Type of Extract /compound**	**Effective Compound**	**Cancer Cell Lines**	**References**
Spirulina platensis	Methanolic extract	Not specified	L20B (Mouse intestine adenocarcinoma), MCF-7 (Human breast adenocarcinoma)	Fayyad et al., 2019
***Chlorella* spp.**	Aqueous extract	Carotenoids (Lutein, β-carotene, zeaxanthin, violaxanthin)	Caco-2 (Human colorectal adenocarcinoma)	Cha et al., 2008
Dunaliella tertiolecta	Aqueous extract	Violaxanthin	MCF-7 (Human breast adenocarcinoma)	Pasquet et al., 2011
Navicula incerta	Ethanol extract	Stigmasterol	HepG2 (Human hepatocellular adenocarcinoma)	Kim et al., 2014
Phaeodactylum tricornutum	Organic solvent extracts (hexane,	Polyunsaturated aldehydes, Fatty	Caco-2 and HT-29 (Human colorectal adenocarcinoma),	Andrianasolo et al., 2008; Samarakoon et

	EA), Methanolic extracts	alcohol ester, Galactolipids	A549 (Human lung adenocarcinoma), HL-60 (Human promyelocytic leukemia), B16F10 (Mouse melanoma)	al., 2013; Sansone et al., 2014
Chaetoceros calcitrans	EA extract, Ethanol extract	Not specified	MDA-MB-231 (Human breast adenocarcinoma), MCF-7 (Human breast adenocarcinoma)	Ebrahimi Nigjeh et al., 2013; Loh et al., 2014
Chlorella ovalis	Ethyl acetate extract, Chloroform extract, Hexane extract, Aqueous extract	Not specified	HL-60 (Human promyelocytic leukemia), B16F10 (Mouse melanoma), A549 (Human lung adenocarcinoma)	Samarakoon et al., 2013
Chlorella vulgaris	Methanolic extract	Myristic acid, Palmitic acid, Butanedioic acid	HepG2 (Human hepatocellular adenocarcinoma)	Qasem et al., 2016
Nannochloropsis oculata	Methanolic extract, Hexane extract, Chloroform extract, Ethyl acetate extract, Aqueous extract	Not specified	HepG2 (Human hepatocellular adenocarcinoma)	Qasem et al., 2016; Samarakoon et al., 2013
Amphidinium carterae	Methanolic extract, Chloroform extract, Ethyl acetate extract, Hexane extract, Aqueous extract	Not specified	HL-60 (Human promyelocytic leukemia), B16F10 (Mouse melanoma), A549 (Human lung adenocarcinoma)	Samarakoon et al., 2013

Amphora coffeaeformis	Methanolic extract	Not specified	HepG2 (Human hepatocellular adenocarcinoma)	Qasem et al., 2016
Thalassiosira rotula, Skeletonema costatum, Pseudonitzschia delicatissima	Polyunsaturated aldehydes	Not specified	Caco-2 (Human colorectal adenocarcinoma), A549 (Human lung adenocarcinoma), COLO 205 (Human colorectal adenocarcinoma)	Miralto et al., 1999; Sansone et al., 2014
Chlorella ellipsoidea	Carotenoid extract	Not specified	HCT-116 (Human colorectal adenocarcinoma)	Cha et al., 2008
Synedra acus	Chrysolaminaran	Not specified	HT-29, DLD-1 (Human colorectal adenocarcinoma)	Kusaikin et al., 2010
Cocconeis scutellum	EPA	Not specified	BT20 (Human breast adenocarcinoma)	Nappo et al., 2012
Chaetoceros* sp., *Cylindrotheca closterium, Odontella aurita, Phaeodactylum tricornutum	Fucoxanthin	Not specified	HL-60 (Human promyelocytic leukemia), Caco-2, HT-29, DLD-1 (human colorectal adenocarcinoma), PC-3, DU145, LNCaP (Human prostate adenocarcinoma)	Peng et al., 2011
Skeletonema costatum, Skeletonema marinoi	Hydrophobic fraction and PUAs Hydrophobic fraction	Not specified	Caco-2 (Human colorectaladenocarcinoma), A2058 (Skin melanoma)	Lauritano et al., 2016; Miralto et al., 1999

Canadian marine microalgal pool	Aqueous extract	Not specified	A549 and H460 (Human lung adenocarcinoma), PC-3 and DU145 (Human prostate adenocarcinoma), N87 (Human stomach adenocarcinoma), MCF-7 (Human breast adenocarcinoma), BxPC-3 (Human pancreas adenocarcinoma), MNNG (Osteosarcoma)	Somasekharan et al., 2016
Chlorella sorokiniana	Aqueous extract	Not specified	A549 and CL1-5 (Human lung carcinoma)	Lin et al., 2017

The bioactivity of microalgae can vary significantly between different strains and it is influenced by culturing conditions such as nutrient availability, temperature, and light intensity, as well as the growth phase (Ingebrigtsen et al., 2016; Lauritano et al., 2016; Ribalet et al., 2007).

1.1.3. Eustigmatophyceae class and their bioactive compounds

The Coimbra Collection of Algae (ACOI) at the University of Coimbra, Portugal, houses 80 strains belonging to the Eustigmatophyceae class, known for their unique cytological characteristics (Martins et al., 2021a). Eustigmatophyceae, or eustigmatophytes, are a distinct group within phylum Heterokontophyta (stramenopile) algae, comprising around 30 described species, with much taxonomic diversity still to be explored (Eliáš et al., 2016). These unicellular coccoid algae are typically spherical or ovoid, but can also exhibit distinctive shapes like stipitate, tetrahedral, or branched projections (Eliáš et al., 2016). They are primarily found in freshwater environments,

though some species are terrestrial, and one subgroup is mainly marine (Eliáš et al., 2016).

Reproduction in eustigmatophytes occurs primarily through autosporogenesis, forming zoospores with an anterior long flagellum bearing mastigonemes and occasionally a posterior short bare flagellum (Eliáš et al., 2016). While sexual reproduction has not been directly observed, genomic evidence suggests its presence in some species (Eliáš et al., 2016). These algae are distinguished by unique cytological features, although not all are present in every species. Key features include a pigmented lipidic body (reddish globule), a swelling at the base of the anterior flagellum linked to an extraplastidial stigma (eyespot), lamellate vesicles (potentially a reserve product), and plastids lacking a girdle lamella and continuity with the nuclear envelope. They lack chlorophyll b and c, with violaxanthin as the dominant xanthophyll (Eliáš et al., 2016).

Eustigmatophytes are particularly noted for their ability to accumulate significant amounts of lipids, including polyunsaturated fatty acids, making them valuable for biotechnological applications. However, despite their potential, the pharmaceutical applications of eustigmatophytes remain largely unexplored (Martins et al., 2021a).

The taxonomy of Eustigmatophyceae, based on comprehensive molecular datasets, particularly the 18S rRNA gene phylogeny, is presented in Table 2. Higher taxa are informally named as groups or clades, and these names have been used since their initial descriptions, pending formal validation through further studies (Amaral, 2020)

Table 2. Taxonomy of Eustigmatophyceae inferred from 18S rRNA gene phylogeny and comprehensive molecular datasets (Amaral, 2020)

Order	Families	Genera
Eustigmatales	Eustigmataceae group	*Vischeria* (syn. *Eustigmatos*) Clade Ia *Chlorobotrys* *Characiopsis(*syn. *Pseudocharaciopsis*)
	Monodopsidaceae	*Monodopsis* *Pseudotetraëdriella* *Nannochloropsis* *Microchloropsis*
	Neomonodaceae, fam. nov.	*Neomonodus, comb. nov.*

		Pseudellipsoidion *Characiopsiella, gen. nov.* *Munda, gen. nov.*
Goniochloridales clade	clade IIa	*Trachydiscus* *Tetraedriella subglobosa* *´Microtalis aquatica´*
	clade IIb	*Goniochloris (G. sculpta)* *Goniochloris (G. mutica)*
	clade IIc	*Vacuoliviride* *Goniochloris (G. tripus)*
	Pseudostaurastrum clade	*Pseudostaurastrum*
Incertae sedis	Incertae sedis	*Botryochloropsis* *Tetraedriella (T. tumidula)* *Tetraedriella (T. verrucosa)*

The bioeconomic interest in Eustigmatophyceae has significantly increased due to their ability to produce high levels of extraplastidial carotenoids. This interest was sparked by Antia and Cheng's study on *N. oculata*, which documented substantial production of astaxanthin and astacene, highlighting these algae's capacity to synthesize high oxidation level keto-carotenoids (Antia & Cheng, 1982; Stoyneva-Gärtner, et al., 2019b).

Carotenoids, responsible for red, yellow, and orange colors, are found in various organisms. These isoprenoids have a 40-carbon polyene chain and are divided into carotenes and xanthophylls (Barredo, 2012; Croteau et al., 2000). Carotenoids are crucial in photosynthesis and chemosystematics due to their specific distribution across algal classes (Britton, 1995; Stoyneva-Gärtner, et al., 2019a). They provide health benefits, including antioxidant properties and reduced disease risks (Stoyneva-Gärtner, et al., 2019a). β-Carotene, known for its provitamin A activity and immune system benefits, and astaxanthin are in high demand in various industries due to their antioxidant and antitumor properties (Z. Li, Ma, et al., 2012; Z. Li, Sun, et al., 2012; Stoyneva-Gärtner, et al., 2019b). Eustigmatophyceae also produce violaxanthin, which has shown strong radical scavenging activity and other health benefits, including inhibition of lipid peroxidation and red blood cell hemolysis, as well as anti-proliferative, anti-inflammatory, and proapoptotic activity against human cancer cell

lines (Talero et al., 2015a; F. Wang, Huang, et al., 2018). Lutein and zeaxanthin are also important carotenoids found in Eustigmatophyceae, beneficial for eye health and reducing cancer and cardiovascular disease risks (Bernstein et al., 2016; B. Li et al., 2010; Stoyneva-Gärtner, et al., 2019b; Yu et al., 2022). Canthaxanthin, another valuable carotenoid, is used as a food additive and in pharmaceuticals due to its health benefits, including its role in modifying lipid membranes and potential use as a chemosensitizer in chemotherapy (Eid et al., 2012; Koller et al., 2014; Mourelle et al., 2017; Stoyneva-Gärtner, et al., 2019b; Sujak et al., 2005). Chlorophyll *a*, the primary photosynthetic pigment in Eustigmatophyceae, is known for its detoxification, antioxidant effects, immune system enhancement, wound healing, weight loss, skin healing, and anti-cancer properties (Stoyneva-Gärtner, et al., 2019b).

Eustigmatophyceae are also rich in vitamins B, C, D, and K, making them valuable for health and nutrition (Liu & Lin, 2005). Tocopherols (vitamin E), particularly α-tocopherol, are synthesized exclusively by photosynthetic organisms and are valued for their antioxidant activity and health benefits, including preventing light-induced pathologies and degenerative disorders (Durmaz, 2007).

Most biotechnology-oriented studies have been performed on the minute marine species of the genus *Nannochloropsis* , that have also long been used as a food source in aquaculture (Eliáš et al., 2016). However, not much is known about the potential applications of eustigmatophytes as pharmaceuticals. Studies have identified several *Nannochloropsis* species as promising sources of bioactive compounds with significant anti-cancer potential. In particular, *N. gaditana* has been found to produce oxylipins, which show significant cytotoxic activity against cancer cell lines such as UACC-62 (melanoma) and HT-29 (colon adenocarcinoma). These effects have been linked to a reduction in ATP levels, highlighting the potential of oxylipins as therapeutic agents in cancer treatment (Ávila-Román et al., 2016; De Los Reyes et al., 2014). Additionally, studies on lipid extracts from *N. gaditana*, especially omega-3-rich extracts, have demonstrated selective cytotoxicity against human colon carcinoma cells (HCT-116), without harming non-tumorigenic cells. This suggests that these extracts may be an effective source of bioactive compounds (Castejón & Marko, 2022). Polyphenol and carotenoid-rich extracts from *N. gaditana* have also exhibited significant antiproliferative activity against colon cancer cells, possibly due to their antioxidant properties (Martínez et al., 2022).

Research on *N. oculata* has similarly revealed a range of biologically active compounds with cytotoxic and antitumor effects. For example, polysaccharides isolated from *N. oculata* have demonstrated potent inhibition of HeLa cervical cancer cells (Ben et al.,

2017). Sterol-rich fractions from this strain have also shown remarkable cytotoxic effects on human promyelocytic leukemia cells (HL-60) (Sanjeewa et al., 2016). Furthermore, essential oils and sulfate polysaccharides from *N. oculata* have exhibited promising anti-leukemic activity against K562 cells (Atasever-Arslan et al., 2016). In another study, hexanedioic acid bis(2-ethylhexyl) ester was identified as a major compound in *N. oculata*, demonstrating strong cytotoxicity against MCF-7 and 4T1 breast cancer cells when combined with silver nanoparticles (Hussein et al., 2020). Methanolic extracts from *N. oculata* have also been studied for their antitumor effects against HepG2 liver cancer cells (Venkatraman et al., 2022). Saturated fatty acids, such as stearic and palmitic acids derived from this species, have shown cytotoxicity against MCF-7 human breast cancer cells (Elkhateeb et al., 2020).

In addition to these findings, *N. oceanica* has been recognized for its rich composition of saturated fatty acids, including stearic acid and palmitic acid, which have exhibited selective cytotoxic effects against MCF-7 human breast cancer cells (Elkhateeb et al., 2020).

Other eustigmatophyte strains have also been studied for their bioactive compounds and antitumor properties. For example, both *Munda aquilonaris* and *Chlorobotrys regularis* demonstrated cytotoxic effects against MCF-7 breast cancer cells when extracted with DCM:MeOH as solvents (Martins et al., 2021a). Notably, *M. aquilonaris* also exhibited higher antioxidant activity, which is likely due to its carotenoid and phenolic content. The information outlined above, detailing the bioactive compounds from various eustigmatophyte strains and their cytotoxic effects on different cancer cell lines, is summarized in Table 3.

