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Uso de extratos da microalga *Planktochlorella nurekis* anti-coronavírus para fins clínicos e ambientais

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Tese submetida ao Programa de Biotecnologia e Biociências da Universidade Federal de Santa Catarina como requisito parcial para a obtenção do Título de Doutora em Biotecnologia e Biociências.

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O presente trabalho em nível de doutorado foi avaliado e aprovado por banca examinadora composta pelos seguintes membros:

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Certificamos que esta é a **versão original e final** do trabalho de conclusão que foi julgado adequado para obtenção do título de doutor em Biotecnologia e Biociências.

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Florianópolis, 2022

Dedico a tese a minha mãe Janisse, pelas incansáveis orações, por todo o amor, carinho e atenção a mim dedicados e a meu filho Gustavo, inspiração da minha luta diária para construir um futuro melhor no qual ele possa realizar seus sonhos com mais facilidade e sabedoria.

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Algae are an alternative resource to synthetic drugs, because algae are very low toxicity and some are non-toxic at doses that have a broad antiviral spectrum against several viruses and minimal side effects (BESEDNOVA et al., 2021).

RESUMO

O desenvolvimento de produtos com novos compostos bioativos com atividade antiviral e virucida tem sido explorado pela indústria farmacêutica, especialmente nos últimos anos, devido ao surgimento de epidemias e pandemias. Nesse sentido, as micro e macroalgas são importantes candidatos para aplicações biotecnológicas e farmacêuticas, pois apresentam uma rica composição bioquímica, como lipídios, proteínas e carboidratos. Assim, o objetivo desse estudo é avaliar o potencial virucida e antiviral de extratos da microalga *Planktochlorella nurekis* frente ao gênero Coronavirus (murino e humano), visando aplicação clínica e ambiental. Nesse escopo, o manuscrito está dividido em três capítulos, sendo o primeiro voltado a uma revisão da literatura, acerca das potencialidades das algas, abordando mecanismos de ação e a atividade frente ao vírus envelopados e não envelopados. No segundo capítulo, trata-se da avaliação *in vitro* da potencial ação virucida de diferentes extratos da microalga *Planktochlorella nurekis* frente ao o coronavírus murino – 3 (MHV-3), um modelo seguro do coronavírus para ensaio laboratorial. Extratos de *P. nurekis* com metanol (MeOH), hexano (HEX) e diclorometano (DCM) foram testados quanto à citotoxicidade e a inativação viral. Além disso, sendo esses caracterizados por espectrofotometria ultravioleta e visível (UV-vis), por espectroscopia de ressonância magnética nuclear (RMN) e cromatografia líquida de ultraperformance acoplado à espectrometria de massas (UPLC-MS). Os resultados apontam que o extrato da microalga com DCM foi o mais promissor como agente virucida, inativando no mínimo 4 Log (99,99%) dos vírions. A ação anti-coronavírus observada se mostrou diretamente correlacionada com a presença de polifenóis, carboidratos e derivados de isopreno (terpenos e carotenoides). Considerando os compostos encontrados, no terceiro capítulo testou-se inicialmente a eficiência dos extratos de microalgas (MeOH, DCM e HEX) frente ao SARS-CoV-2 e posteriormente pelos resultados obtidos com o DCM, onde verificou-se menor toxicidade (concentração de CC_{50}) e maior índice de seletividade frente o vírus (IS) testou-se a inativação do coronavírus na presença de esgoto humano. Os resultados mostraram redução de 4, 5 e 6 Log após 30, 45 e 60 min de exposição em esgoto humano, respectivamente. Extratos HEX e especialmente de DCM da microalga *P. nurekis* mostraram-se promissores para fins de exploração do controle viral, como agente virucida e antiviral, podendo ser estes considerados para que novos produtos biotecnológicos possam ser empregados na área clínica e ambiental.

RESUMO EXPANDIDO

Introdução

A família Coronaviridae surgiu por transmissão zoonótica e nos últimos anos tem causado doenças respiratórias graves em humanos e animais, como a "Doença do Coronavírus" (COVID-19) e raros são os medicamentos e antissépticos capazes de controlar os vírus em suas vias de infecção, isso é uma preocupação mundial, tendo em vista que as partículas de SARS-CoV-2 são excretadas nas fezes dos pacientes e podem sobreviver por mais de 30 dias em esgoto humano, por exemplo. A indústria farmacêutica está atualmente tentando desenvolver novos compostos bioativos para inativar vírus envelopados e não envelopados para fins terapêuticos. Consequentemente, compostos bioativos de microalgas e macroalgas estão sendo explorados por indústrias farmacêuticas, biotecnológicas e alimentícias, sendo que essas podem ser estudadas para aplicação de tratamentos clínicos e ambientais que vissem a inativação do vírus, como o SARS-CoV-2. Os vírus possuem alta capacidade replicativa e podem gerar novas cepas em diferentes ambientes. Nesse sentido, a prospecção de compostos de origem biológica como forma alternativa de obtenção de novos virucidas e antivirais tornou-se ainda mais crítica. Microalgas da família Chlorellaceae são fontes de compostos bioativos com atividade antioxidante, antiviral e antitumoral. As microalgas possuem uma gama de compostos com atividade antimicrobiana, mas várias espécies ainda são pouco estudadas, como a *Planktochlorella nurekis*. Dessa maneira, estudos que comprovem o potencial virucida e antiviral dessa espécie é necessário, visando sua aplicação clínica e ambiental.

Objetivos

O objetivo geral da tese foi realizar análises acerca do potencial virucida e antiviral de extratos da microalga *Planktochlorella nurekis* frente ao gênero *Coronavirus* (murino e humano), visando aplicação clínica e ambiental. Para o Capítulo I intitulado "Broad spectrum algae compounds against viruses: a review" o objetivo foi discutir como compostos produzidos por algas podem ser candidatos importantes para aplicações de controle viral. No Capítulo II "Characterization of *Planktochlorella nurekis* extracts and virucidal activity against a coronavirus model, the murine coronavirus 3" o objetivo foi avaliar condições *in vitro* de extratos de *P. nurekis* com metanol (MeOH), hexano (HEX) e diclorometano (DCM) frente o coronavírus murino – 3 (MHV-3), além de caracterizar bioquimicamente esses extratos. O Capítulo III "Cytotoxic assay and acceleration of inactivation of murine coronavirus in human sewage by the application of *Planktochlorella nurekis* extract" teve-se como objetivo avaliar a citotoxicidade dos extratos de MeOH, HEX e DCM de *P. nurekis* nas linhagens celulares L929 e Vero e a partir desses resultados buscou-se promover a inativação do MHV-3 no esgoto humano.

Metodologia

A fim de responder os objetivos pretendidos para cada um dos capítulos da tese, será realizada a descrição metodológica separadamente. Assim, no Capítulo I realizou-se uma revisão narrativa da literatura selecionando artigos que tratavam do tema em questão, i.e., verificando-se quais algas possuem capacidade virucida para aplicações nos setores fitoquímico, farmacêutico e sanitizante. No Capítulo II, os extratos de *P. nurekis* com MeOH, HEX e DCM foram testados em células infectadas com MHV-3 e posteriormente caracterizados por espectrofotometria ultravioleta e visível (UV-vis) e de ressonância magnética nuclear (RMN), e via cromatografia líquida de ultraperformance acoplada à espectrometria de massas (UPLC-MS). A fim de analisar os resultados obtidos realizou-se a análise de variância (ANOVA) e o

teste *post hoc* e aplicou-se a quimiometria através da análise de componentes principais (PCA). Para tal, foram desenvolvidos algoritmos para análise estatística utilizando o programa MATLAB. No Capítulo III, os extratos com MeOH, HEX e DCM foram testados quanto à citotoxicidade nas linhagens celulares L929 e Vero. Os parâmetros medidos em cada um dos poços de microplacas foram: o número total de células e o número de células infectadas. Com o resultado dos testes de citotoxicidade, o extrato de DCM de *P. nurekis* foi testado em esgoto humano. Assim, o vírus MHV-3 foi inoculado em esgoto humano e tratado com 10 µg/mL do extrato por 5, 30, 45 e 60 minutos a 25 ± 2 ºC. Realizou-se o teste t e a ANOVA para avaliar as diferenças na taxa de inativação viral entre o controle tratado e não tratado. Do mesmo modo, os valores de Log foram analisados por regressão linear.