Table 3. Eustigmatophycean species, active fraction/compounds tested and cell lines against which these have proven to be effective

Strains	Compounds	Cell lines	Solvents	Reference
N. gaditana	Oxylipins	UACC-62 (melanoma), HT-29 (colon adenocarcinoma)	acetone- methanol	Ávila-Román et al., 2016; De Los Reyes et al., 2014
	omega-3 lipid extract	human colon carcinoma cells	chloroform:methanol (2:1)	Castejón & Marko, 2022

	polyphenol and carotenoid content	T84 colon cancer cell	ethanol	Martínez et al., 2022
N. oculata	mainly polysaccharides	human HeLa cervical cancer cell	methanol	Ben et al., 2017
	essential oils Polysaccharides, especially sulfate polysaccharides	K562 Leukemia cell	Not specified	Atasever-Arslan et al., 2016
	sterol-rich fractions	Human promyelocytic leukemia cell (HL-60)	80% methanol extracts	Sanjeewa et al., 2016
	hexanedioic acid, bis(2-ethylhexyl) ester as the major compound	MCF-7 and 4T1 breast cancer cells	AgNPs + *N. oculata*-chloroform	Hussein et al., 2020
	Not specified	HepG2 cells	methanol	Venkatraman et al., 2022
	saturated fatty acids, stearic acid, palmitic acid	human breast cancer MCF7	methanol	Elkhateeb et al., 2020
N. oceanica	saturated fatty acids, stearic acid, palmitic acid	human breast cancer MCF7	methanol	Elkhateeb et al., 2020
M. aquilonaris	Not specified	MCF-7	DCM:MeOH	Martins et al., 2021b
C. regularis	Not specified	MCF-7	DCM:MeOH	Martins et al., 2021b

Prior studies showed that half of the strains were rich in polyunsaturated fatty acids (PUFAs), which play a significant role in health (Patterson et al., 1994; Suen et al., 1987; Volkman et al., 1999). PUFAs reduce cholesterol levels and lower the risk of various diseases (Stoyneva-Gärtner, et al., 2019b). Fish, the primary source of PUFAs, acquire these through the food chain, starting with algae (Pilátová, 2013; Spolaore et al., 2006). Marine *Nannochloropsis* species have a high content of EPA, highlighting their potential for human dietary applications (Stoyneva-Gärtner, et al., 2019b). Research focuses on optimizing growth conditions to maximize EPA yield (Stoyneva-Gärtner, et al., 2019b). Techniques such as random and ultraviolet mutagenesis are used to enhance UFA and EPA content (Stoyneva-Gärtner, et al., 2019b). The application of *Nannochloropsis* in cosmetics is increasing due to its high lipid content, particularly PUFAs, and tanning effects of canthaxanthin (Koller et al., 2014; Mourelle et al., 2017). Extracts of *N. oculata* and *Microchloropsis gaditana* protect against oxidative stress and enhance collagen synthesis (Letsiou et al., 2017; Stolz & Obermayer, 2005). An ingredient from *N. oculata*, known for skin elasticity and tightening, was launched by Pentapharm (Stoyneva-Gärtner, et al., 2019b). Eustigmatophyceae are expected to see broader use in skincare products for anti-aging and other benefits.

Eustigmatophyceae have advantages as feedstock, including effective land and CO_2 utilization, high growth rates, and self-purification when used with wastewater treatment. They contain valuable bioactive compounds for various products.

Despite their potential, many aspects of applied research on Eustigmatophyceae remain underexplored. Potential applications include their use as biofertilizers, producers of antibiotic metabolites, and ingredients in molecular cooking. The bioeconomic potential of these microalgae is recognized, but still in its infancy (Stoyneva-Gärtner, et al., 2019b).

2. Glioblastoma

1.2.1. Definition, epidemiology, and etiology

The global burden of brain and central nervous system (CNS) tumors is increasing, which globally accounts for 321,731 new cases and 248,500 deaths according to GLOBOCAN 2022 data (International Agency for Research on Cancer, 2022). Among these, GB, an adult-type diffuse glioma, represents the most aggressive and prevalent form of primary glioma of the central nervous system. Classified as a grade 4 by the World Health Organization (WHO), GB is distinguished by its highly heterogeneous profile and invasive nature, rapid proliferation, and resistance to conventional therapies

(Maher & Bachoo, 2020). This malignancy represents nearly half of all primary brain cancers and primarily affects older individuals (aged 50 to 70), but can arise at any age, with a higher prevalence in men (Ahmed et al., 2021). GB is characterized by extensive angiogenesis, invasion of normal brain regions, and necrosis, leading to a typical survival time of about one year (Ahmed et al., 2021).

The global incidence rate for all CNS malignancies is 3.5 per 100,000 people, with the highest cases in Asia (55.1%) followed by Europe (21.0%) and North America (8.7%) (International Agency for Research on Cancer, 2022). The epidemiology of GB has been extensively studied, revealing global incidence rates ranging from 0.59 to 5 per 100,000 persons (Grech et al., 2020). This increase in incidence highlights the need for continuous surveillance and investigation into contributing factors.

The etiology of GB remains incompletely understood, though several risk factors have been identified, including genetic predispositions, environmental exposures, and potentially modifiable lifestyle factors (Yoshikawa et al., 2023). The role of cancer stem cells in the pathogenesis of GB has gained significant attention, with evidence suggesting these cells contribute to the tumor's initiation, progression, and recurrence (Biserova et al., 2021). Furthermore, epidemiological studies have explored various associations, including the impact of age, gender, and geographical factors on the onset and progression of GB, complicating our understanding of its etiology (Colopi et al., 2023).

GB presents a formidable challenge in neuro-oncology due to its complex biology, variable epidemiology, and elusive etiology. Despite the high costs of treatment, including surgery, radiation, and chemotherapy, GB remains incurable, with limited FDA-approved medications that are not universally effective (Ahmed et al., 2021). Continued research into the molecular underpinnings and environmental influences of GB is crucial for developing more effective therapeutic strategies and improving patient outcomes.

1.2.2. Conventional treatment and its limitations

Despite advancements in medical research, the prognosis for GB patients remains dismal, with median survival rates often extending to no more than 15 months following diagnosis (Davis, 2016; Stupp et al., 2005). Conventional treatment strategies for GB include surgical resection, radiotherapy, and chemotherapy with temozolomide (TMZ), along with other chemotherapeutic agents such as carmustine

and lomustine. However, these approaches are associated with significant limitations, including incomplete surgical resection, severe side effects, high recurrence rates, and the challenges posed by the blood-brain barrier (BBB) that restricts effective drug delivery to the brain (Ahmed et al., 2021; Fisher & Adamson, 2021).

One of the primary challenges in treating GB is its heterogeneity, which fosters resistance to standard therapies. For instance, while TMZ is initially effective, it often fails to prevent recurrence due to the tumor's ability to adapt and develop resistance mechanisms (Bahadur et al., 2019). This resistance, coupled with the complexity and invasiveness of GB tumors, underlies the limited progress in improving treatment options over the past several decades (Grochans et al., 2022).

Furthermore, innovative approaches such as tumor treating fields (TTF) have been explored to complement existing treatments. TTF is a non-invasive treatment that disrupts cancer cell division through electric fields. Although promising, TTF presents challenges, including high costs and accessibility issues, which restrict its widespread adoption and impact on patient survival (Rick et al., 2018).

Moreover, the identification of specific tumor cell gene mutations offers significant predictive and prognostic value, facilitating the development of targeted therapies. However, the variability in epidemiological reports and risk studies, due to differences in population demographics and study designs, complicates the reproducibility and comparison of findings across different studies (Grochans et al., 2022).

While conventional treatments for GB provide a foundational approach to managing this disease, their limitations highlight the need for ongoing research and innovation. Improving outcomes for GB patients requires addressing molecular diversity, enhancing drug delivery methods, and incorporating new therapeutic approaches. (Rick et al., 2018; Sotelo et al., 2006). The relentless pursuit of understanding and overcoming these challenges continues to drive advancements in GB therapy.

1.2.3. Natural products with antitumor potential against glioblastoma

Natural products have emerged as a significant area of interest in cancer therapy, offering vast chemical diversity and the ability to target multiple cellular pathways, which is crucial for addressing the complex biology of GB (Abbas et al., 2020; M. N. Park et al., 2017; Vengoji et al., 2018). These compounds, derived from plants, marine organisms, and microorganisms, have shown promising antitumor activity, either alone or in combination with standard therapies (Vengoji et al., 2018).

Various plant-derived natural compounds have demonstrated significant antitumor effects against GB. Carotenoids such as crocetin, derived from saffron, reduce tumor size by inducing cell cycle arrest and promoting apoptosis in GB cells (Colapietro et al., 2020; Tsuji et al., 2020). Flavonoids like quercetin and rutin suppress GB cell proliferation and invasion by modulating signaling pathways and reducing inflammatory mediators (Da Silva et al., 2020). Curcumin from turmeric inhibits GB cell proliferation and induces apoptosis by suppressing NF-κB signaling and causing mitochondrial dysfunction (K.-S. Park et al., 2019). Additionally, terpenes like betulinic acid, along with lignans and tannins, target DNA replication and promote apoptosis, contributing to their antitumoral effects (Bache et al., 2019; Jiang et al., 2020).

The marine environment, covering 71% of the Earth's surface, is a rich source of bioactive compounds with significant antitumor properties, including those derived from microalgae. These compounds have shown potential as therapeutic agents against GB (Khalifa et al., 2019; E. Wang et al., 2020). For example, fucoxanthin, a marine xanthophyll from algae, has demonstrated potent antitumoral effects in GB cells by triggering oxidative stress and inducing cell death with minimal impact on normal cells (Mohamed Abdoul-Latif et al., 2024). As mentioned before, extracts from *N. oculata* have exhibited both anti-inflammatory and antitumor properties, highlighting the therapeutic potential of marine-derived natural products (Sanjeewa et al., 2016).

Additionally, carotenoids like astaxanthin and adonixanthin from the microalga *Haematococcus pluvialis* inhibit tumor growth, reduce tumor volume, and induce apoptosis in GB cells by targeting key signaling pathways such as PI3K/Akt and ERK. These compounds also reduce the invasive potential of GB cells by downregulating matrix metalloproteinases (MMPs), further emphasizing the role of marine and microalgae-derived compounds in GB therapy (Abd El-Hack et al., 2019; Bouyahya et al., 2024; Tsuji et al., 2020).

The synergy between natural compounds and conventional therapies has been another focal point of research. For example, icariin, a natural compound, enhances the effectiveness of TMZ (Yang et al., 2018). Despite these promising findings, the clinical application of these natural compounds is challenged by issues such as bioavailability and the ability to cross the BBB. Ongoing research is focused on developing formulations to enhance their therapeutic potential, and further studies are needed to validate their efficacy and safety in clinical settings (Zhai et al., 2021).

The exploration of natural products offers a promising avenue for the development of new therapeutic strategies against GB. Plant-derived compounds such as carotenoids, flavonoids, and terpenes, along with marine-derived compounds, particularly from microalgae, present novel and potent alternatives (Abbas et al., 2020; M. N. Park et al., 2017; Vengoji et al., 2018). Ongoing research is focused on overcoming challenges such as bioavailability and BBB permeability to enhance the clinical application of these natural products in GB treatment (Zhai et al., 2021). The synergy between natural compounds and conventional therapies could offer a multifaceted approach to tackling this formidable cancer (Bouyahya et al., 2024; Mohamed Abdoul-Latif et al., 2024).

2. Aims

The primary objective of this research was to determine the antitumor potential of carotenoid-rich microalgae strains from the class Eustigmatophyceae against GB. This study aimed to optimize the extraction process of bioactive compounds from microalgae by selecting suitable solvents and comparing their effectiveness in yielding high concentrations of bioactive compounds.

The research evaluates the cytotoxic effects of extracts from four specific microalgae strains (*N. oculata* SAG 38.85*, M. aquilonaris* ACOI 2424*, Vischeria helvetica* ACOI 299*, and C. regularis* ACOI 307) on GB cell lines (U87 and A172). It also assesses the selectivity of these extracts by testing their cytotoxicity on NHDF (non-tumoral cells) to ensure potential therapeutic efficacy with minimal side effects on healthy cells.

Further, the extracts with high cytotoxic effects on GB cells and low toxicity on non-tumoral cells were selected for further concentration-effect analysis. Additionally, the study analyzes the pigment and fatty acid profiles of the microalgae extracts to identify specific bioactive compounds that could have antitumoral properties, thereby highlighting their promise for developing new antitumor agents. Finally, the research aimed to provide a foundation for further studies of optimizing extraction processes, understanding the mechanism of action of the extracts and compounds and conducting *in vivo* studies to validate the findings and advance the development of microalgae-based therapeutics for GB treatment.

By achieving these objectives, this first-step seeks to contribute to the discovery of promising, sustainable and effective natural compounds for future therapies, specifically targeting the challenging treatment landscape of GB.

3. Material and Methods

3.1. Microalgae strains and extraction

3.1.1. Strains cultivation

Three different Eustigmatophyceae strains from the ACOI (http://acoi.ci.uc.pt) and one strain originally from the Culture Collection of Algae at Göttingen University (SAG) (https://sagdb.uni-goettingen.de), maintained at ACOI for many years, were studied (Table 4). The biomass was obtained from a 2-step batch system cultures using 3 different media, F/2 20ppm adapted from Guillard & Ryther, 1962, BG11 adapted from Allen & Stanier, 1968 and M7 adapted from Schlösser, 1994. They were maintained for 10 days under controlled conditions of 18:6 (h:h, light:dark) photoperiod, at 23°C temperature and 51 µmol.m-2. s-1 light intensity.

Table 4. Studied strains with respective codes and cultivation media.

Strains	Strain Code Number	Cultivation Media
Nannochloropsis oculata	SAG 38.85	F/2 20ppm
Munda aquilonaris	ACOI 2424	BG11
Vischeria helvetica	ACOI 299	BG11
Chlorobotrys regularis	ACOI 307	M7

3.1.2. Preparation of extracts

The disruption of freeze-dried biomass (250 mg) was achieved through maceration using liquid nitrogen. Subsequently, two distinct extractions were performed using separate batches of biomass. The first extraction utilized a Dichloromethane:Methanol (DCM:MeOH) solvent mixture (1:1, v/v), (Leão et al., 2013), while for the second extraction, a separate batch of biomass underwent a sequential extraction with Hexane (Hx), EA, and methanol (MeOH) (Cepas et al., 2021). Each extract was individually collected in separate flasks. After the addition of the respective solvent (20 ml), the samples underwent vortexing for 2 minutes, followed by centrifugation at 4500 rpm for 15 minutes. The resulting supernatants were carefully recovered and subjected to rotary evaporation under reduced pressure at 30°C using a Buchi Rotavapor R 200, aimed at minimizing solvent content. This process, including the addition of solvent, vortexing, centrifugation, supernatant recovery and subsequent rotary evaporation was repeated until the recovered supernatant attained a clear appearance. The steps of the extraction process are illustrated in Figure 1. Following the evaporation step, the

extracts were resuspended with their respective solvents, transferred to Eppendorf tubes, and centrifuged at 12000 rpm for 15 minutes.

The resulting supernatants were subsequently recovered and subjected to drying using a speed-vacuum system (Gyrozen). The dried extracts were then stored in a refrigerator at 4°C until further analysis and utilization in bioactivity studies.

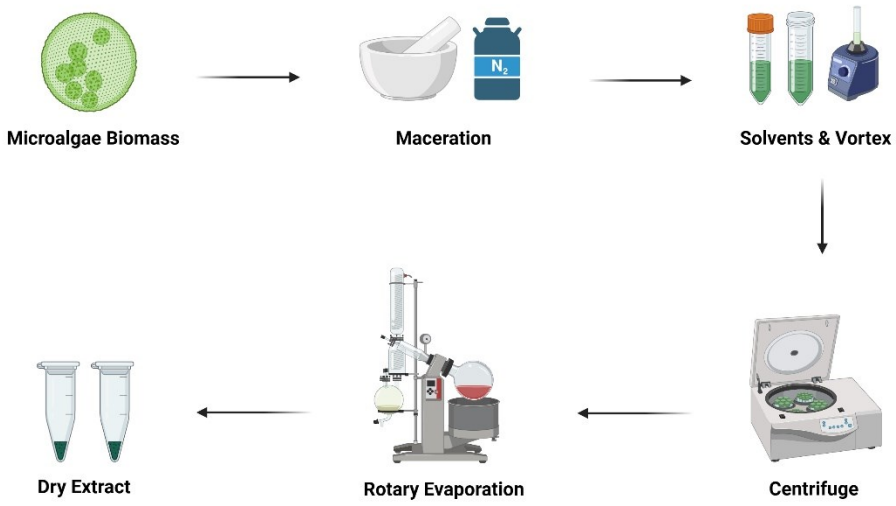

Figure 1. Preparation of the extracts

3.2. Chemical characterization of the extracts

3.2.1. Pigment profile

EA extracts were resuspended with 1 mL MTBE (>99.5%), centrifuged at 12 000 rpm for 10 min, filtered (PTFE filter membrane, 0.45µm pore size) and immediately injected in the HPLC. Pigment extracts were analysed using a HPLC Nexera XR (Shimadzu) comprised of a solvent deliver module (LC-2AD XR) with system controller (CBM-20A), a photodiode array (SPD-M20A) and a column oven (CTO-20AC). A monomeric Telos C18 column (150mm x 4.6mm) was used. The HPLC program was adapted from Mendes et al. (2007) and Van Heukelem &; Thomas (2001) with a flow rate of 1.1 mL/min, an injection volume of 100 µL, a column temperature of 60°C and a run duration of 35 min. The gradient profile and mobile phase composition are shown in Table 5.

Table 5. Gradient profile and mobile phase composition of HPLC method.

Time (min)	Solvent		
	A **Methanol:water*** **(85:15 v/v)** **%**	**B** **Acetonitrile:water** **(90:10 v/v)** **%**	**C** **Ethyl acetate** **%**
0	60	40	0
2	0	100	0
7	0	80	20
17	0	50	50
21	0	30	70
28.5	0	30	70
29.5	0	100	0
30.5	60	40	0
35	60	40	0

*** Buffered with 0.5M ammonium acetate (final concentration)**

3.2.2. Fatty acids profile

The fatty acid profile of the EA extracts was analyzed by gas chromatography according to Assunção et al., (2017), with some modifications. To the dried extracts, 1 mL of Hx and 0.5mL of MeOH were added, the sample was then vortexed until the lipid was totally dissolved. Then the combined fatty acids were converted into fatty acids methyl ethers by transesterification with the addition of 400 µL of sodium methoxide. This mixture was then vortexed for 5 min (Fig. 1). Then the upper phase was collected and filtered through a nylon syringe filter (pore size: 0.22µm) and analyzed. The gas chromatography was performed in a NEXIS GC-2030 (Shimadzu) chromatograph equipped with flame ionization detector and a TR-CN 100 capillary column (60m x 0.25µm x 0.20µm). Helium was used as carrier gas at a pressure of 150 kPa at the top of the column. The temperature of the injector and detector was 260°C and the split ratio was 1:25. The initial temperature of the column was maintained at 90°C for 7 min after the injection, increasing 5°C/min to 220°C and held for more 15min. Fatty acid (FA) was identified by comparing the relative retention times (RRT) with an authentic external standard, the Supelco 37 component FAME mix. The quantification of FA was based on the internal standard method described by Lim, et al. (2012). Internal standard used was methyl nonadecanoate (C19:0) with a final concentration of 0.3 mg/mL.

3.3. Cell culture

3.3.1. Cell lines

GB human cell lines (U87 and A172) were provided by Professor Carla Vitorino (Faculty of Pharmacy, University of Coimbra) and purchased from the American Type Culture Collection (ATCC), while the NHDF cell line was provided by Professor Paulo J. Oliveira (Center for Neuroscience and Cell Biology, University of Coimbra (CNC-UC) and purchased from Lonza Group AG (Basel, Switzerland). All cell lines were monitored for mycoplasma contamination and stored at -80°C.

3.3.2. Glioblastoma cell lines

U87 and A172 cell lines were grown in monoculture and cultivated in Dulbecco's Modified Eagle's Medium-high glucose (DMEM-HG) (Biowest, Nuaillé, France) supplemented with 10% (v/v) of heat-inactivated fetal bovine serum (FBS) (Biowest, Nuaillé, France) and 1% (v/v) of penicillin-streptomycin (Sigma, St. Louis, MO, United States). All cell lines were cultivated in a controlled environment at 5% of CO_2 and at a temperature of 37°C, within a humidified atmosphere.

3.3.3. Non-tumoral cells

NHDF cells were cultured in monolayer at 37 °C in a humidified atmosphere of 5 % CO_2, in low-glucose DMEM (D5030; Sigma, St. Louis, MO, United States), pH=7.2. Medium was supplemented with 5 mM glucose, 1 mM sodium pyruvate, 4 mM L-glutamine, 21 mM sodium bicarbonate, 10% FBS and 1% (v/v) of penicillin-streptomycin. According to the supplier's recommendations, cells were used until a maximum of 15 passages.

3.3.4. Cells maintenance

All cells were grown in T75 flasks, and the sub-culture was performed by trypsinization when reaching 80-90% confluence. For sub-culture, it was performed a dilution of 1:2 and 1:3 for U87 and A172 and NDHF cells, respectively. Before the biological assays, it was determined the cell density using Trypan Blue (Sigma, St. Louis, MO, United States). Trypan blue is used to distinguish live cells from dead cells, once in dead cells the membrane is disrupted allowing the dye to enter the cytoplasm, rendering these

cells visibly blue under light microscopy. This selective staining property is critical for accurately distinguishing dead cells from live cells (Fang & Trewyn, 2012).

Regarding this, a solution of 10 µL of Trypan Blue and 10 µL of cell suspension was prepared. After, 10 µL of this solution was loaded into a Neubauer Chamber and cell counting was executed, followed by the calculation of the density of the cell suspension.

3.3.5. Metabolic activity – Alamar Blue® assay

The extracts were resuspended in Dimethyl Sulfoxide (DMSO) at a final concentration of 10 mg/mL. Briefly, the solutions were homogenized using vortex and sonication, followed by filtration through a dual-material filter with a pore size of 0.22 nm. The filtration system is comprised of a glass fiber prefilter and a secondary filter made of Cellulose Acetate.

The tubes were stored protected from light and maintained at 4°C, ensuring the stability of the prepared extracts samples. When required for experiments, the extracts were thawed.

Metabolic activity was assessed using the Alamar Blue® assay (Resazurin). Resazurin is a non-fluorescent blue compound, that in metabolically active cells is reduced into resorufin, a fluorescent pink compound. This conversion is detected through a colorimetric change and evaluated via spectrophotometry.

Resazurin stock solution was prepared by dissolving in Phosphate Buffered Saline (PBS), achieving a final concentration of 0.1 mg/mL. For the metabolic activity assay, a 10% Resazurin solution was prepared in cell medium.

U87, A172, and NHDF cell lines were seeded in 96-well plates with a cell density of 10 x 10^3 cells per well, 24 hours before the treatment. After, cells were treated with the DCM:MeOH (v/v) and Hx and EA and MeOH extracts, using a range of concentrations of 0.01 to 100 µg/mL, which were prepared in medium using a serial dilution method, and further incubated for 24, 48 and 72 hours.

After the desired incubation period (24h, 48h, and 72h), the metabolic activity was assessed by Alamar Blue® assay (Magalhães et al., 2022). Briefly, a solution of cell medium with 10% (v/v) of a stock solution of resazurin (0.1 mg/mL) was prepared and further added to each well. Cells were incubated for 3 hours at 37ºC and 5% CO_2. Spectrophotometric readings were obtained using a Synergy™ HT Multi-Mode

Microplate Reader (BioTek Instruments) at wavelengths of 600 nm and 570 nm. The results were normalized to the control, and the effect of extracts was calculated using the following equation:

$$Cell\ viability\ (\%) = \frac{Abs\ sample\ (570-600)}{Abs\ Ctl\ (570-600)} \times 100\%$$

The steps involved in the metabolic activity assay are depicted in Figure 2.

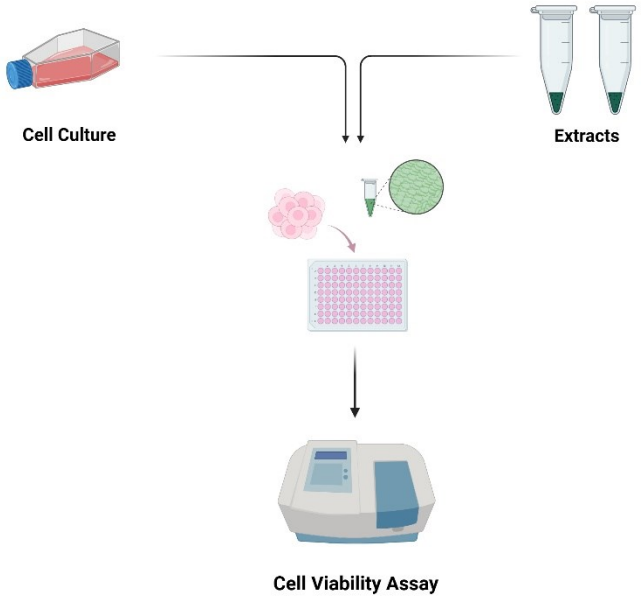

Figure 2. Scheme of the metabolic activity experimental assay.

3.3.6. Statistical analysis

Results represent the average of three independent experiments and are presented as mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism edition 10 (version 10.1.2324 for Windows; GraphPad Software, Inc., San Diego, CA, USA). The data was analyzed by two-way ANOVA, to compare the time points and the concentration of each extract in each strain and cell line. The differences were accessed by Tukey's multiple comparison post hoc test. Statistical significance was considered for $p<0.05$.

4. Results

4.1. Extracts yields

The extraction yields of various solvents for different microalgal strains show notable variations, as presented in Table 6. The extraction yields are expressed as the weight of extracts per weight of biomass (mg/g) for four different solvents: Hx, EA, MeOH, and a mixture of DCM:MeOH.

Table 6. Extraction yields (mg/g) of various solvents for different microalgal strains

Sample Code	Strain	Extraction yield (*m*g/g)			
		Hx	**EA**	**MeOH**	**DCM:MeOH**
ACOI 2424	*Munda aquilonaris*	49.2	52.8	118.4	330.4
SAG 38.85	*Nannochloropsis oculata*	25.2	52.82	133.8	171.2
ACOI 299	*Vischeria helvetica*	30.2	60	84	238
ACOI 307	*Chlorobotrys regularis*	34.4	25.2	52	129.6

The DCM:MeOH solvent mixture produced the highest yields, with *M. aquilonaris* ACOI 2424 yielding 330.4 mg/g, and other strains such as *V. helvetica* ACOI 299 and *N. oculata* SAG 38.85 showing high yields of 238 mg/g and 171.2 mg/g, respectively.

MeOH also resulted in moderate to high yields, reaching 133.8 mg/g for *N. oculata* SAG 38.85 and 118.4 mg/g for *M. aquilonaris* ACOI 2424. However, EA displayed more variable results, with moderate yields for some strains. The highest yield using EA was 60 mg/g for *V. helvetica* ACOI 299, while both *M. aquilonaris* ACOI 2424 and *N. oculata* SAG 38.85 yielded around 52.8 mg/g. The lowest yield with EA was recorded for *C. regularis* ACOI 307, at 25.2 mg/g.