Resultados e Discussão

No Capítulo I inicialmente realizou-se uma ampla discussão acerca dos mecanismos de ação e atividade virucida frente à vírus envelopados e não envelopados, incluindo aqueles que causam infecções por vias entérica, parenteral e respiratória. As etapas de obtenção de compostos com bioatividades de interesse biotecnológico são simples e envolvem 4 principais etapas, são elas: (1) coleta e identificação taxonômica de amostras; (2) secagem; (3) extração pelo uso de solvente e; (4) filtração e concentração por evaporação. Posteriormente a essa extração, são necessários ensaios citotóxicos de forma a avaliar se os extratos serão tóxicos para células viáveis. Se o extrato passar pelos ensaios de citotoxicidade, a ação virucida e antiviral é testada. Os ensaios de ação virucida identificam compostos que matam vírus, por exemplo, inativando proteínas de reconhecimento, enquanto ensaios antivirais identificam compostos que, por exemplo, inibem a replicação viral. A partir da revisão da literatura verifica-se que as macro e microalgas são uma fonte natural de importantes compostos que possuem propriedades antivirais, sendo de baixo custo de obtenção, portanto, trata-se de um recurso alternativo às drogas sintéticas, com baixa ou nenhuma toxicidade para uso humano. Pela diversidade de moléculas encontradas, essas possuem capacidade de inativar o vírus e bloquear sua ação sem causar resistência ou seletividade. Assim, pelas diferentes espécies encontradas, vários são os compostos presentes nas algas que possuem atividades potentes frente à diversos tipos de vírus, sendo assim, fortes candidatos para controle e tratamento, em humanos e animais. Compostos simples e complexos são descritos e já identificados em algas, como, por exemplo, polissacarídeos (fucoidano, alginato, laminarina e carragenina), compostos fenólicos e florotaninos (flavonoides, lignanas e taninos), proteínas e peptídeos (aminoácidos, incluindo leucina, glutâmico ácido, triptofano), lipídios, terpenoides e esteroides (carotenoides), vitaminas e minerais. Esses compostos bioativos possuem propriedades além de virucida e antiviral, antitumorais, anticoagulantes e antioxidantes, o que aponta para a ampla aplicação das algas tanto na área clínica como ambiental. No Capítulo II, verificou-se que o perfil espectral dos extratos de *P. nurekis* foram muito similares frente à absorção na região UV-Vis. Os picos de absorção na região UV apresentaram um platô nas bandas 240-260 nm e em 280 nm. Quando considerada a região Visível, é possível identificar duas principais bandas em 410 nm, região característica de carotenoides, e 660 nm, região que absorve as clorofilas. Essas bandas são características de algas verdes e apesar de similares, em extratos de *P. nurekis* com DCM é possível verificar maior intensidade das bandas. Foram quantificados β-caroteno, fenóis e aminoácidos. Os extratos de *P. nurekis* em diclorometano apresentaram altas concentrações de carotenoides totais (16,6 µg/g) quando comparado aos extratos hexânicos (10.1 µg/g) e metanólicos (7.14 µg/g). Os compostos polifenóicos foram extraídos de melhor forma em extratos metanólicos (84 mg/g), obtendo-se mais que o dobro dos demais, que apresentaram concentração de 40 mg/g para o DCM e 29 mg/g HEX. Dentre os aminoácidos quantificados,

destaca-se a valina que diferiu entre os extratos, sendo o HEX o que apresentou maior concentração. O tipo do extrator e sua polaridade influenciou a tendência de extração, dependendo da solubilidade de cada molécula, a sua permeabilidade entre as membranas e potencial do analito de ser transportado entre as membranas. Os carotenoides luteína (polar) e β-caroteno (relativamente não polar) são solúveis em DCM, o que explica a tendência de altas concentrações de carotenoides neste extrator. Ao analisar os compostos por RMN verificou-se que o extrato de DCM foi o que apresentou maior concentração de metabólitos, seguido pelo extrato de HEX. O MeOH foi o que apresentou menor número de picos e com menor intensidade. Os extratos de DCM e HEX apresentaram perfil semelhante, com presença de vitamina B5, que pertence à zeaxantina e de valina e violaxantina. Alguns compostos foram encontrados apenas em um dos extratos, como, por exemplo, no extrato de HEX que verificou-se um pico de vitamina B2 enquanto no DCM de luteína. Todavia, a principal diferença entre esses dois extratos está na presença dos ácidos orgânicos, sendo que o extrato de DCM apresentou maior quantidade de compostos e em maiores concentrações. Compostos bioativos como o ácido pirúvico, piroglutâmico e succínico foram encontrados somente no extrato de DCM. A presença desses compostos, assim como de hipoxantina, por exemplo, que é precursor de xantina, indica para uma ação antiviral já comprovada em outros estudos, incluindo do SARS-CoV-2. Em relação à análise em UPLC-ESIMS, os resultados apontam que os extratos de HEX e DCM apresentam o mesmo perfil, todavia, na fração hexânica foram encontrados 11 metabólitos secundários e no diclorometano 18. No extrato diclorometânico foram encontrados diversos ácidos graxos, enquanto os ácidos hexadecatrienoico e palmítico não foram encontrados nos extratos hexânicos. Na classe lipídica, os ácidos palmítico e estearidônico, presentes apenas no extrato de DCM se destacaram quanto a atividade virucida. O extrato de DCM, que apresenta polaridade intermediária permitiu um melhor isolamento dos lipídios devido à existência de uma região alifática e oxigenada no metabólito. Todas as amostras foram submetidas à testes de citotoxicidade na linhagem celular L929 por 48 h. Todos os extratos apresentaram baixa citotoxicidade, e o extrato diclorometânico apresentou efeito citotóxico essencial nas células L929 com valor de CC_{50} de 53,19 µg/mL. Os resultados das análises virucidas mostraram que o tratamento com o extrato de MeOH da microalga resultou em uma redução da infecção por coronavírus MHV-3 de 6 Log10 PFU e 8 Log10 PFU a 24 ± 2 °C e 35 ± 2 ° C. O tratamento com o extrato de HEX resultou em uma redução de 7 Log10 PFU (12,5 a 50 µg/mL) a 24 °C, mas não houve redução na infecção por coronavírus MHV-3 a 35 ± 2 °C. Os extratos de DCM levaram a uma redução de 6 Log10 PFU para 8 Log10 PFU (3,1 para 50 µg/mL) a 24 °C, apontando que o extrato de *P. nurekis* com DCM foi o mais eficaz e reduziu mais de 99,99% da infecção. O Capítulo III demonstrou que o extrato de *P. nurekis* com DCM não possui alto efeito citotóxico na linhagem celular L929, apresentando 53,19 µg/mL considerando o valor médio de CC_{50}. Com a metodologia de HCS avaliou-se o efeito antiviral dos três extratos na sobrevivência de células Vero CCL-81 frente ao vírus SARS-CoV-2. Na fração metanólica nas células, foram observados EC_{50} e CC_{50} acima de 100 µg/mL e atividade máxima para inibição de 42%. Nos extratos diclorometânico, o EC_{50}, o CC_{50} e a atividade máxima foram de 57,50 µg/mL, 100 µg/mL e 64%, respectivamente. Os resultados da fração de hexano mostram EC_{50}, CC_{50} e atividade máxima de 41,27 µg/mL, 51,92 µg/mL e 89%, respectivamente. Os dados *in vitro* apontam que o extrato hexânico e o diclorometânico apresentaram potencial virucida superior ao extrato metanólico. Os dados de EC_{50} nas concentrações testadas, descartam o uso de extrato metanólico visando ação viricida, apesar de apresentar baixa toxicidade celular (CC_{50}). Dentre os dois extratos com potencial virucida (EC_{50}), o extrato de *P. nurekis* com diclorometano apresentou menor toxicidade e maior índice de seletividade (SI) contra o vírus comparado ao hexano. O controle apresentou valores de SI

de 22,9, enquanto os extratos hexânico e diclorometânico apresentaram valores de 1,3 e 1,7, respectivamente. Em relação à aceleração da inativação do MHV-3 em esgoto humano com extrato de diclorometano de *P. nurekis* (PNDE) verificou-se uma inativação significativa ($p = 0,0016$), havendo redução de 4, 5 e 6 Log10 após 30, 45 e 60 min de exposição, respectivamente. Salienta-se que não há inativação do MHV-3 durante os primeiros 60 min sem tratamento. Todavia, na presença de PNDE, houve inativação acelerada do MHV-3, obtendo um coeficiente de inativação (k) de 0,1023 PFU/min, prospectivo por regressão linear ($R^2 = 0,93$), demonstrando uma taxa de redução do vírus de 90% (T90) aos 9,7 min após tratamento de esgoto humano com PNDE (T90 min = $1/k\ min^{-1}$).

Considerações Finais

As algas são promissoras fonte de compostos para uso como sanitizantes de baixa toxicidade e apresentam alta capacidade virucida. Os resultados na pesquisa abrem novas possibilidades biotecnológicas para explorar a biomassa de *P. nurekis*, que por ser um extrato de organismo natural e que possui baixa citotoxicidade, associado à sua alta eficácia e baixo custo de produção, tem potencial para desenvolvimento de novos medicamentos ou tratamento de coronavírus, como SARS-CoV-2. Além disso, a partir dos testes realizados verifica-se que o extrato de *P. nurekis* em DCM apresentou maior potencial para elaboração de produtos à base de microalgas de forma ecologicamente correta. O aumento da área voltada a bioeconomia circular tem estimulado o uso de microalgas na formulação de produtos de base biológica, como ração animal, produtos químicos e biocombustíveis, especialmente porque viabilizam a combinação do tratamento de águas residuais em confluência com a biorremediação de águas residuais.

Palavras-chave: *Planktochlorella nurekis*; SARS-CoV-2; Potencial virucida; MHV-3.

ABSTRACT

The development of products with new bioactive compounds with antiviral and virucidal activity has been explored by the pharmaceutical industry, especially in recent years, due to the emergence of epidemics and pandemics. In this sense, micro and macroalgae are important candidates for biotechnological and pharmaceutical applications, as they have a rich biochemical composition, such as lipids, proteins, and carbohydrates. Thus, the aim of this study is to evaluate the virucidal and antiviral potential of extracts from the microalgae *Planktochlorella nurekis* against the genus Coronavirus (murine and human), aiming at clinical and environmental application. In this scope, the manuscript is divided into three chapters, the first of which is devoted to a literature review, of the potential of algae, addressing mechanisms of action and activity against enveloped and non-enveloped viruses. The second chapter deals with the *in vitro* evaluation of the potential virucidal action of different extracts of the microalgae *Planktochlorella nurekis* against the murine coronavirus – 3 (MHV-3), a safe model of the coronavirus for laboratory testing. Extracts of *P. nurekis* with methanol (MeOH), hexane (HEX), and dichloromethane (DCM) were tested for cytotoxicity and viral inactivation. Furthermore, these are characterized by ultraviolet and visible spectrophotometry (UV-vis), nuclear magnetic resonance spectroscopy (NMR), and ultra-performance liquid chromatography coupled with mass spectrometry (UPLC-MS). The results indicate that the microalgae extract with DCM was the most promising as a virucidal agent, inactivating at least 4 Log (99.99%) of the virions. The observed anti-coronavirus action was directly correlated with the presence of polyphenols, carbohydrates, and isoprene derivatives (terpenes and carotenoids). Considering the compounds found, in the third chapter, the efficiency of microalgae extracts (MeOH, DCM, and HEX) was initially tested against SARS-CoV-2 and later by the results obtained with DCM, where lower toxicity (concentration of CC_{50}) and higher selectivity index against the virus (SI) tested the inactivation of the coronavirus in the presence of human sewage. The results showed a reduction of 4, 5, and 6 Log after 30, 45, and 60 min of exposure to human sewage, respectively. HEX extracts and especially DCM from the microalgae *P. nurekis* have shown to be promising for the purpose of exploring viral control, as a virucidal and antiviral agent, and these may be considered for new biotechnological products to be used in the clinical and environmental areas.

Keywords: *Planktochlorella nurekis*; SARS-CoV-2; Virucidal potential; MHV-3.

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LISTA DE ABREVIATURAS E SIGLAS

SLE	Solid-liquid extraction
SPE	Solid-phase extraction
SFE	Supercritical fluid extraction
UAE	Ultrasound-assisted extraction
MAE	Microwave-assisted extraction
PLE	Pressurized liquid extraction
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PFU	Plaque-formed units
CHIKV	Chikungunya virus
EMCV	Encephalomyocarditis
WSSV	White spot syndrome virus
RNA	Ribonucleic acid
ISA	Salmon anaemia virus
NDV	Newcastle virus
CDV	Canine Distemper Virus
HIV	Human immunodeficiency virus
HCV	Hepatitis C virus
HSV-2	Herpes simplex virus 2
JEV	Japanese encephalitis virus
GRFT	Griffithsin protein
IBV	Infectious bronchitis virus
MNV	Murine norovirus
HMPV	Human metapneumovirus
RSV	Respiratory syncytial virus
DENV-2	Dengue virus type 2
DENV-3	Dengue virus type 3
IRF-3	Interferon regulatory factor 3
Ca-SP	Called calcium spirulana
HCoV	Coronaviruses
PNDE	Dichloromethane extract of *Planktochlorella nurekis*

IFN-α	Mediated interferon-alfa
HAdV-5	Adenovírus humano tipo 5
HSV-1	Herpes simples tipo 1
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus-2
MHV	Murine
MHV-3	Mouse hepatitis virus strain 3
DMSO	Dimethyl sulphoxide
CC50	Cell viability by 50%
EC50	Half maximal effective concentration
L929	Fibroblast cells
MOI	Multiplicity of the assay
FDA	Food and Drug Administration
SI	Selectivity index
HCS	High Content Screening
NMR	Nuclear Magnetic Resonance
UPLC-MS	Ultraperformance liquid chromatography-mass
PCA	Principal component analysis
MERS-CoV	Middle Eastern coronavirus respiratory syndrome
QTOF	Quadrupole orthogonal acceleration time-of-flight
ESI	Electrospray ionization
ANOVA	Analysis of Variance
UV-VIS	Ultraviolet-visible spectroscopy
GAE	Gallic acid equivalents

SUMÁRIO

1 INTRODUÇÃO GERAL

As microalgas são organismos unicelulares aquáticos e fotossintetizantes autotróficos que utilizam a luz solar para fixar carbono em vários compostos orgânicos e, do ponto de vista ecológico, são muito importantes para o equilíbrio dos ecossistemas, contribuindo na produtividade primária do ambiente, na produção de oxigênio e na remoção de CO_2 da água. Estes microrganismos podem estar presentes em diferentes *habitats*, como regiões polares, hipersalinos, ácidos, alcalinos e de água doce. A adaptação à heterogeneidade de ambientes que estes seres habitam propiciam a este grupo uma rica diversidade de espécies com perfis bioquímicos diversos (SILVA, 2010).

A biomassa microalgal é composta majoritariamente por lipídios, proteínas e carboidratos, mas também são capazes de sintetizar pigmentos, como clorofilas e compostos carotenoídicos, além de uma gama de compostos secundários (SUTHERLAND; RALPH, 2021). Esta diversidade de compostos produzidos pelas microalgas são enfoque para a área da biotecnologia, mostrando-se atrativos frente a presença de bioativos com novas estruturas e combinações de grupos funcionais incomuns e diferentes dos encontrados em metabólitos de plantas terrestres (JHA; ZI-RONG, 2004). Os compostos bioativos de microalgas já foram relatados em diversas pesquisas com ações antimicrobiana, anti-inflamatória, antiviral, anticoagulante, anti-helmíntica, antiprotozoária, dentre outras ações farmacológicas (MAYER; HAMANN, 2005).

Por apresentarem esses compostos, estudos demonstram que as microalgas possuem atividade antiviral e virucida com aplicações clínicas, tais como, o exopolissacarídeo sulfatado p-KG03, produzido pela microalga *Gyrodinium impudicum*, que apresentou atividade antiviral frente o vírus da encefalomiocardite (YIM et al., 2003), dos polissacarídeos presentes em *Chlorella vulgaris* frente o vírus do herpes simples tipo 1 (HSV-1) (SANTOYO et al., 2010), o glicoglicerolipídio monogalactosil diacilglicerídeo isolado da microalga *Coccomyxa* sp. responsável pela atividade frente o norovírus murino e o vírus do herpes simples tipo 2 (HAYASHI et al., 2019; HAYASHI et al., 2022). Da mesma maneira, as microalgas apresentam eficácia em aplicação ambientais, como verificado por Michelon et al. (2022) ao testar a ficorremediação de águas residuais de produção de suínos com consórcio de microalgas, predominantemente com *Chlorella* spp., possibilitando a remoção de amônia-N e fosfato-P,

além de otimizar os metabólitos com atividade virucida frente o vírus envelopado e não envelopado.

No ano de 2020, a população global foi abruptamente lembrada que os vírus podem representar uma ameaça à saúde pública e devido à globalização, rapidamente se alastram por todos os continentes. Schmeller, Courchamp e Killen (2020) descrevem que epidemias como a H1N1 e a H5N1 ou pandemias como a SARS-CoV-2, podem ocorrer com ainda mais frequência e com consequências cada vez maiores. A família Coronaviridae, e.g., que compreende grandes vírus de RNA de fita simples e envelopados, podem provocar doenças leves a graves do trato respiratório em humanos, e como visto na pandemia de COVID-19 tornou-se urgente o desenvolvimento de novos antivirais e/ou virucidas para fins clínicos e ambientais (HEYDARI et al., 2021).

O SARS-CoV-2 até junho de 2022 causou 6,24 milhões de mortes e continua infectando pessoas diariamente (OUR WORLD IN DATA, 2022) por estar presente em todos os tipos de ambiente, como em água, esgoto, lodo, ar e em superfícies (CARRATURO et al., 2020). Assim como o SARS-CoV-2, outros patógenos virais infecciosos já foram detectados, e.g., em água utilizada para consumo humano mesmo após tratamento com cloração e em águas subterrâneas de poços profundos (FONGARO et al., 2015), o que reforça a necessidade de tratamentos eficazes para descontaminação da água nesses ambientes.

Nesse sentido, os extratos de microalgas possuem atividade antiviral e virucida, e por essa razão, avançar nessa área de estudo é de extrema importância. Dentre as algas pouco estudadas está a *Planktochlorella nurekis*, uma alga verde unicelular, autotrófica com células vegetativas esféricas e plantônicas, definida em um novo gênero e espécie pertencente ao clado Parachlorella em Chlorellaceae, Chlorophyta. Sua origem é mapeada em Nurek Dam, Tajiquistão, sendo isolada em 1977. As características principais dessa espécie são a taxa de sedimentação reduzida e a tolerância a temperaturas altas. Esse gênero difere-se dos demais da família pela ordem de nucleotídeos em suas sequências únicas do gene SSU e ITS 18S rRNA (ČERMÁK et al., 2015).