In contrast, Hx consistently resulted in the lowest yields across all strains, with the highest being 49.2 mg/g for *M. aquilonaris* ACOI 2424.

4.2. Screening of cytotoxic effect of the extracts in glioblastoma and NHDF cell lines

4.2.1. A172 cell line

The effect of treatment with the extracts obtained from *N. oculata* SAG 38.85, *V. helvetica* ACOI 299, *M. aquilonaris* ACOI 2424 and *C. regularis* ACOI 307 on the metabolic activity of A172 cell line (GB cells) was assessed and the results are presented below.

Treatment with EA extract showed the most pronounced effect, significantly decreasing the metabolic activity of tumor cells from 78.50% at 24 hours to 65.49% at 48 hours, and further to 54.19% at 72 hours (p<0.05) (Figure 3B). In contrast, treatment with DCM:MeOH extract initially reduced metabolic activity to 72.18% at 24 hours, followed by a slight increase to 77.63% at 48 hours, and its effect diminished by 72 hours (p<0.05) (Figure 3A). MeOH and Hx extracts had no significant influence on the metabolic activity of tumor cells (p<0.05) (Figures 3C and 3D).

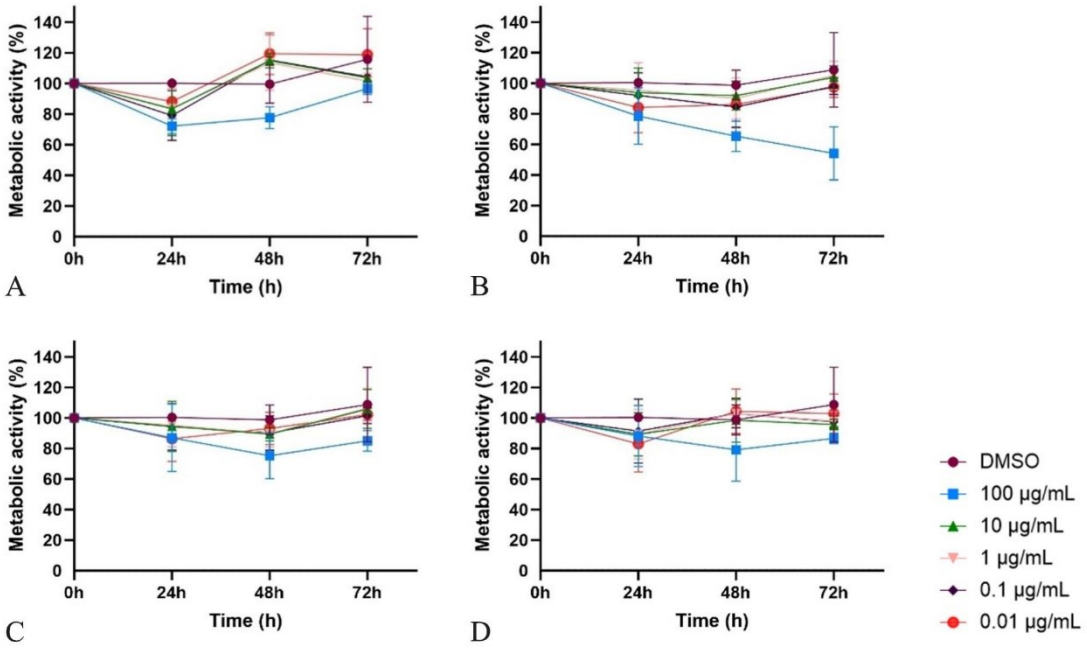

Figure 3. Impact of *N. oculata* SAG 38.85 extracts on the metabolic activity of A172 GB cells. A172 cells were treated with different solvent extracts of *N. oculata* SAG 38.85: A) DCM:MeOH, B) EA, C) MeOH, and D) Hx for 24, 48, and 72 hours. Metabolic activity was measured using the Alamar Blue® assay and expressed as a percentage of control (CTL, untreated cells). Data are representative of three independent experiments and are presented as mean ± SD.

The DCM:MeOH extract of *V. helvetica* ACOI 299, at three different concentrations, had distinct effects on the metabolic activity of A172 cells. At 100 µg/mL, this extract led to a decrease of metabolic activity to 62.38% and 46.89% after 24 and 48 hours of treatment, respectively, but this effect was less pronounced after 72 hours (59.53%) (p<0.05). At 1 µg/mL, the extract induced a reduction of metabolic activity to 75.81% after 24 hours (p<0.05). Similarly, at 0.1 µg/mL, the extract lowered metabolic activity

to 75.16% after 24 hours (p<0.05). (Figure 4A). Treatment with the MeOH extract of *V. helvetica* ACOI 299 resulted in a decrease in metabolic activity to 65.94% after 48 hours (p<0.05) (Figure 4C), while treatment with the Hx extract of *V. helvetica* ACOI 299, across all tested concentrations, showed no significant impact on the metabolic activity of the cells (Figure 4D). Similarly to what was observed with the EA extract of *N. oculata* SAG 38.85, treatment with the EA extract of *V. helvetica* ACOI 299 also produced the best results, leading to a significant reduction in the metabolic activity of cells (72.81%, 64.69%, and 47.49% after 24, 48, and 72 hours, respectively) (p<0.05) (Figure 4B).

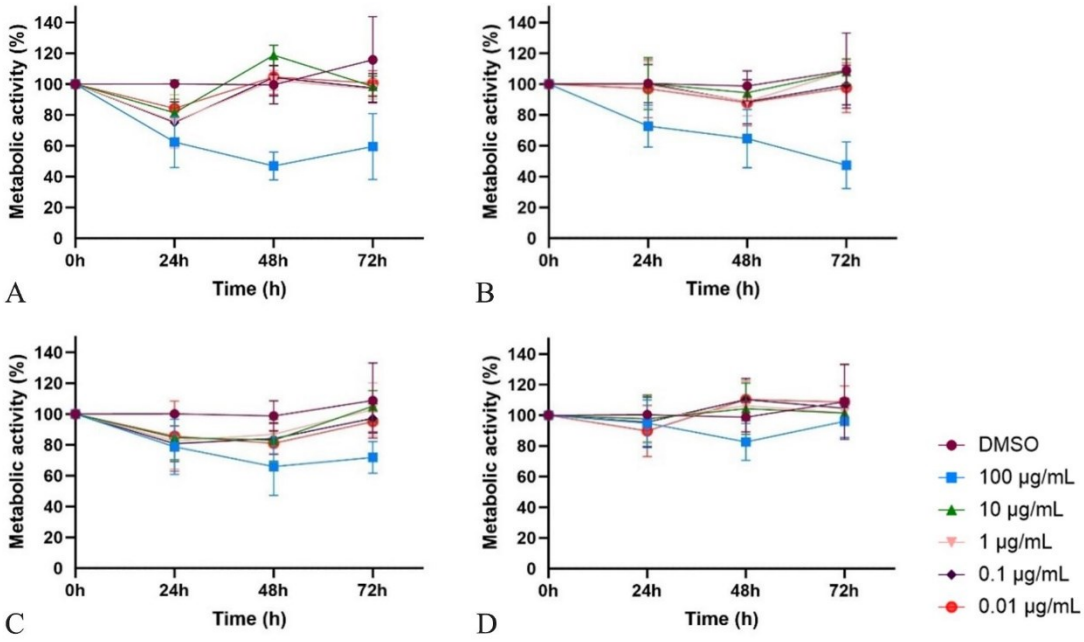

Figure 4. Impact of *V. helvetica* ACOI 299 extracts on the metabolic activity of A172 GB cells. A172 cells were treated with different solvent extracts of *V. helvetica* ACOI 299: A) DCM:MeOH, B) EA, C) MeOH, and D) Hx for 24, 48, and 72 hours. Metabolic activity was measured using the Alamar Blue® assay and expressed as a percentage of control (CTL, untreated cells). Data are representative of three independent experiments and are presented as mean ± SD.

The EA extract of *M. aquilonaris* ACOI 2424 at 100 µg/mL caused a decrease in the metabolic activity of A172 cells after 72 hours of treatment (65.12%) (p<0.05) (Figure 5A). While the MeOH and Hx extracts of *M. aquilonaris* ACOI 2424 did not affect the viability of GB cells (Figure 5B and 5C).

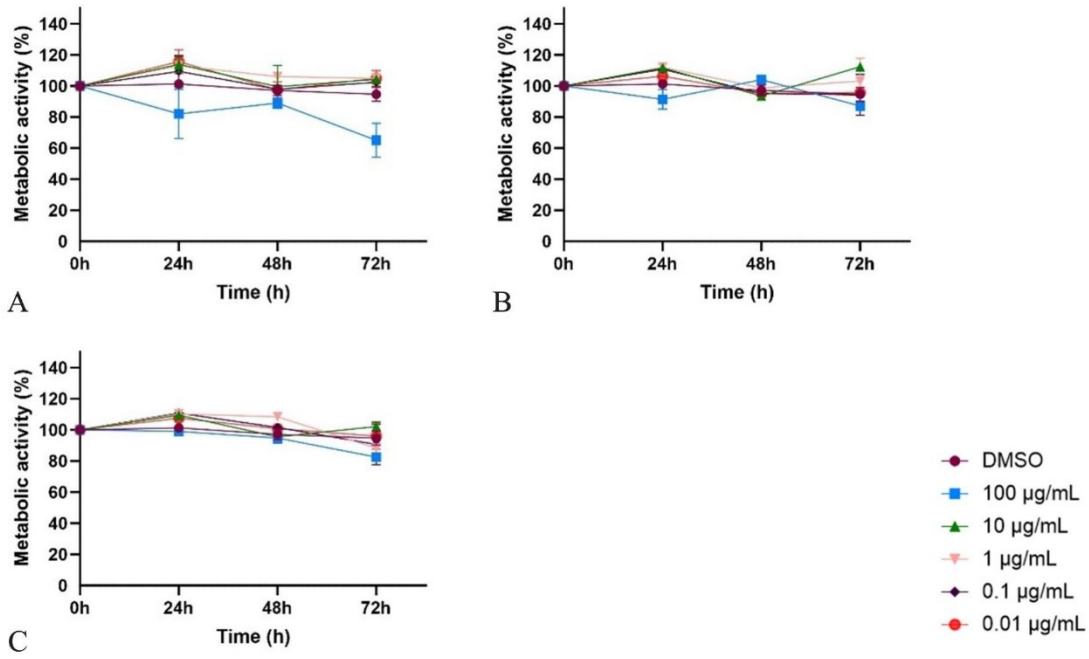

Figure 5. Impact of *M. aquilonaris* ACOI 2424 extracts on the metabolic activity of A172 GB cells. A172 cells were treated with different solvent extracts of *M. aquilonaris* ACOI 2424: A) EA, B) MeOH, and C) Hx for 24, 48, and 72 hours. Metabolic activity was measured using the Alamar Blue® assay and expressed as a percentage of control (CTL, untreated cells). Data are representative of three independent experiments and are presented as mean ± SD.

Treatment with DCM:MeOH extract of *C. regularis* ACOI 307 at a concentration of 100 µg/mL led to a slight reduction in the metabolic activity of A172 cells after 72 hours (73.45%) (p<0.05) (Figure 6).

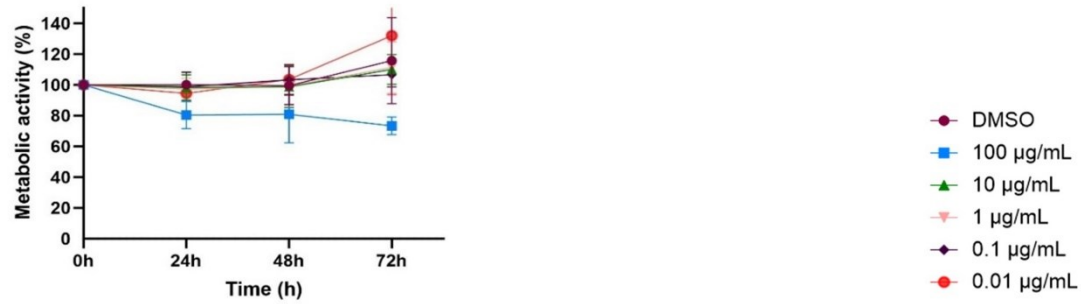

Figure 6. Impact of *C. regularis* ACOI 307 extract on the metabolic activity of A172 GB cells. A172 cells were treated with DCM:MeOH extract of *C. regularis* ACOI 307 for 24, 48, and 72 hours. Metabolic activity was measured using the Alamar Blue® assay and expressed as a percentage of control (CTL, untreated cells). Data are representative of three independent experiments and are presented as mean ± SD.

4.2.2. U87 cell line

The results below illustrate the effect of the extracts on the metabolic activity of the U87 cell line.

Treatment with the DCM:MeOH extract presented a pronounced effect, significantly reducing the metabolic activity of U87 cells at different concentrations. At 100 µg/mL, the extract initially reduced metabolic activity to 59.64% after 24 hours, followed by a slight increase to 65.04% at 48 hours, and then a further reduction to 47.60% at 72 hours *(p*≤0.05). At 10 µg/mL, the metabolic activity decreased to 68.72% after 24 hours (P≤0.05), while at 1 µg/mL, it was reduced to 68.28% after 24 hours (P≤0.05) (Figure 7A). The EA extract exhibited a stronger inhibitory effect on U87 cells. At 100 µg/mL, it significantly led to a reduction of the metabolic activity to 57.02% after 24 hours, which further decreased to 48.86% at 48 hours, and dropped to 24.94% at 72 hours (P≤0.05). Even at 1 µg/mL, the extract showed a notable effect, reducing activity to 63.63% after 24 hours (P≤0.05) (Figure 7B). MeOH extract significantly reduced metabolic activity in U87 cells. At 100 µg/mL, it decreased activity to 77.32% after 48 hours and further to 37.83% at 72 hours (P≤0.05). Additionally, at 0.1 µg/mL, the extract reduced activity to 77.14% after 72 hours (P≤0.05), and at 0.01 µg/mL, it lowered activity to 69.01% after 24 hours (P≤0.05) (Figure 7C). The Hx extract reduced U87 cell metabolic activity, lowering it to 70.13% at 100 µg/mL after 24 hours, and further to 48.07% after 72 hours (P≤0.05). At 10 µg/mL, it decreased activity to 69.94% after 24 hours, and at 1 µg/mL, it reduced activity to 74.13% after the same period (P≤0.05) (Figure 7D).