Estudo realizado por Potocki et al. (2021) avaliando extratos etanólico e aquoso da biomassa da espécie *P. nurekis* identificou inibição no crescimento das bactérias patogênicas *Enterococcus faecalis*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Escherichia coli* e o fungo *Candida* spp., o que demonstra sua eficiência na substituição de antibióticos e de produtos químicos nocivos ao meio ambiente, i.e,

organossolventes. Além disso, a microalga demonstrou potencial para tratamento antienvelhecimento da pele após melhoria do perfil bioquímico, com otimização no teor de lipídeos, pigmentos previamente selecionados e das vitaminas do complexo B (ADAMCZYK-GROCHALA et al., 2020). Pesquisa desenvolvida por Sasi et al. (2020) ao utilizar extrato de microalgas *P. nurekis* e *Chlamydomonas reinhardtii* a fim de tratar efluente coletado de uma indústria de papel e celulose, destacam que o extrato de *P. nurekis* exibiu maior capacidade de ficorremediação e de produção de lipídeos.

Em trabalho anterior realizado pelo nosso grupo de pesquisa, ao cultivar microalgas, predominantemente de *P. nurekis*, em um biorreator de escala piloto alimentado com águas residuais de suínos como extrato de crescimento, verificou-se que houve atividade virucida frente o vírus HSV-1 e adenovírus humano tipo 5 (HAdV-5). Os resultados apontaram que extratos de microalgas com diclorometano e metanol apresentaram atividade de inibição em concentrações mais baixas do que o verificado com hexano (MICHELON et al., 2022). Dessa maneira, a partir dos dados do grupo de pesquisa criou-se um projeto visando analisar o potencial biotecnológico da microalga *P. nurekis*, com foco na sua ação virucida frente ao vírus Sars-CoV-2.

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2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar o potencial virucida e antiviral de extratos da microalga *Planktochlorella nurekis* frente ao gênero *Coronavirus* (murino e humano) visando aplicação clínica e ambiental.

2.2 OBJETIVOS ESPECÍFICOS

- Realizar uma revisão bibliográfica acerca do potencial de algas com ação virucida, relatando os possíveis mecanismos de ação envolvidos no processo de inativação;
- Avaliar a citotoxicidade dos extratos de *P. nurekis* na linhagem celular L929;
- Analisar a atividade virucida dos extratos de *P. nurekis* em coronavírus murino;
- Caracterizar bioquimicamente os compostos presentes nos extratos de microalga *P. nurekis* obtidos com diclorometano, metanol e hexano;
- Testar a inativação de coronavírus murino em esgoto bruto não tratado com aplicação de extrato de diclorometano de *P. nurekis*;
- Avaliar a atividade virucida e antiviral dos extratos de *P. nurekis* em coronavírus, usando MHV-IV e SARS-CoV-2, respectivamente, visando aplicações ambientais e clínicas.

3 ORGANIZAÇÃO DA TESE DOUTORAL

A presente tese doutoral foi dividida em três capítulos. Todos os manuscritos foram escritos em inglês, visando a publicação em revistas conceituadas da área.

No primeiro artigo intitulado "Broad spectrum algae compounds against viruses: a review", buscou-se apresentar diferentes compostos algais com potencial para controle de vírus, visando a aplicabilidade nos setores fitoquímico, farmacêutico e sanitizante. Esse manuscrito foi publicado na revista Frontiers in Microbiology (FI: 6,064) em janeiro de 2022.

O segundo manuscrito "Characterization of *Planktochlorella nurekis* extracts and virucidal activity against a coronavirus model, the murine coronavirus" teve como objetivo caracterizar o perfil químico dos extratos de microalga *P. nurekis* obtidos pela extração com diclorometano, metanol e hexano, bem como avaliar sua atividade biológica frente a infecção *in vitro* do coronavírus murino (MHV-3). Esse manuscrito encontra-se publicado na revista International Journal of Environmental Research and Public Health (FI: 4,614).

No terceiro capítulo intitulado "Cytotoxic assay and acceleration of inactivation of murine coronavirus in human sewage by the application of *Planktochlorella nurekis* extract" buscou-se avaliar a citotoxicidade dos extratos de diclorometano, metanol e hexano de *P. nurekis* nas linhagens celulares L929 e Vero, a partir dos resultados obtidos, testou-se a inativação do MHV-3 no esgoto humano com uso do extrato de diclorometano de *P. nurekis.* O artigo será submetido à revista Journal of Medicine and Public Health, estando sob análise dos revisores.

Além dos artigos neste documento, foi realizado o pedido de patente intitulado "Produto para inativação viral obtido a partir de extrato de *Planktochlorella nurekis*" com número do processo BR 10 2021 024541 7 depositada no Instituto Nacional da Propriedade Industrial (INPI).

Ao participar do XXXI Congresso de Virologia em conjunto com XV Encontro de Virologia do Mercosul, no ano de 2020, foram apresentados os trabalhos "Accelerating coronavirus inactivation in human sewage by *Chlorella* spp. extract" e "Viral Brazil: Brazilian virology network focused on one heath approach" do grupo de do Laboratório de Virologia Aplicada da UFSC. Ademais, houve participação no capítulo "Emergência e vigilância do SARS-CoV-2" publicado em 2021 no Ebook "COVID-19 a pandemia do século: evidências

científicas relacionadas aos aspectos evolutivos, moleculares e patológicos" de organização de Fernanda Barbisan e Ivana Beatrice Mânica da Cruz e no artigo "Virucidal activity of microalgae extracts harvested during phycoremediation of swine wastewater" publicado na revista Environmental Science and Pollution Research em janeiro de 2022.

Ademais, durante o doutorado realizei o curso básico de boas práticas em cultura celular no ano de 2021 ofertado pela Universidade Federal de Santa Catarina e o curso Biossegurança em Foco na Fundação Oswaldo Cruz.

CAPÍTULO I

BROAD SPECTRUM ALGAE COMPOUNDS AGAINST VIRUSES: A REVIEW

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Broad Spectrum Algae Compounds Against Viruses

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The pharmaceutical industry is currently trying to develop new bioactive compounds to inactivate both enveloped and non-enveloped viruses for therapeutic purposes. Consequently, microalgal and macroalgal bioactive compounds are being explored by pharmaceutical, as well as biotechnology and food industries. In this review, we show how compounds produced by algae include important candidates for viral control applications. We discuss their mechanisms of action and activity against enveloped and non-enveloped viruses, including those causing infections by enteric, parenteral, and respiratory routes. Indeed, algal products have potential in human and animal medicine.

Keywords: virucidal, algae, antiviral, health, mechanisms

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INTRODUCTION

Many significant zoonotic pathogens are viruses and they have substantial infection-related impacts on public health worldwide. Non-enveloped enteric viruses are persistent in the environment (in water, soil and sewage, and on surfaces), resisting pH ranges of up to 3 to 9, temperature variations, and radiation (Evans, 1982; Taylor et al., 2001; Olival et al., 2017). Such viruses are a common cause of foodborne contamination (Carter, 2005; Elizondo-Gonzalez et al., 2012), which is responsible for 1.1 million hospitalizations and 218,000 deaths/year of children in countries with poor sanitary conditions (Patel et al., 2008). Enveloped viruses, with their environmental dissemination by air and aerosols, are responsible for pandemic events such as those involving Influenza and Coronavirus (Medina and García-Sastre, 2011; Chiu et al., 2012; Wang et al., 2018; Glass et al., 2020). Some enveloped viruses demonstrated high capability to cause epidemic episodes, such as Dengue virus on 2019 infecting 6,162,394 and causing death of 3,930 people. Ebola virus on Uganda/Congo region between 2018/2020, causing 3,453 infections and 2,273 deaths, other viruses as Nipah, Yellow fever and Zika caused epidemics through the worldwide (Agca et al., 2021; Anand et al., 2021; Yoon et al., 2021).

Enteric and respiratory viruses present enormous challenges both for human and animal medicine, and there is therefore a need for new antiviral solutions for fighting viruses with their worldwide economic and social consequences. Recent respiratory viral epidemics such as the worldwide outbreaks of swine flu, avian flu, and coronavirus has increased interest in the development of antiviral drugs (Prasse et al., 2010; Feng et al., 2018; Ye et al., 2020). Exploring natural compounds is an important approach to obtaining new virucides and antivirals. Macroalgal marine polysaccharides are potential candidates for human and animal medicine and have attracted

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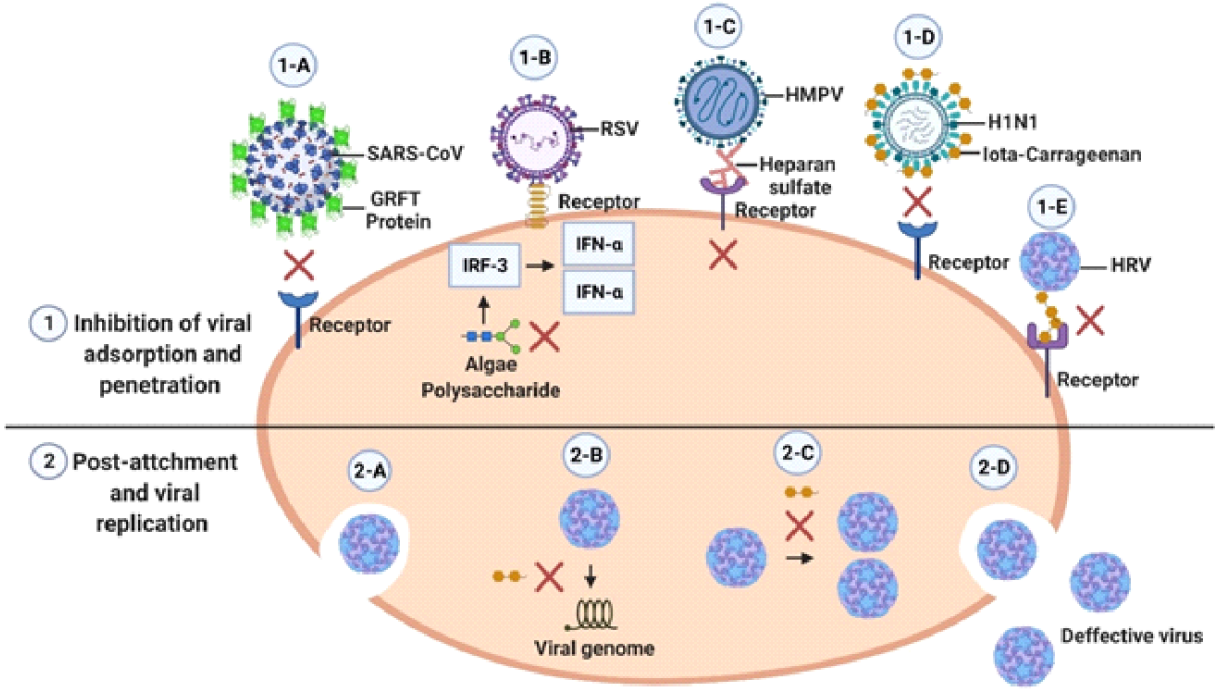
ABSTRACT

The pharmaceutical industry is currently trying to develop new bioactive compounds to inactivate both enveloped and non-enveloped viruses for therapeutic purposes. Consequently, microalgal and macroalgal bioactive compounds are being explored by pharmaceutical, as well as biotechnology and food industries. In this review, show how compounds produced by algae include important candidates for viral control applications. Discuss their mechanisms of action and activity against enveloped and non-enveloped viruses, including those causing infections by enteric, parenteral, and respiratory routes. Indeed, algal products have potential in human and animal medicine.

Keywords: virucidal, algae, antiviral, health, mechanisms.



1 INTRODUCTION

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Macroalgae are a phylogenetically artificial group of multicellular, macroscopic, eukaryotic, photoautotrophic organisms, mostly benthic (many being commonly known as seaweed), which are classified into three large groups: Chlorophyta (green algae), Rhodophyta (red algae) and Phaeophyceae (brown algae) (LEANDRO et al., 2019).

The components of macroalgae vary from simple to complex compounds, including polysaccharides (e.g. fucoidan, alginate, laminarin, carrageenan), phenolics and phlorotannins (e.g. flavonoids, lignans, tannins), protein and peptides (themselves made up of amino acids including leucine, glutamic acid, tryptophan), lipids, terpenoids and steroids (e.g. carotenoids), vitamins and minerals (SPOLAORE et al., 2006; ONOFREJOVÁ et al., 2010; BALBOA et al., 2013). Freshwater and marine microalgae contain compounds of high relevance to health, for example vitamins, proteins with essential amino acids, fatty acids, polysaccharides, minerals, enzymes, fibres and photosynthetic pigments such as carotenoids and chlorophylls (SILVA et al., 2018; MONTALVÃO et al., 2016). Various bioactive compounds from algae are attracting growing interest because of their antitumor, antiviral, anticoagulant, and antioxidant properties (WANG et al., 2012; WANG et al., 2018; BESEDNOVA et al., 2019).

Algae have a variety of broad-spectra activities against viruses and low cytotoxicity, both *in vitro* and *in vivo* (BESEDNOVA et. al, 2019). However, their potential is still underexplored by the pharmaceutical industry, and only 9% of medicines of natural origin come from algae (KUMAR JHA; ZI-RONG, 2004). Besides that, there are many *in vitro* studies proving the antiviral effects of algae, there have been few studies of their efficiency *in vivo* and in the environment. Indeed, there is an urgent need for further investigations of this type (ROSA et al., 2020).

This review presents selected compounds from algae as candidates for the control of viruses with applications in the phytochemical, pharmaceutical, and sanitizing sectors.

2 OBTAINING CRUDE AND FRACTIONATED EXTRACTS FOR ANTIVIRAL AND VIRUCIDAL ASSAYS FROM ALGAE

Bioactive compounds can be extracted from macroalgae or microalgae using various methods. The most common for extracting bioactive compounds from marine samples is solid-liquid extraction (SLE), involving solvents to extract soluble constituents from a solid or semisolid matrix. The downside of this method is its long extraction time and high solvent consumption (WANG et al., 2018; JACOBSEN et al., 2019). Other relevant techniques are solid-phase extraction (SPE), supercritical fluid extraction (SFE), ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), and pressurized liquid extraction

(PLE); SFE, UAE, and MAE are the most commonly used for macroalgae (KAUFMANN; CHRISTEN, 2002; CIKO et al., 2018).

Obtention of compounds of interest from marine macroalgae and microalgae involves the following major steps: (1) collection and taxonomic identification of samples; (2) drying; (3) extraction through the use of solvent; (4) filtration and concentration by evaporation; (FAYZUNNESSA et al., 2011; EL-BAZ et al., 2013; CHOI et al., 2014).

Cytotoxic, antivirus, and virucide assays and other cell culture techniques are used to screen candidates. Cytotoxic assays identify compounds that are toxic to healthy cells. A typical protocol is to cultivate an appropriate cell line in an Eagle medium at 30º with 5% of CO_2 in a layer in a 96-well plate and applying serial dilutions of compounds of interest. After 7 days of incubation, viable cells are revealed by using chemicals such as sulphorhodamine B and tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (CHACON et al., 1996; VICHAI, 2006).

Antiviral assays identify compounds that for example inhibit viral replication. Such assays generally involve placing viruses in contact with permissive cells and allowing the cycle of replication to begin. The antiviral compound being tested is then applied and successfully replicated viruses counted as plaque-formed units (PFU) after revelation by staining cells with crystal violet. Percentages of viral inhibition and of cells free of infection can then be calculated (ZHU et al., 2004).

Virucidal assays identify compounds that kill viruses, by for example, inactivating recognition proteins. Viruses are exposed to the sample under study and then a known quantity of virus is used to inoculate cells in 6-well plates; in some protocols, agarose is used to stabilize neighbouring cells to favour the transmission of viruses. Viral neutralization is quantified by counting PFU after viable cells are coloured with crystal violet (JOTHIKUMAR et al., 2005). This assay is important for evaluating compounds that may be able to inactivate viruses in the environment (VICTORIA et al., 2009; RIGOTTO et al., 2011; LANNA et al., 2019).

3 DISCUSSIONS

Algae and their extracts have numerous applications and have historically stimulated significant economic interest, mainly as a source of new drugs such as antivirals. therefore, report crude extracts and compounds isolated from algae that showed antiviral activity against

both enveloped and non-enveloped viruses. Some examples of these compounds and extracts from algae with antiviral activity are listed in Table 1.

Table 1 – Species and compounds from algae used as antivirals.

Species	Compounds	Applications	References
	Macroalgae		
-	Iota-carrageenan	HRV1A, HRV2, HRV8, HRV14, HRV16, HRV83, HRV84	GRASSAUER et al., 2008
Stypopodium zonale	Meroditerpene epitaondiol	HMPV	MENDES et al., 2011
Laminaria japonica	Sulphated polysaccharide fucoidan	H5N1, RSV	MAKARENKOVA et al., 2009; CAO et al., 2016
Chondrus armatus *Laminaria cichorioides,* *Laminaria japonica*	ι-carrageenans, fucoidan	Hantavirus	PAVLIGA et al., 2016
Griffithsia sp.	Protein griffithsin	SARS-CoV (Urbani strain), HIV, HCV, HSV-2, JEV, PEDV	O'KEEFE et al., 2010; O'KEEFE et al., 2009; MORI et al., 2005; TAKEBE et al., 2013; MEULEMAN et al., 2011; LEVENDOSKY et al., 2015; ISHAG et al., 2013;
Griffithsia sp.	Grifonin-1	HIV-1	MICEWICZ et al., 2010
Laurencia obtusa	Polysaccharide	HCV	GHEDA et al., 2016
Cladosiphon okamuranus	Fucoidan	DENV-2	HIDARI et al., 2008
-	λ-carrageenans, ι-carrageenans	DENV-2, DENV-3	TALARICO; DAMONTE, 2007;
-	Fucoidan	NDV	ELIZONDO-GONZALEZ et al., 2012
Cladosiphon okamuranus	Fucoidan	CDV	TREJO-AVILA et al., 2014
Eisenia bicyclis	Dieckol/phlorofucofuroeckol-A	FCV, murine norovirus	CHOI et al., 2014, EOM et al., 2015
Nicotiana benthamiana	Griffithsin/ Carrageenan	HSV-2/human papillomavirus	LEVENDOSKY et al., 2015