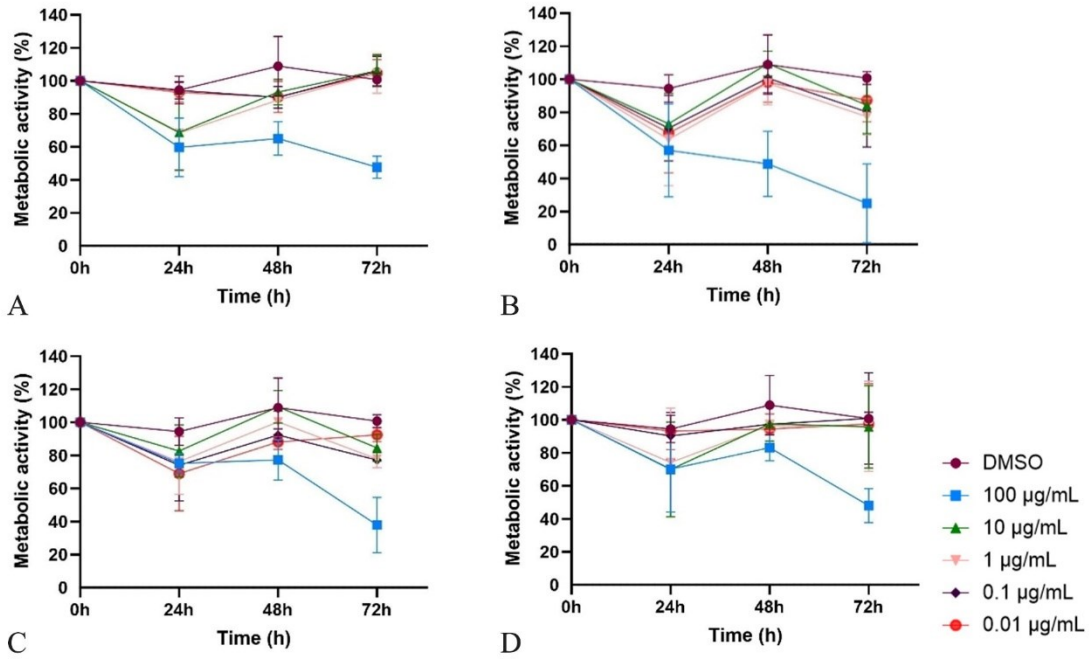

Figure 7. Impact of *N. oculata* SAG 38.85 extracts on the metabolic activity of U87 GB cells. U87 cells were treated with different solvent extracts of *N. oculata* SAG 38.85: A) DCM:MeOH, B) EA, C) MeOH, and D) Hx for 24, 48, and 72 hours. Metabolic activity was measured using the Alamar Blue® assay and expressed as a percentage of control (CTL, untreated cells). Data are representative of three independent experiments and are

The DCM:MeOH extract had a significant impact on the metabolic activity of U87 cells. At 100 µg/mL, the extract drastically reduced activity to 42.82% after 24 hours, further declining to 33.62% at 48 hours, and reaching 25.24% at 72 hours ($P \leq 0.05$). At 10 µg/mL, it initially decreased metabolic activity to 68.28% after 24 hours, but the effect diminished at 48 hours ($P \leq 0.05$). At 1 µg/mL, the metabolic activity was reduced to 70.75% after 24 hours, followed by an increase at 48 hours ($P \leq 0.05$) (Figure 8A). The EA extract had a more pronounced effect causing a significant decrease in metabolic activity at 100 µg/mL, lowering it to 56.78% after 24 hours, further to 44.74% at 48 hours, and dramatically to 11.20% at 72 hours ($P \leq 0.05$). At 0.1 µg/mL, it decreased activity to 68.74% after 24 hours, but this effect was partially reversed at 72 hours ($P \leq 0.05$) (Figure 8B). The MeOH extract at 100 µg/mL led to a reduction in metabolic activity, bringing it down to 69.18% after 48 hours and further to 30.23% at 72 hours ($P \leq 0.05$) (Figure 8C). The Hx extract at 100 µg/mL initially reduced metabolic activity to 63.96% after 24 hours and then decreased again to 59.37% at 72 hours ($P \leq 0.05$) (Figure 8D).

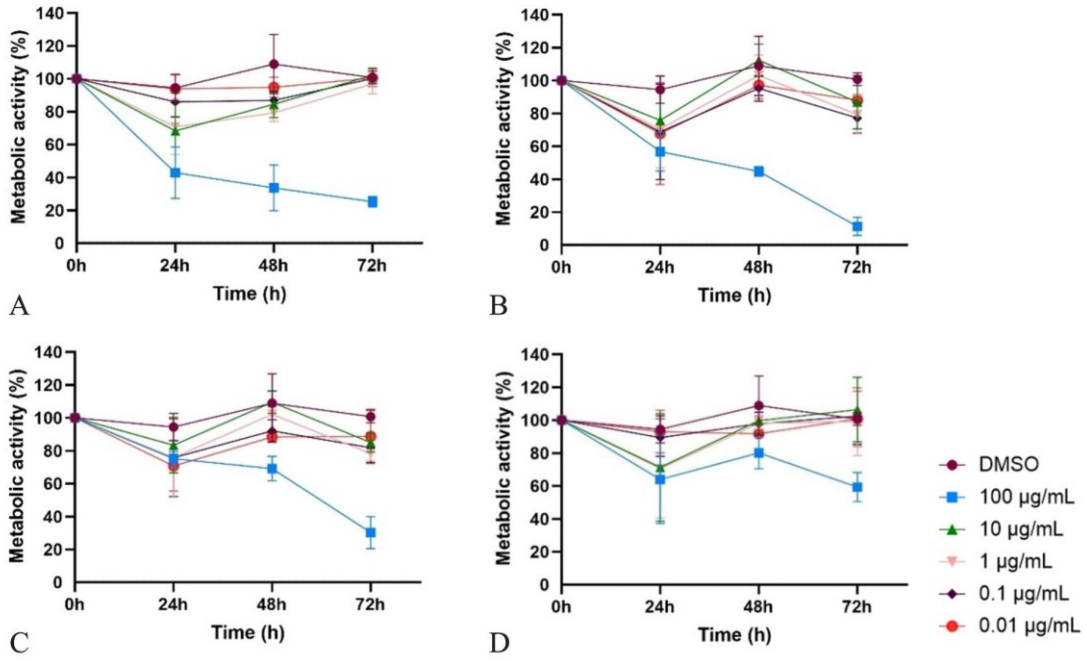

Figure 8. Impact of *V. helvetica* ACOI 299 extracts on the metabolic activity of U87 GB cells. U87 cells were treated with different solvent extracts of *V. helvetica* ACOI 299 : A) DCM:MeOH, B) EA, C) MeOH, and D) Hx for 24, 48, and 72 hours. Metabolic activity was measured using the Alamar Blue® assay and expressed as a percentage of control (CTL, untreated cells). Data are representative of three independent experiments and are presented as mean ± SD.

The EA extract significantly reduced metabolic activity at 100 µg/mL, lowering it to 77.17% after 24 hours, further decreasing to 72.82% at 48 hours, and reaching 59.47% at 72 hours ($P \leq 0.05$) (Figure 9A). In contrast, both the MeOH and Hx extracts did not show any significant effect on metabolic activity (Figures 9B and 9C).

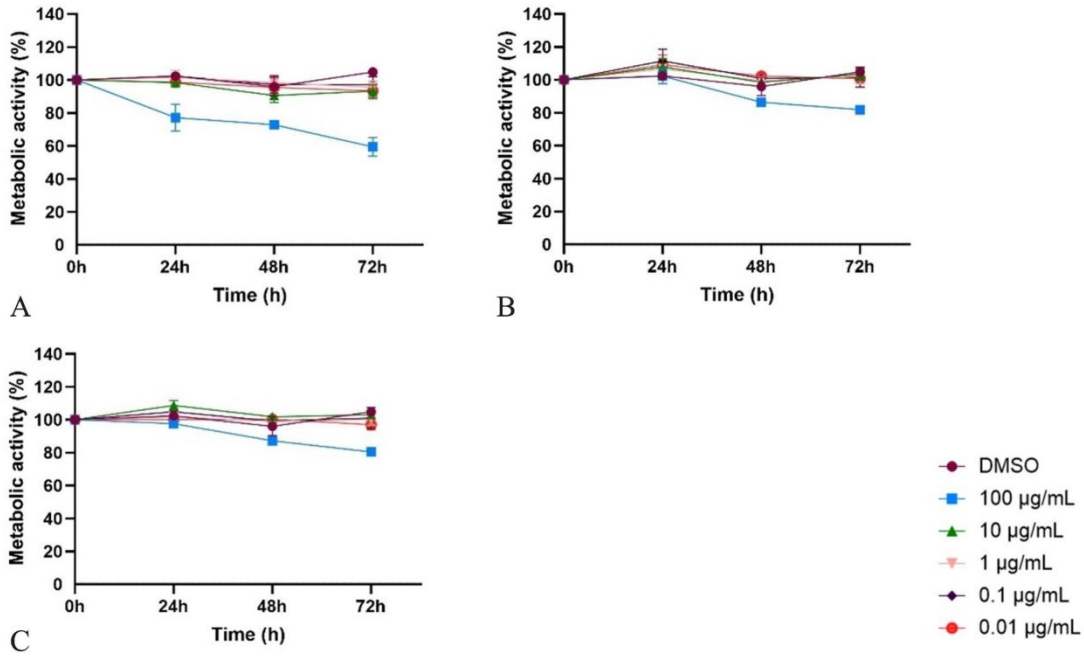

Figure 9. Impact of *M. aquilonaris* ACOI 2424 extracts on the metabolic activity of U87 GB cells. U87 cells were treated with different solvent extracts of *M. aquilonaris* ACOI 2424: A) EA, B) MeOH, and C) Hx for 24, 48, and 72 hours. Metabolic activity was measured using the Alamar Blue® assay and expressed as a percentage of control (CTL, untreated cells). Data are representative of three independent experiments and are presented as mean ± SD.

At a concentration of 100 µg/mL, the DCM:MeOHextract reduced metabolic activity to 69.49% after 48 hours, with a further decrease to 46.28% after 72 hours (P≤0.05) (Figure 10).

Figure 10. Impact of *C. regularis* ACOI 307 extract on the metabolic activity of U87 GB cells. U87 cells were treated with DCM:MeOH extract of *C. regularis* ACOI 307 for 24, 48, and 72 hours. Metabolic activity was measured using the Alamar Blue® assay and expressed as a percentage of control (CTL, untreated cells). Data are representative of three independent experiments and are presented as mean ± SD.

4.2.3. NHDF cell line

The results of the effect of the extracts on the metabolic activity of the NHDF cell line are presented below, illustrating the effect of the treatment on cellular viability.

None of the tested extracts from *N. oculata* SAG 38.85 had a significant effect on the metabolic activity of cells. The DCM:MeOH, EA, MeOH, and Hx extracts showed no impact across all tested concentrations (Figure 11A-D).

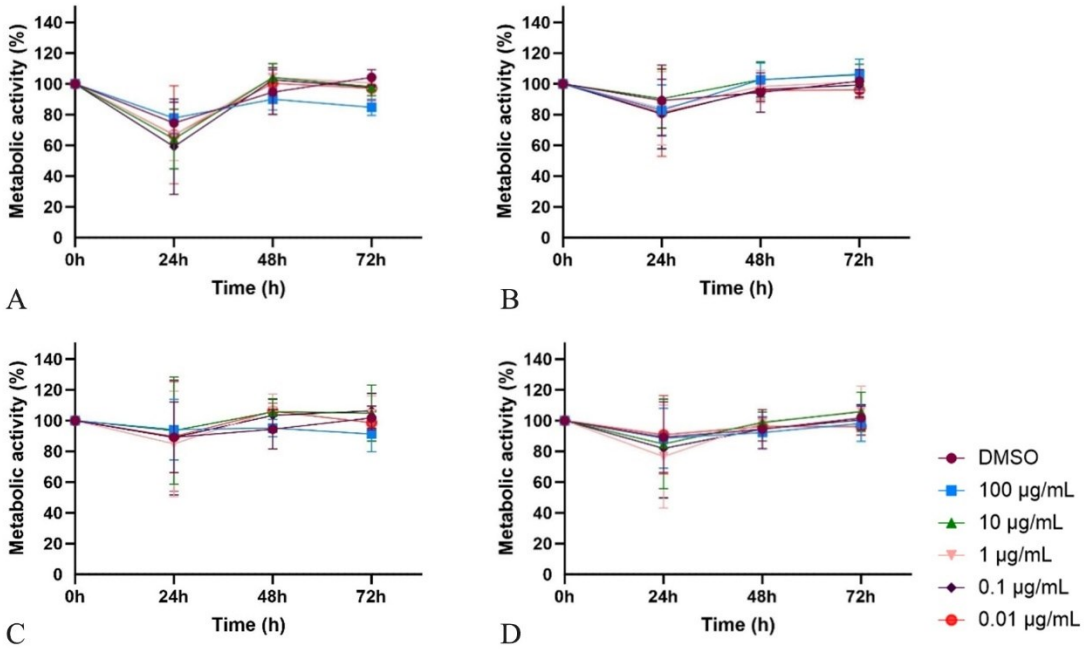

Figure 11. Impact of *N. oculata* SAG 38.85 extracts on the metabolic activity of NHDF cells. NHDF cells were treated with different solvent extracts of *N. oculata* SAG 38.85: A) DCM:MeOH, B) EA, C) MeOH, and D) Hx for 24, 48, and 72 hours. Metabolic activity was measured using the Alamar Blue® assay and expressed as a percentage of control (CTL, untreated cells). Data are representative of three independent experiments and are presented as mean ± SD.