Schizymenia binderi	Sulphated galactan	HSV-1, HSV-2	MATSUHIRO et al., 2005
Gracilaria corticata	Sulphated galactan	HSV-1, HSV-2	MAZUMDER et al., 2002
Padina pavonica, Sargassum vulgare, Pterocladia capillace, Laurencia obtusa	Sulphated polysaccharides	HCV	GHEDA et al., 2016
	Microalgae		
Chlorella vulgaris	Polysaccharide	HSV-1	SANTOYO et al., 2010
Gyrodinium impudium	Sulphated polysaccharide p-KG03	IAV, EMCV	KIM et al., 2012; YIM et al., 2007
Spirulina platensis	Sulphoquinovosyl diacylglycerol,	Adenovirus 40-7, Coxsackievirus B4, Astrovirus type 1, Rotavirus Wa, HSV-1	ABDO et al., 2012 EL-BAZ et al., 2013;
Cochlodinium polykrikoides	Sulphated polysaccharides A1 and A2	Influenza A and B viruses, RSV-A, RSV-B	HASUI et al., 1995
Spirulina platensis	Calcium spirulan	HIV1, HIV2, HSV1, HSV2, HCMV, MuV, IAV	HAYASHI et al., 1996
Porphyridium cruentum	-	HH3, VV, ASFV, VHSV	FABREGAS et al., 1999
Chlorella autotrophica	-	VHSV, ASFV	FABREGAS et al., 1999

3.1 CRUDE EXTRACTS FROM ALGAE AGAINST VIRUSES

Red seaweed *Osmundaria obtusiloba* has been reported to have higher antiviral and virucidal effects against the Chikungunya virus (CHIKV) than ribavirin, which is used as a drug to control the virus (CIRNE-SANTOS et al., 2019). The λ- and ι-carrageenans obtained from *Osmundaria obtusiloba* have potent antiviral activity against dengue virus type 2 (DENV-2) and type 3 (DENV-3) (TALARICO; DAMONTE, 2007).

An ethanol extract from the microalgae *Spirulina platensis* has antiviral effects against Adenovirus type 40, a non-enveloped virus (ABDO et al., 2012). This virus causes

gastroenteritis and mortality especially in children. *S. platensis* also has antiviral effects against Adenovirus type 7, Astrovirus type 1, Coxsackievirus B4 and Rotavirus Wa strain (EL-BAZ et al., 2013), all of which cause gastroenteritis in humans.

Gyrodinium impudium is a marine microalga with antiviral effects against encephalomyocarditis (EMCV) non-enveloped viruses. EMCV infection causes death among pigs in production units, primates in research centres, and animals in zoos (KIM et al., 2012).

Extracts of some algae have been fed to some types of shrimp to reduce the impact of the white spot syndrome virus (WSSV); this macroalgae and microalgae diet appears to have improved innate immunity and increased the resistance of shrimp to infection by WSSV (CHOTIGEAT et al., 2004; IMMANUEL et al., 2012; SIVAGNANAVELMURUGAN et al., 2012; CHAROONNART et al., 2019). Seaweed extracts have also been used in fish diets and have shown promising antiviral effects against the salmon anaemia virus (ISA) and the enveloped RNA virus (LOZANO et al., 2016).

3.2 BIOCOMPOUNDS ISOLATED FROM ALGAE AGAINST VIRUSES

A wide variety of compounds obtained of micro and macroalgae has already been explored and tested against viruses and their cell infection capabilities.

Several studies of macroalgae compounds have shown promising antiviral effects against viruses that cause animal diseases causing serious economic losses. There are no effective treatments, either antivirals or vaccines, currently available against many of these diseases. A study in 2012 reported that the brown algae compound fucoid had an antiviral effect, albeit weak, against the Newcastle virus (NDV), which causes serious diseases in poultry and thereby substantial financial loss (ELIZONDO-GONZALEZ et al., 2012).

The first seaweed compound with antiviral activity against Canine Distemper Virus (CDV), a morbillivirus related to the measles virus that infects dogs and other carnivores (TREJO-AVILA et al., 2014), was reported in 2014. In the same year, another report showed the antiviral potential of brown algae products against norovirus infections using the Feline Calicivirus as a model (CHOI et al., 2014).

The Griffithsin protein (GRFT protein), produced by a red alga called *Griffithsin* sp., has activity against viruses such as the human immunodeficiency virus (HIV), hepatitis C virus (HCV), human papillomavirus, herpes simplex virus 2 (HSV-2) and the Japanese encephalitis

virus (JEV), the most frequent cause of viral encephalitis in Asia (ISHAG et al., 2013; TAKEBE et al., 2013). GRFT also shows antiviral activity against respiratory viruses including SARS-CoV (Urbani strain), coronaviruses (HCoV-NL63 group and HCoV-OC43 group) and infectious bronchitis virus (IBV) (MORI et al., 2005; O'KEEFE et al., 2009; O'KEEFE et al., 2010; MEULEMAN et al., 2011; ISHAG et al., 2013; TAKEBE et al., 2013; LEVENDOSKY et al., 2015; LUSVARGHI; BEWLEY, 2016). Studies with SARS-COV suggest that the GRFT also can reduce the overall viral load, and it acts by binding to the peak glycoprotein at the start of an infection and as an immunomodulator (O'KEEFE et al., 2009; O'KEEFE et al., 2010). The GRFT protein of red algae is also effective against the porcine diarrhoea virus (PEDV), which causes deaths and large economic losses in the swine industry (LI et al., 2019).

Human noroviruses cause gastroenteritis and phlorotannins from macroalgae have been reported to be active against murine norovirus (MNV), a model for human noroviruses (EOM et al., 2015).

The red seaweed compound carrageenan prevents the replication of Rhinoviruses (HRV1A, HRV2, HRV8, HRV14, HRV16, HRV83, and HRV84), which are more common causes of infection than any other respiratory virus. Carrageenan has also been reported to help improve symptoms of common cold and reduce high viral loads (GRASSAUER et al., 2008; ECCLES et al., 2010). The meroditerpene epitaondiol extracted from brown macroalgae has virucidal activity the human metapneumovirus (HMPV), another respiratory virus, and inhibits the penetration of viral particles into cells (MENDES et al., 2010).

Fucoidan and another polysaccharide from a brown seaweed have virucide activity against Influenza A (H5N1), Hantavirus and Respiratory syncytial virus (RSV), and also other non-respiratory viruses (DERYABIN et al., 2014; CAO et al., 2016; PAVLIGA et al., 2016).

A sulfated polysaccharide was isolated from the microalgae *Spirulina platensis* and called calcium spirulana (Ca-SP), this isolate showed antiviral activity against replication of several enveloped viruses, including Herpes simplex virus type 1, human cytomegalovirus, measles virus, mumps virus, influenza A virus, and HIV-1 (HAYASHI et al., 1996).

3.3 VIRAL ACTION MECHANISM FROM BIOACTIVE ALGAE

Viruses are obligatorily intracellular parasites; they need to invade cells and hijack cellular machinery to replicate. Enveloped viruses tend to fuse their membrane to the cell

membrane to release the genome into the cytoplasm of the host using cytoplasmic endosomes. Fusogenic peptides active at low pH facilitate access to cytoplasmic endosomes; thus, preventing pH lowering by molecules released by cells inhibits virion fusion. Non-enveloped viruses, such as enteroviruses, accumulate in endosomes which are highly acidic. Recognition depends on the activity of receptors on the surface of the cells, where the virus binds. Most enteroviruses bind α2β1 integrin and adenoviruses and coxsackieviruses use adenovirus and coxsackie receptors (BELGERSON et al., 1997; MARJOMÄKI et al., 2015). Viruses with an RNA genome initiate their translation and transcription in the cytoplasm, such that they are specific potential targets for viral inhibitors inside the cell. DNA viruses need to penetrate the nucleus to start the process of replication. During translation and transcription, there is an abundance of proteins and viral polymerases, which are also potential targets for inhibition. Non-enveloped viruses are assembled in the cytoplasm in general, and this is followed by cell lysis and thus release of infectious viral particles (LINNAKOSKI et al., 2018).

The antiviral mechanism of compounds obtained from algae is generally related to their specific structure and type of virus. Thus, each algae biomolecule may have a distinct mechanism to inactivate different types of viruses. Some studies suggest that one of the mechanisms of action involved in viral inactivation by algae is due to algal cells having a negatively charged surface which, interacting with the positive charge present in viruses or their cell surfaces, can prevent entry and cellular virus replication (BUCK et al., 2006; GRASSAUER et al., 2008; BRANYIKOVA et al., 2018; ECCLES, 2020). Furthermore, this antiviral mechanism that algae present is due to the synergistic effect that can occur with the combined use of different compounds present in these algae (ROSALES-MENDOZA et al., 2020). Some mechanisms of action of distinct and several compounds from algae with antiviral potential are described below.

Sulphated polysaccharides from seaweed have antiviral effects by acting at the beginning of the virus infection, interfering with virus adsorption and internalization (MAZUMBER et al., 2002; TALARICO et al., 2004; MATSUHIRO et al., 2005; WANG et al., 2012).

The antiviral action of fucoidan consists of blocking viral adsorption, inhibiting viral penetration and replication, and heavily suppressing virus-induced syncytium (PONCE et al., 2003; MANDAL et al., 2007; TREJO-AVILA et al., 2014).