The DCM:MeOH extract of *V. helvetica* ACOI 299 at a concentration of 100 µg/mL reduced metabolic activity to 65.69% after 48 hours, with a slight increase to 69.08% after 72 hours ($P \leq 0.05$) (Figure 12A). In contrast, the EA, MeOH, and Hx extracts of *V. helvetica* ACOI 299 showed no significant effect on the metabolic activity of the cells across all tested concentrations (Figure 12B-D).

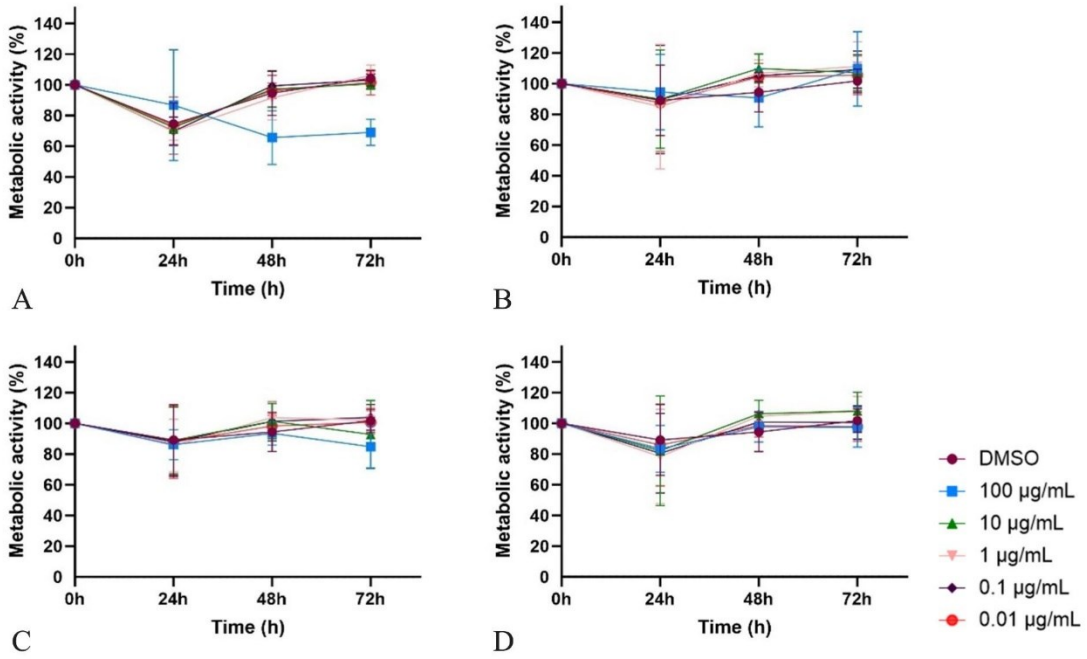

Figure 12. Impact of *V. helvetica* ACOI 299 extracts on the metabolic activity of NHDF cells. NHDF cells were treated with different solvent extracts of *V. helvetica* ACOI 299: A) DCM:MeOH, B) EA, C) MeOH, and D) Hx for 24, 48, and 72 hours. Metabolic activity was measured using the Alamar Blue® assay and expressed as a percentage of control (CTL, untreated cells). Data are representative of three independent experiments and are presented as mean ± SD.

The EA extract of *M. aquilonaris* ACOI 2424 at a concentration of 100 µg/mL significantly reduced metabolic activity to 63.64% after 24 hours ($P \leq 0.05$) (Figure 13A). The MeOH extract of *M. aquilonaris* ACOI 2424 showed varying effects across different concentrations. At 100 µg/mL, it did not significantly reduce metabolic activity, while at lower concentrations, the effects were primarily increases in metabolic activity (Figure 13B). Similarly, the Hx extract of *M. aquilonaris* ACOI 2424 showed no significant reduction in metabolic activity at the tested concentrations, with primary increases observed (Figure 13C).

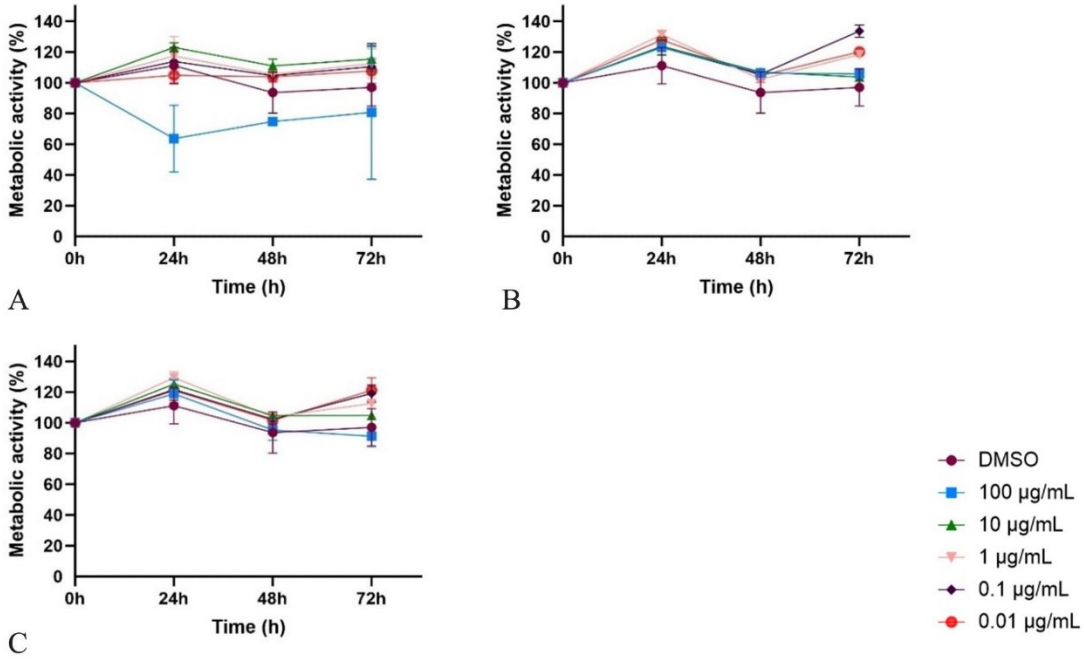

Figure 13. Impact of *M. aquilonaris* ACOI 2424 extracts on the metabolic activity of NHDF cells. NHDF cells were treated with different solvent extracts of *M. aquilonaris* ACOI 2424: A) EA, B) MeOH, and C) Hx for 24, 48, and 72 hours. Metabolic activity was measured using the Alamar Blue® assay and expressed as a percentage of control (CTL, untreated cells). Data are representative of three independent experiments and are presented as mean ± SD.

The DCM:MeOH extract of *C. regularis* ACOI 307, at all tested concentrations, did not exhibit any significant effect on the metabolic activity of the cells (Figure 14).

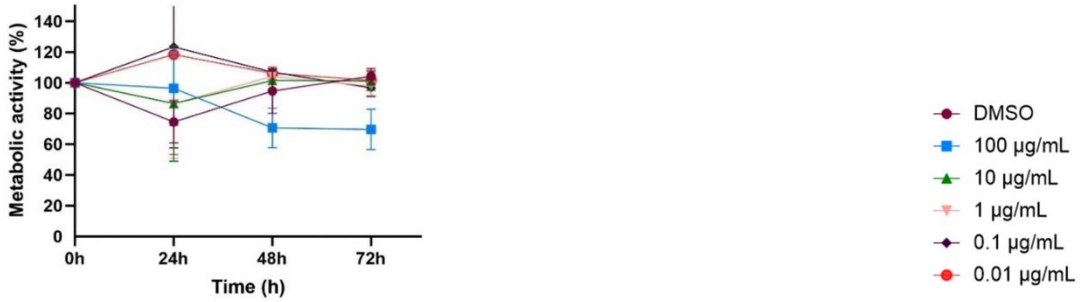

Figure 14. Impact of *C. regularis* ACOI 307 extract on the metabolic activity of NHDF cells. NHDF cells were treated with DCM:MeOH extract of *C. regularis* ACOI 307 for 24, 48, and 72 hours. Metabolic activity was measured using the Alamar Blue® assay and expressed as a percentage of control (CTL, untreated cells). Data are representative of three independent experiments and are presented as mean ± SD.

4.3. Evaluation of the cytotoxic effect of the best extracts

4.3.1. A172 cell lines

The results of the effect of the best extracts on metabolic activity of the A172 cell line are presented below, illustrating the effect of the treatment on cellular viability.

The DCM:MeOH extract of *N. oculata* SAG 38.85, across all tested concentrations, did not affect the metabolic activity of the cells (Figure 15A). In contrast, EA extract of *N. oculata* SAG 38.85 at a concentration of 100 µg/mL significantly reduced metabolic activity, lowering it to 37.53% after 48 hours and further to 23.23% after 72 hours (P≤0.05) (Figure 15B).

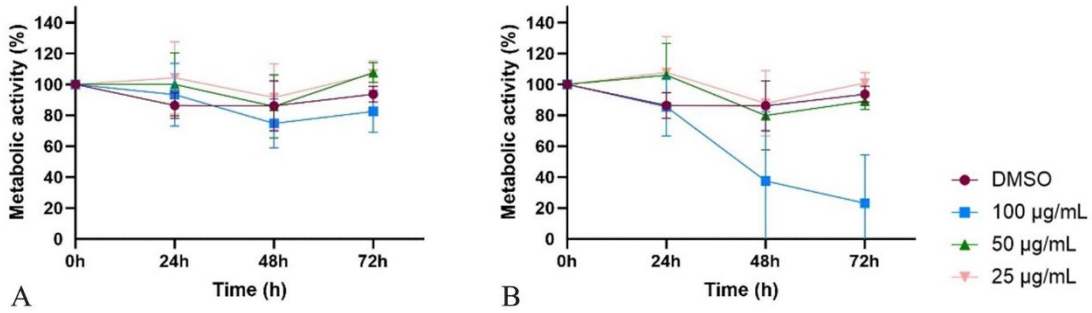

Figure 15. Impact of *N. oculata* SAG 38.85 extracts on the metabolic activity of A172 cells. A172 cells were treated with different solvent extracts of *N. oculata* SAG 38.85: A) DCM:MeOH, B) EA, for 24, 48, and 72 hours. Metabolic activity was measured using the Alamar Blue® assay and expressed as a percentage of control (CTL, untreated cells). Data are representative of three independent experiments and are presented as mean ± SD.

The DCM:MeOH extract of *V. helvetica* ACOI 299 at a concentration of 100 µg/mL led to a decrease in metabolic activity, reducing it to 55.41% after 48 hours, followed by a slight increase to 66.82% after 72 hours (P≤0.05) (Figure 16A). The EA extract, also at 100 µg/mL, reduced metabolic activity to 71.02% after 72 hours (P≤0.05) (Figure 16B).

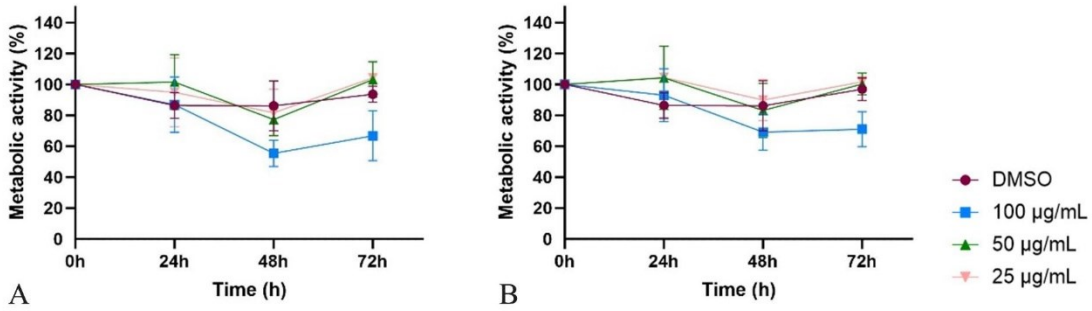

Figure 16. Impact of *V. helvetica* ACOI 299 extracts on the metabolic activity of A172 cells. A172 cells were treated with different solvent extracts of *V. helvetica* ACOI 299 : A) DCM:MeOH, B) EA, for 24, 48, and 72 hours. Metabolic activity was measured using the Alamar Blue® assay and expressed as a percentage of control (CTL, untreated cells). Data are representative of three independent experiments and are presented as mean ± SD

The results of the metabolic activity of the extracts in U87 cell line are presented below, illustrating the effect of the treatment on cellular viability.

4.3.2. U87 cell line

The results of the influence of the extracts on the metabolic activity of the U87 cell line are presented below.

The DCM:MeOH extract of *N. oculata* SAG 38.85 at a concentration of 100 µg/mL decreased metabolic activity to 62.17% after 72 hours (P≤0.05) (Figure 17A). In contrast, the EA extract of *N. oculata* SAG 38.85 at 100 µg/mL significantly reduced metabolic activity, bringing it down to 49.60% after 48 hours and further decreasing it to 33.61% after 72 hours (P≤0.05) (Figure 17B).

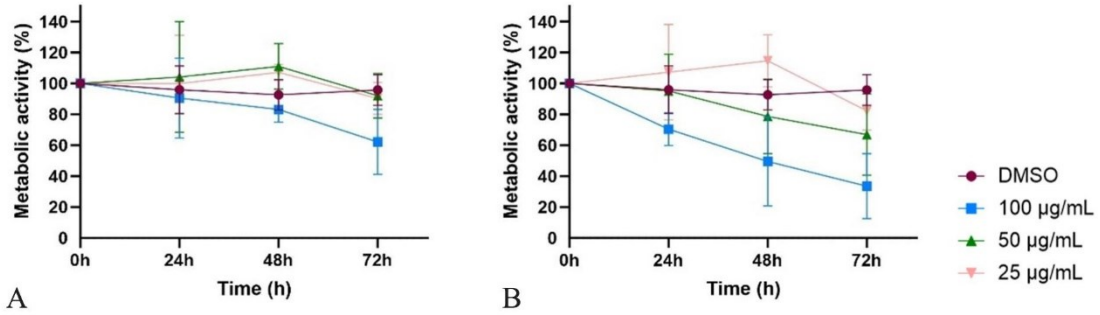

Figure 17. Impact of *N. oculata* SAG 38.85 extracts on the metabolic activity of U87 cells. U87 cells were treated with different solvent extracts of *N. oculata* SAG 38.85: A) DCM:MeOH, B) EA, for 24, 48, and 72 hours. Metabolic activity was measured using the Alamar Blue® assay and expressed as a percentage of control (CTL, untreated cells). Data are representative of three independent experiments and are presented as mean ± SD

The DCM:MeOH extract of *V. helvetica* ACOI 299 at a concentration of 100 µg/mL led to a decrease in metabolic activity, reducing it to 66.40% after 48 hours and further to 46.79% after 72 hours (P≤0.05) (Figure 18A). The EA extract of *V. helvetica* ACOI 299, also at 100 µg/mL, reduced the metabolic activity of the cells, bringing it down to 56.85% after 72 hours (P≤0.05) (Figure 18B).

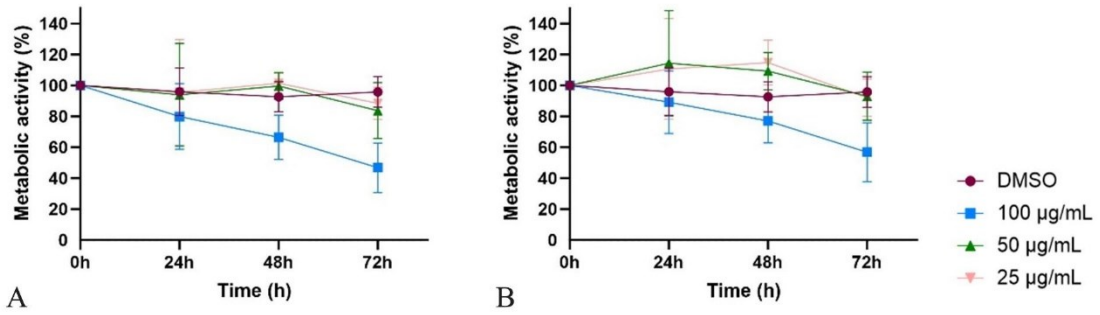

Figure 18. Impact of *V. helvetica* ACOI 299 extracts on the metabolic activity of U87 cells. U87 cells were treated with different solvent extracts of *V. helvetica* ACOI 299 : A) DCM:MeOH, B) EA, for 24, 48, and 72 hours. Metabolic activity was measured using the Alamar Blue® assay and expressed as a percentage of control (CTL, untreated cells). Data are representative of three independent experiments and are presented as mean ± SD

4.3.3. NHDF cell line

The results presented below demonstrate the impact of the extracts on the metabolic activity of the NHDF cell line.

The DCM:MeOH extract of *N. oculata* SAG 38.85 showed no significant effect on metabolic activity (Figure 19A). In contrast, the EA extract of *N. oculata* SAG 38.85, at a concentration of 100 µg/mL, significantly reduced the metabolic activity of the cells to 70.08% after 72 hours (P≤0.05) (Figure 19B).

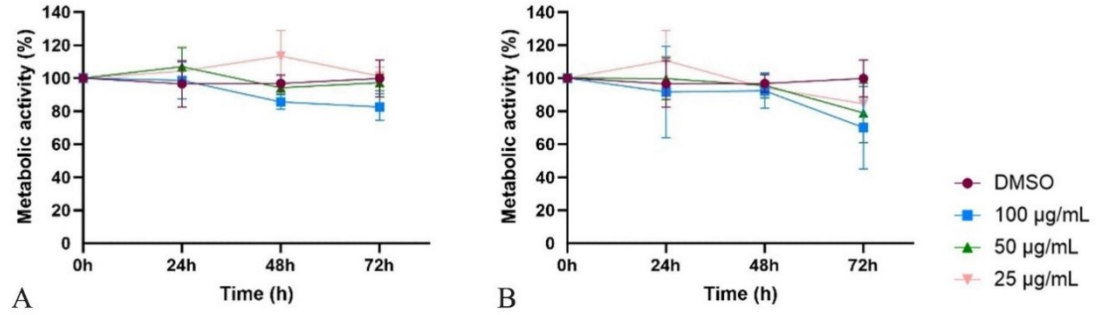

Figure 19. Impact of *N. oculata* SAG 38.85 extracts on the metabolic activity of NHDF cells. NHDF cells were treated with different solvent extracts of *N. oculata* SAG 38.85: A) DCM:MeOH, B) EA, for 24, 48, and 72 hours. Metabolic activity was measured using the Alamar Blue® assay and expressed as a percentage of control (CTL, untreated cells). Data are representative of three independent experiments and are presented as mean ± SD

The DCM:MeOH extract of *V. helvetica* ACOI 299 at a concentration of 100 µg/mL decreased metabolic activity to 74.53% after 72 hours (P≤0.05) (Figure 20A). Similarly, the EA extract of *V. helvetica* ACOI 299 at 100 µg/mL reduced the metabolic activity of the cells, bringing it down to 78.46% after 72 hours (P≤0.05) (Figure 20B).

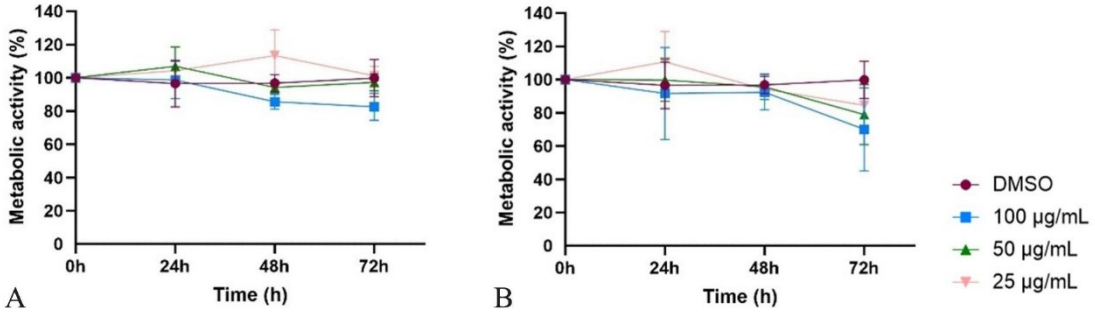

Figure 20. Impact of *V. helvetica* ACOI 299 extracts on the metabolic activity of NHDF cells. NHDF cells were treated with different solvent extracts of *V. helvetica* ACOI 299: A) DCM:MeOH, B) EA, for 24, 48, and 72 hours. Metabolic activity was measured using the Alamar Blue® assay and expressed as a percentage of control (CTL, untreated cells). Data are representative of three independent experiments and are presented as mean ± SD

4.4. Characterization of extracts

4.4.1. Pigment profile

The pigment profile of the EA extract of the two strains *N. oculata* SAG 38.85 and *V. helvetica* ACOI 299 was analyzed (Table 7).

Table 7. Pigment profile of the EA extracts of strains *N. oculata* SAG 38.85 and *V. helvetica* ACOI 299.

Pigment	**% of total pigments**	
	***N. oculata* SAG 38.85**	***V. helvetica* ACOI 299**
Neoxanthin	9.99	12.46
Violaxanthin	15.81	19.27
Lutein	4.06	4.37
Zeaxanthin	2.94	3.29
Chlorophyll *a*	54.83	16.10
Phaeophytin *a*	9.98	25.67
β -carotene	2.35	18.81
Total carotenoids	35.17	58.22
Total chlorophylls	64.83	41.78

The pigment profile analysis reveals that in *N. oculata* SAG 38.85 the most abundant pigment is chlorophyll *a* (54.83%), followed by violaxanthin (15.81%), phaeophytin *a* (9.98%) and neoxanthin (9.99%). In contrast, the pigment profile of *V. helvetica* ACOI 299 is characterized by a significantly different distribution, with phaeophytin *a* being

the most abundant (25.67%), followed by violaxanthin (19.27%), β-carotene (18.81%), chlorophyll *a* (16.10%) and neoxanthin (12.46%).

4.4.2. Fatty acid profile

The fatty acid profile of the EA extract of the two strains, *N. oculata* SAG 38.85 and *V. helvetica* ACOI 299, was analyzed (Table 8).

Table 8. Fatty acid profile of the EA extracts of strains *N. oculata* SAG 38.85 and *V. helvetica* ACOI 299

Fatty acids		**% of total fatty acids**	
		***N. oculata* SAG 38.85**	***V. helvetica* ACOI 299**
Lauric acid	(C12:0)	n.d.	1.10 ± 0.00
Myristic acid	(C14:0)	7.00 ± 0.01	12.95 ± 0.02
Pentadecylic acid	(C15:0)	0.96 ± 0.00	1.40 ± 0.00
Palmitic acid	(C16:0)	34.01 ± 0.05	20.24 ± 0.03
Stearic acid	(C18:0)	5.92 ± 0.01	10.68 ± 0.02
Arachidic acid	(C20:0)	0.67 ± 0.00	1.06 ± 0.00
Behenic acid	(C22:0)	n.d.	1.86 ± 0.00
Sum SFA		48.56 ± 0.07	49.29 ± 0.08
Palmitoleic acid	(C16:1)	24.64 ± 0.06	9.99 ± 0.01
Oleic acid	(18:1ω9 cis)	9.49 ± 0.02	10.15 ± 0.01
Sum MUFA		34.12 ± 0.08	20.14 ± 0.02
Linolelaidic acid	(C18:2ω6 trans)	0.95 ± 0.00	n.d.
Linoleic acid	(C18:2ω6 cis)	2.51 ± 0.01	4.38 ± 0.01
α-Linolenic acid	(C18:3ω3)	n.d.	2.69 ± 0.00
Eicosatrienoate acid	(C20:3ω5)	0.59 ± 0.00	n.d.
Tricosylic acid + Arachidonate acid	(C23:0) + (C20:4ω6)	2.64 ± 0.01	4.93 ± 0.00
EPA acid	(C20:5ω3)	10.62 ± 0.02	18.57 ± 0.01
Sum PUFA		17.32 ± 0.03	30.57 ± 0.02
Sum ω3		10.62 ± 0.02	21.25 ± 0.01
Sum ω6		6.11 ± 0.01	9.31 ± 0.01

Note: n.d. = not detected

In terms of fatty acid composition, *N. oculata* SAG 38.85 has a total saturated fatty acids (SFA) content of 48.56%, while the monounsaturated fatty acids (MUFA) content is 34.12%, and the polyunsaturated fatty acids (PUFA) content is 17.32%. In *V. helvetica* ACOI 299, (SFA) content is 49.29%, the (MUFA) content is 20.14%, and the (PUFA) content is 30.57%.

In the strain *N. oculata* SAG 38.85, the six most abundant fatty acids are palmitic acid (34.01%), palmitoleic acid (24.64%), EPA acid (10.62%), oleic acid (9.49%), stearic acid (5.92%), and myristic acid (7.00%). Comparatively, in the strain *V. helvetica* ACOI 299, the six most abundant fatty acids are EPA (18.57%), palmitic acid (20.24%), myristic acid (12.95%), stearic acid (10.68%), oleic acid (10.15%), and palmitoleic acid (9.99%).

5. Discussion

This study explored the extraction of bioactive compounds from some microalgal strains, focusing on their potential antitumor properties. Various solvents were used to extract these compounds, each with different efficiencies and effects.

5.1. Extraction yields

The extraction yields varied based on the solvents used, which had different polarities. The solvents employed ranged from non-polar Hx to polar MeOH, with EA representing an intermediate polarity, and a semi-polar mixture of DCM:MeOH (1:1). These differences in polarity influenced both the extraction efficiency and the types of bioactive compounds isolated (Gangadhar et al., 2016; Gnanakani et al., 2019; Jeon et al., 2013; Susanto et al., 2024).

5.1.1. Dichloromethane:Methanol (1:1) extraction

The DCM:MeOH (1:1) mixture proved to be the most efficient solvent in terms of extraction yield, consistently producing the highest amount of extract across all microalgae strains. This solvent system is increasingly favored over the traditional chloroform:methanol mixture due to its reduced toxicity and environmental impact (Jeon et al., 2013). It efficiently extracted both polar and non-polar bioactive compounds, including fatty acids and lipophilic pigments, which are known to contribute to antioxidant and antitumor activities (Figueiredo et al., 2019; Hassouani et al., 2017; Stranska-Zachariasova et al., 2016). The high yield of DCM:MeOH is due to its ability to extract compounds of varying polarities, making it an efficient solvent for obtaining a large quantity of extract. However, despite its efficiency in terms of yield, the cytotoxic potency of the DCM:MeOH extracts in biological assays was lower compared to that of EA extracts. EA, although it yielded a smaller amount of extract, demonstrated stronger and more selective cytotoxic activity against GB cells. Therefore, while DCM:MeOH was more efficient in terms of extraction yield, EA was the most potent solvent in terms of biological activity.