Iota-carrageenan from red marine algae acts against infection by influenza virus through direct binding of the polymer to the viral particles, thereby preventing adsorption onto cell receptors and subsequent internalization, iota-carrageenan has a long chain of negatively charged molecules that attract and capture positively charged viruses and prevent them infecting cells (LEIBBRANT et al., 2010; ECCLES, 2020). Iota-carrageenan also can bind to the surface of the rhinovirus and causes inhibition of the virus's binding to cell receptors (GRASSAUER et al., 2008). Iota-carrageenan has an inhibitory effect also after the virus enters the cell, blocking mandatory conformational changes of rhinovirus, Iota-carrageenan acts on the occlusion of the virion surfaces involved in binding to the cellular proteins involved in the infectious process, which can prevent replication and generate defective viral particles (BUCK et al., 2006; GRASSAUER et al., 2008).

Carrageenan from red seaweed adsorbs Enterovirus 71 particles, consequently preventing the viruses from entering cells (CHIU et al., 2012). Both λ- and ι-carrageenans can effectively interfere with the adsorption and internalization of DENV when added at the same time as the virus or shortly after infection.

Griffithsin protein from the macroalga *Griffithsia* sp. has the ability to bind to specific oligosaccharides in the glycoproteins in the virus envelope and block viral entry, GRFT was active against SARS-CoV and HCoV-NL63 using protein-protein interactions for viral targeting, and for HCoV-OC43 and IBV-CoV utilize protein-carbohydrate interactions for viral attachment (O'KEEFE et al. 2010).

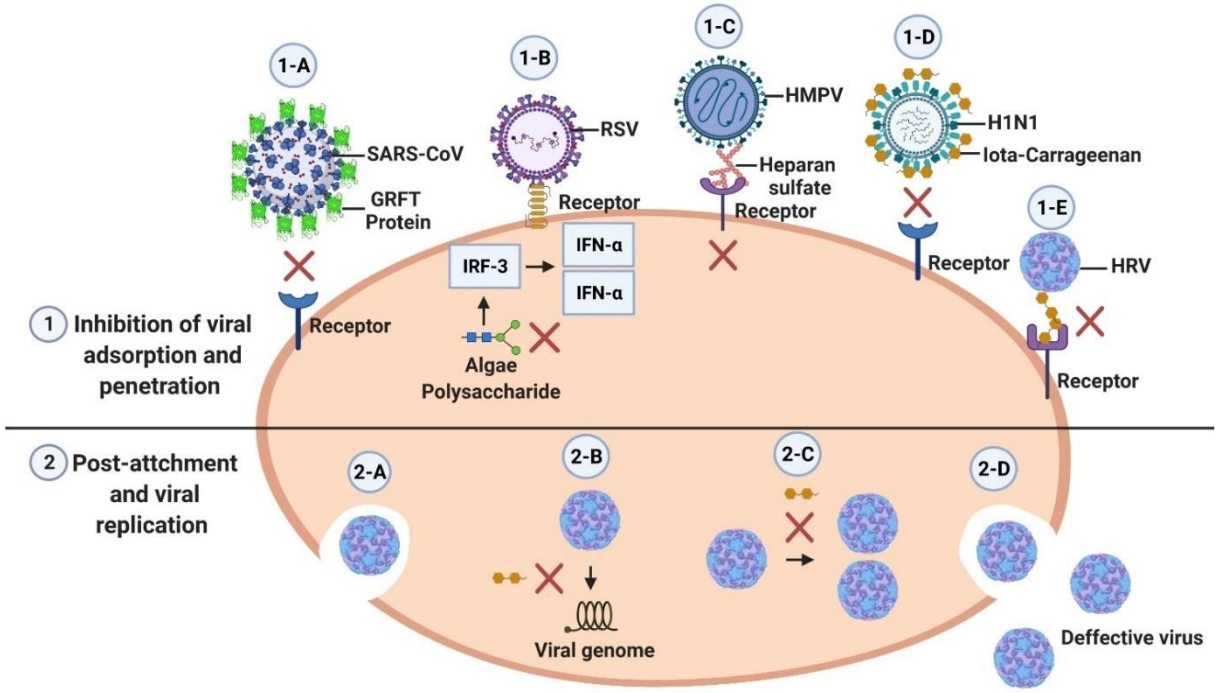
An extract from the brown algae *Laminaria japonica* has a polysaccharide that efficiently inhibits RSV replication, and the mechanism of action depends on interferon regulatory factor 3 (IRF-3)-mediated interferon-alfa (IFN-α) secretion (CAO et al., 2016).

The antiviral effects of macroalgae extracts against the HMPV virus involve interaction with the viral particles outside the cells and thereby preventing infection. Meroditerpenoids of this species have both virucidal effects and the capacity to inhibit the penetration of viral particles into cells. The binding of HMPV to heparan sulfate involves charge-charge, heparan sulfate blocks the binding of HMPV to the receptor and this occlusion inhibits infection of cells (KLIMYTE et al., 2016).

Many groups who have demonstrated that compounds and extracts from macroalgae have antiviral effects report the need for studies to elucidate the mechanisms of action of the

bioactive compounds (CHOI et al., 2014; EOM et al., 2015). Figure 1 illustrates various mechanisms of antiviral activity of compounds derived from algae.

Figure 1 – The mechanisms of the action of natural compounds can be divided into two phases: before and after viral entry.



1-A: GRFT Protein from the *Griffithsia* sp. macroalgae binds to specific oligosaccharides in the virus envelope glycoproteins and block viral entry (O'KEEFE et al., 2010). 1-B: Polysaccharides from *Laminaria japonica* enhance the expression level of IRF3 and the secretion of IFN alpha that results an antiviral activity against RSV. 1-C: The binding of HMPV to heparan sulphate involves charge-charge interactions; this blocks the binding of HMPV to the receptor and consequently inhibits the infection of cells (KLIMYTE et al., 2016).1-D: Iota-carrageenan from red algae has a long chain of negatively charged molecules that attract and capture positively charged viruses and prevent them from infecting cells (LEIBBRANT et al., 2010; RONALD ECCLES, 2020). 1-E: Iota-carrageenan binds to the surface of rhinovirus and inhibits virus binding to cell receptors. (GRASSAUER et al., 2008). 2-A: Viral entry. 2-B Iota-carrageenan also has an inhibitory effect after the virus enters the cell, blocking the conformational changes of rhinovirus necessary for infection (2-B: uncoating and 2-C: replication) Iota-carrageenan acts occludes virion surfaces involved in binding to cellular proteins required for the infectious process; this prevents replication and results in the viral particles produced being defective (BUCK et al., 2006; GRASSAUER et al., 2008). 2-D: Exit of defective viral particles.

3.4 ANTIVIRAL DRUGS FROM ALGAE AS ALTERNATIVE TO SYNTHETIC DRUGS

Algae are a natural source of compounds with antiviral properties, have proven efficiency against enveloped and non-enveloped viruses, this compounds and extracts from algae have inexpensive to obtain, especially those of marine origin (ALAM et al., 2021). Furthermore, algae are an alternative resource to synthetic drugs, because algae have very low

toxicity and some are non-toxic at doses that have a broad antiviral spectrum against several viruses and minimal side effects (BESEDNOVA et al., 2021). Among the benefits of algae, can still mention that due to the diversity of molecules and their mechanisms of action, it inactivates viruses and block their action without causing resistance or selection of these organisms (HAMED et al., 2015).

4 CONCLUSIONS

Various compounds from algae have potent activities against viruses and are strong candidates for the control and treatment of viruses that affect humans and animals. These bioactive compounds should be further explored for health applications, both in clinicals and the environmental. They are also promising for use as low toxicity sanitizers of high virucidal capacity. More studies are required both for prospecting algae for active molecules and for the development of products suitable for applications in viral control.

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CAPÍTULO II

CHARACTERIZATION OF *PLANKTOCHLORELLA NUREKIS* EXTRACTS AND VIRUCIDAL ACTIVITY AGAINST A CORONAVIRUS MODEL, THE MURINE CORONAVIRUS 3

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Characterization of *Planktochlorella nurekis* Extracts and Virucidal Activity against a Coronavirus Model, the Murine Coronavirus 3