5.1.2. Hexane extraction

Hx, being a non-polar solvent, was used first in the sequential extraction process to isolate lipophilic compounds, such as neutral lipids, fatty acids, sterols, and carotenoids (Susanto et al., 2024; F. Wang, Gao, et al., 2018). These compounds have demonstrated

antitumor potential, particularly through mechanisms like apoptosis induction (Wali et al., 2020) and immune modulation (Silva et al., 2022). Although these lipophilic molecules can play a role in antitumor activity, Hx's non-polar nature limits its ability to extract a broader spectrum of bioactive molecules, particularly those with polar characteristics, resulting in generally lower extraction yields compared to other solvents (Susanto et al., 2024; F. Wang, Gao, et al., 2018). This explains why Hx had the lowest extraction yields, as it primarily extracted non-polar molecules, and its extracts showed limited cytotoxicity in the screening, likely due to the absence of more potent polar bioactive compounds.

5.1.3. Ethyl acetate extraction

Following Hx extraction, EA was used to extract a wider range of bioactive compounds due to its intermediate polarity. EA is particularly effective at isolating phenolics, flavonoids, carotenoids, and moderately polar fatty acids (Gangadhar et al., 2016; Gnanakani et al., 2019). These compounds, especially phenolics and flavonoids, are known for their strong antioxidant properties and selective cytotoxicity against cancer cells (Gnanakani et al., 2019). In this study, the EA extracts showed the most potent cytotoxic effects on GB cells, significantly reducing metabolic activity in U87 and A172 cells, while having minimal toxicity on NHDF cells. This selective cytotoxicity makes EA a promising solvent for isolating compounds with targeted antitumor effects (Gnanakani et al., 2019; Loh et al., 2014). EA generally yielded lower amounts compared to DCM:MeOH, the bioactive compounds it extracted were likely more concentrated and selective, accounting for its stronger cytotoxic performance.

5.1.4. Methanol extraction

MeOH, a polar solvent, was employed in the final step of sequential extraction to capture the remaining bioactive compounds, targeting both polar and slightly non-polar substances. MeOH was particularly effective at isolating a wide spectrum of bioactive molecules, such as fatty acids, carotenoids, phenolic acids, and sterols (Gnanakani et al., 2019; Haoujar et al., 2019; Martins et al., 2021b). MeOH demonstrated high extraction efficiency, especially for the strain *N. oculata,* MeOH extracts have shown significant cytotoxic effects in other studies, such as in the MDA-MB-231 cell line, while maintaining low toxicity toward normal cells (Hussein et al., 2020; Wali et al., 2020). However, MeOH's broad-spectrum nature might result in less selective

cytotoxicity compared to the more targeted extraction achieved with EA, as it extracts both active and inactive compounds indiscriminately (Gnanakani et al., 2019).

The correlation between solvent polarity and extraction yield highlights that while DCM:MeOH provides a high extraction yield, EA extracts, despite yielding less, showed stronger and more selective cytotoxic effects due to the nature of the isolated bioactive compounds. Thus, solvent polarity significantly influences both the quantity and quality of the extracted bioactive compounds, as evidenced by their performance in reducing GB cell viability.

The selection of extracts tested in this study was informed by both preliminary results and existing literature, allowing us to focus on the most promising candidates while minimizing unnecessary testing.

For *M. aquilonaris* ACOI 2424, we initially screened the EA, MeOH, and Hx extracts. However, these extracts demonstrated limited cytotoxic activity against the GB cell lines. As a result, we decided not to proceed with the DCM:MeOH extract for this strain, given that the initial results did not warrant further exploration with additional solvents. This decision allowed us to conserve resources and focus on strains and extracts with more promising activity.

Conversely, for *C. regularis* ACOI 307, a prior study had shown a selective cytotoxic effect, particularly on the MCF-7 cell line with the DCM:MeOH extract (Martins et al., 2021b). Based on these findings, we focused our efforts on this extraction method, hypothesizing that it would yield similarly significant results in our study. This decision was aimed at maximizing the potential for positive outcomes based on the known bioactivity of this extract.

By adopting this selective approach, we were able to streamline our investigation and focus on extracts with the highest likelihood of yielding relevant antitumor effects. This strategy not only ensured an efficient use of resources, but also aligned with the goals of identifying promising candidates for further study in glioblastoma therapeutics.

5.2. Comparative analysis of the effects of *N. oculata* SAG 38.85 and *V. helvetica* ACOI 299 extracts on cell metabolic activity and the role of pigments and fatty acids in selective cytotoxicity

The EA and DCM:MeOH extracts of the two microalgal strains, *N. oculata* SAG 38.85 and *V. helvetica* ACOI 299, were selected for detailed investigation due to their superior cytotoxic effects on GB cells compared to other extracts. Both EA and DCM:MeOH extracts demonstrated the most significant reduction in cell viability in A172 and U87 GB cell lines, while showing minimal toxicity toward NHDF cells. This selective cytotoxicity, favoring cancer cells over normal cells, makes these extracts promising candidates for further exploration in cancer therapy.

Among the two extracts, the EA extract was chosen over the DCM:MeOH extract for both *N. oculata SAG 38.85* and *V. helvetica ACOI 299* due to its more consistent and prolonged reduction in cell viability in GB cells. The EA extract also demonstrated greater selectivity, effectively targeting cancer cells while sparing non-tumoral cells, which is crucial for therapeutic applications. As these strains showed the most significant cytotoxic effects in the *in vitro* assays, a detailed analysis of pigments and fatty acids in the EA extracts of *N. oculata SAG 38.85* and *V. helvetica ACOI 299* was conducted. This comprehensive comparison was essential to understand the underlying mechanisms of their antitumor activities and to identify the bioactive compounds responsible for these effects.

5.2.1. Pigment profile analysis

The pigments identified in the EA extracts of both strains play a crucial role in their observed cytotoxicity toward GB cells. Pigments are well-known for their biological activities, particularly in antioxidant defense and modulation of cellular processes, such as apoptosis and cell proliferation (Talero et al., 2015b).

The EA extract of *V. helvetica* ACOI 299 was found to contain high levels of β-carotene, a carotenoid extensively studied for its antitumor potential. β-carotene has strong antioxidant properties, which protect cells from oxidative stress, a factor often linked to cancer progression (Talero et al., 2015b). In cancer cells, β-carotene can selectively induce oxidative stress, leading to cell death. It also enhances immune surveillance, helping the immune system better recognize and target cancer cells (Fiedorowicz & Dobrzynska, 2023). This dual function of β-carotene likely contributes to the observed cytotoxicity in GB cells, particularly in the U87 line, where cell

viability was significantly reduced after treatment with *V. helvetica* ACOI 299 EA extract.

Another critical pigment present in *V. helvetica* ACOI 299 is phaeophytin *a*, a derivative of chlorophyll *a*, which has been shown to trigger apoptosis and inhibit cancer cell migration (Shailaja et al., 2019). The ability of phaeophytin *a* to induce apoptosis aligns with the cytotoxic effects observed in both A172 and U87 cells, supporting its role in enhancing the antitumoral activity of the EA extract.

The EA extract of *N. oculata* SAG 38.85 was characterized by high levels of chlorophyll *a*, a green pigment known for its antitumor properties. Chlorophyll *a* has been shown to inhibit cancer cell proliferation and induce apoptosis. Studies have also linked chlorophyll *a* reduced the tumor size in animal models of pancreatic cancers, further emphasizing its importance in cancer therapy (Vaňková et al., 2018).

Lutein and zeaxanthin, two other carotenoids present in *N. oculata* SAG 38.85, contribute to its antitumor potential by inducing cell cycle arrest and reducing cancer cell proliferation. Zeaxanthin promotes apoptosis and has demonstrated antiproliferative effects in neuroblastoma and breast cancer cells (Cha et al., 2008; Juin et al., 2018). This ability to induce apoptosis likely explains the significant reduction in cell viability observed in GB cells treated with *N. oculata* SAG 38.85's EA extract.

Both strains also contained violaxanthin and neoxanthin, two carotenoids with proven antitumor effects. Violaxanthin has been shown to induce apoptosis in breast and colon cancer cells (Pasquet et al., 2011), while neoxanthin activates caspase-dependent apoptosis pathways and contributes to mitochondrial disruption in cancer cells (Kotake-Nara et al., 2005; Terasaki et al., 2007). The presence of these pigments in both strains can enhance their potential antitumor activity probably by targeting multiple tumor-related pathways, contributing to the observed reduction in GB cell viability.

5.2.2. Fatty acid profile analysis

In addition to pigments, the fatty acid profiles of both strains' EA extracts play a significant role in their cytotoxic effects. Fatty acids, particularly polyunsaturated and monounsaturated types, are known to modulate key cellular processes, such as inflammation, oxidative stress, apoptosis, and cancer cell metabolism, making them essential components in antitumor activity (Huang et al., 2022).

In strain *V. helvetica* ACOI 299, the fatty acid profile revealed EPA as one of the most abundant fatty acids. EPA is known to inhibit tumor growth through multiple mechanisms, including induction of apoptosis and modulation of inflammatory pathways, such as COX-2/PGE2, which is often upregulated in cancer. EPA also reduces angiogenesis, a process critical for tumor growth and metastasis, and mitigates oxidative stress by regulating reactive oxygen species (ROS) levels (Spencer et al., 2009).

Palmitic acid, another significant fatty acid in *V. helvetica* ACOI 299, plays a dual role in cancer biology. While it can promote cancer cell survival under certain conditions, it can also induce apoptosis under others, depending on the cellular environment (X. Wang et al., 2023). This dynamic function of palmitic acid makes it a complex yet valuable component of *V. helvetica* ACOI 299's antitumor activity.

In addition to EPA and palmitic acid, myristic acid, stearic acid, and oleic acid are present in notable amounts in *V. helvetica* ACOI 299. Myristic acid and stearic acid have been linked to cytotoxic effects in various cancer cells by inducing apoptosis and disrupting cancer cell membrane integrity (Qasem et al., 2016). Oleic acid further contributes to antitumor activity by promoting apoptosis and reducing cancer cell proliferation.

In the strain *N. oculata* SAG 38.85, the fatty acids palmitic acid and palmitoleic acid were the most abundant. Palmitoleic acid, a monounsaturated fatty acid (MUFA), is known for its anti-inflammatory and antitumor properties, particularly by inhibiting cancer cell proliferation (Westheim et al., 2023).

EPA was also present in *N. oculata* SAG 38.85, though at a lower concentration compared to *V. helvetica* ACOI 299. Nonetheless, EPA's ability to induce apoptosis and inhibit inflammatory pathways supports its contribution to the overall cytotoxic effects of *N. oculata* SAG 38.85 (Spencer et al., 2009). The presence of oleic acid, stearic acid, and myristic acid adds to the strain's antitumor potential, as these fatty acids have been associated with promoting apoptosis and reducing cancer cell proliferation (Qasem et al., 2016).

5.2.3. Selective cytotoxicity

A critical observation from the experiments is the selective cytotoxicity of the EA extracts toward GB cells, with minimal toxicity toward NHDF cells. This selectivity is a key factor in their therapeutic potential, as it demonstrates the ability of the extracts

to target cancer cells while sparing non-tumoral cells—a major challenge in conventional cancer treatments.

Several compounds identified in the EA extracts, such as zeaxanthin, EPA, myristic acid, and stearic acid, are known for their ability to selectively induce apoptosis in cancer cells without affecting normal cells. For example, zeaxanthin induces apoptosis through mitochondrial dysfunction (Cha et al., 2008), EPA regulates apoptosis via ROS modulation and COX-2 inhibition (Spencer et al., 2009), and myristic acid and stearic acid disrupt the integrity of cancer cell membranes, making them more vulnerable to apoptotic pathways (Qasem et al., 2016). This selective action may be due to the differential regulation of apoptosis pathways and cellular stress responses in cancer versus normal cells.

The combination of metabolic activity assays with detailed pigment and fatty acid analyses has allowed for a comprehensive understanding of the antitumor potential of EA extracts of *N. oculata SAG 38.85* and *V. helvetica ACOI 299*. The selective cytotoxicity observed in GB cells, combined with minimal effects on non-tumoral cells, can be attributed to the specific carotenoids, chlorophyll derivatives, and fatty acids identified in these strains. The observed variations in the extract bioactivity suggest complex interactions of bioactive compounds, warranting further research to isolate and identify the specific agents responsible. To the best of our knowledge, no previous studies have evaluated the cytotoxic effects of these specific microalgae strains using the extraction methods applied in this study on these cell lines. This novel approach adds valuable insights to the existing body of research on the potential antitumor properties of microalgal extracts, particularly in the context of GB treatment. The findings suggest that these microalgal extracts have significant therapeutic potential, warranting further research to isolate and develop specific bioactive agents for cancer treatment.

6. Conclusion and Future Perspectives

This research explored the cytotoxic potential of carotenoid-rich microalgae extracts, specifically focusing on GB cell lines and NHDF cells. The EA extracts from *N. oculata* SAG 38.85 and *V. helvetica* ACOI 299 emerged as the most promising in terms of selective cytotoxicity against GB cells while maintaining minimal toxicity to normal cells.

Among these two, the EA extract of *V. helvetica* ACOI 299 stands out as the most effective. While both extracts showed potential antitumor effects, *V. helvetica* ACOI 299 exhibited more consistent results across both GB cell lines (U87 and A172) and over different time points, reducing cell viability more significantly. This extract not only maintained high cytotoxic activity against GB cells, but also demonstrated a greater margin of selectivity, showing even lower toxicity toward NHDF cells compared to *N. oculata* SAG 38.85.

Therefore, the EA extract of *V. helvetica* ACOI 299 is identified as the best-performing extract, making it the leading candidate for further development in GB treatment. Future research should prioritize isolating and characterizing their active compounds, exploring their mechanisms of action in more cancer cell lines, and conducting *in vivo* studies to support further clinical translation. Optimizing extract concentrations and investigating combination therapies with existing chemotherapeutics could further enhance their therapeutic potential and reduce side effects. These microalgal extracts show a valuable avenue for developing future effective and target treatments for GB and other cancers.

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