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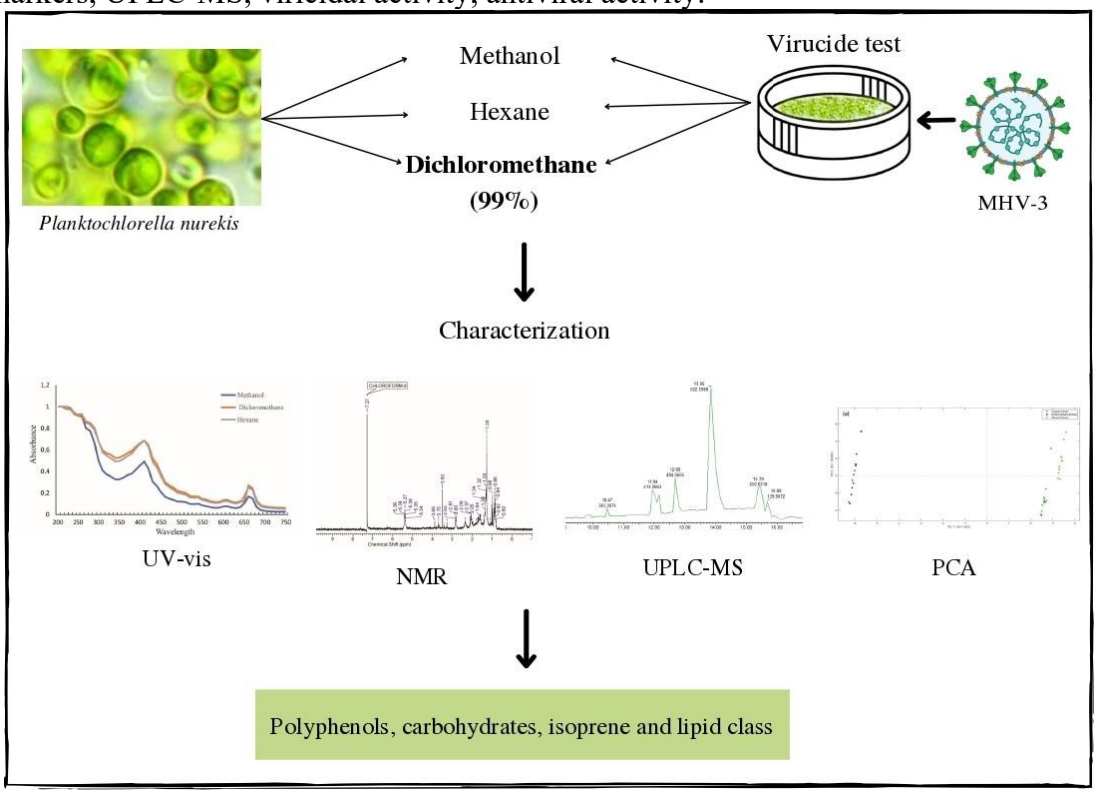
ABSTRACT

Certain members of the Coronaviridae family have emerged as zoonotic agents and have recently caused severe respiratory diseases in humans and animals, such as SARS, MERS, and, more recently, COVID-19. Antivirals (drugs and antiseptics) capable of controlling viruses at the site of infection are scarce. Microalgae from the Chlorellaceae family are sources of bioactive compounds with antioxidant, antiviral, and antitumor activity. In the present study, aimed to evaluate various extracts from *Planktochlorella nurekis in vitro* against murine coronavirus-3 (MHV-3), which is an essential human coronavirus surrogate for laboratory assays. Methanol, hexane, and di-chloromethane extracts of *P. nurekis* were tested in cells infected with MHV-3, and characterized by UV-vis spectrophotometry, nuclear magnetic resonance (NMR) spectroscopy, ultraperformance liquid chromatography-mass spectrometry (UPLC-MS), and the application of chemometrics through principal component analysis (PCA). All the extracts were highly efficient against MHV-3 (more than a 6 Log unit reduction), regardless of the solvent used or the concentration of the extract, but the dichloromethane extract was the most effective. Chemical characterization by spectrophotometry and NMR, with the aid of statistical analysis, showed that polyphenols, carbohydrates, and isoprene derivatives, such as terpenes and carotenoids have a more significant impact on the virucidal potential. Compounds identified by UPLC-MS were mainly lipids and only found in the dichloromethane extract. These results open new biotechnological possibilities to explore the biomass of *P. nurekis*; it is a natural extract and shows low cytotoxicity and an excellent antiviral effect, with low production costs, highlighting a promising potential for development and implementation of therapies against coronaviruses, such as SARS-CoV-2.

Keywords: coronavirus, SARS-CoV-2, murine coronavirus, metabolomic approach, NMR biomarkers, UPLC-MS, viricidal activity, antiviral activity.



1 INTRODUCTION

Coronaviruses (CoVs, subfamily Coronavirinae, order Nidovirales) are enveloped and positive-strand RNA viruses. The CoV virion has a triple protein membrane, containing spike proteins on the surface, responsible for binding of the virus to its receptor and cell entry (BÁRCENA et al., 2009; PEIRIS, 2012; SHARMA et al., 2020). CoVs are human and animal pathogens that cause respiratory, hepatic, and neurological problems (DENIS et al., 2020; JIANG et al., 2020) and have been responsible for epidemics and pandemics, such as severe acute coronavirus respiratory syndrome (SARS-CoV) in 2003, Middle Eastern coronavirus respiratory syndrome (MERS-CoV) in 2012, and SARS-CoV-2 coronavirus disease (COVID-19) in 2019 (JIANG et al., 2020; LEE; HSUEH, 2020).

The Murine coronavirus (mouse hepatitis virus, MHV) has been used as a safe model (it does not infect humans) for SARS-CoV and SARS-CoV-2 because of the structural and genomic similarities between them. MHV causes various types of infection in mice: respiratory, hepatic, enteric, and neurological. Thus, the murine coronavirus has recently be-come widely used as a surrogate model for SARS-CoV-2 in prevention strategies, vaccine development, and new treatment methodologies (GONZALEZ et al., 2003; ZÜST et al., 2007; KÖRNER et al., 2020).

Algae have been used to develop new medicines and treat viruses for many years (BESEDNOVA et al., 2021). Certain species, such as the microalga Spirulina platensis, have demonstrated an anti-viral effect against adenovirus type 40 non-enveloped viruses (ABDO et al., 2012) and proven antiviral effects against adenovirus type 7, adenovirus type 40, astrovirus type 1, coxsackievirus B4, and the rotavirus Wa strain, suggesting a strong potential of algae extracts as antivirals (KIM et al., 2012). Several compounds obtained from microalgae, such as sulphated polysaccharides against influenza A (YIM et al., 2007) and EMCV (SHIH et al., 2003) and phycobiliprotein against enterovirus 71 (O'KEEFE et al., 2010), have already been reported. Other studies have shown algae compounds to have much potential in therapeutic and prophylactic approaches against the group of viruses to which MHV-3 and SARS-CoV belong (EL-BAZ et al., 2013; ROSALES-MENDOZA et al., 2020). The antiviral activity of macroalgae extracts against members of the Coronaviridae family has been already reported, showing the ability to inactivate the virus by binding to the SARS-CoV spike glycoprotein and thus inhibiting viral entry into the cell, with minimal toxicity (ROSALES-MENDOZA et al.,

2020). In addition to macroalgae, studies with microalgae have also reported an antiviral effect against respiratory viruses, such as adenovirus type 40 (ABDO et al., 2012), adenovirus type 7 and adenovirus type 40 (KIM et al., 2012), influenza A (IAV), an enveloped virus (YIM et al., 2007), and influenza A and B viruses, RSV-A, and RSV-B (HASUI et al., 1995).

Chlorellaceae is a family of unicellular green freshwater or marine microalgae commonly studied for a wide range of components that have antioxidant, antibacterial, anti-viral, and antitumor potential (KATHARIOS et al., 2005; SANTOYO et al., 2010; WANG; ZHANG, 2013). The compounds formed by the unique metabolic pathways of microalgae arise from the processes that allow them to adapt to an environment in which they are subjected to a broad spectrum of stresses, such as variations in temperature, humidity, salinity, pressure, the availability of oxygen, and UV. These biotic and abiotic factors lead to differentiation in the production of compounds, such as carotenoids, fatty acids, proteins, and polysaccharides, which may vary between microalgae species and may also vary within the same community during the year depending on seasonal variation (CONNAN; STENGEL, 2011). *Planktochlorella nurekis* is a coccoid shaped planktonic uninuclear organism consisting of vegetative cells with pot-shaped chloroplasts that contain pyrenoids and a cell wall composed of two layers (ŠKALOUD et al., 2014).

Aimed to characterize the chemical profile of the dichloromethane, methanol, and hexane extracts obtained from *P. nurekis* and evaluate their biological activity against murine coronavirus (MHV-3) infection *in vitro*.

2 MATERIAL AND METHODS

2.1 OBTENTION OF THE MICROALGAE BIOMASS

The microalgae used as inoculum was obtained directly from a facultative open pond used as tertiary treatment process to remove nutrients from a previously digested swine wastewater effluent. *P. nurekis* obtained from swine wastewater was grown in 10 L photobioreactors exposed to light (99 μmol m^{-2} s^{-1}) under mixotrophic conditions (12 h:12 h, light:dark) with continuous agitation using a mechanical recirculation pump 1200 $L.h^{-1}$ (Sarlobetter) at room temperature (23 °C). The photobioreactors were operated in a fed-batch mode using effluents from an anaerobic treatment system, which was diluted by adding 1.0 L

to 6.0 L of chlorine-free tap water. The photobioreactors were inoculated with 70 ± 0.6 mg DW microalgae L^{-1}. After 11 days, the biomass reached a fresh weight of 0.4 ± 0.1 g L^{-1}. This biomass was then harvested by centrifugation at 3000× g, frozen immediately (−20 °C), and then lyophilized for further analysis and assays. In total, 20 g of *P. nurekis* biomass was exhaustively extracted with hexane, dichloromethane, and methanol at a 1:5 ratio (w:v; g:mL). The extracts were dried using a rotary evaporator maintained under vacuum at 50 °C and then stored at −20 °C).

2.2 CHEMICAL CHARACTERIZATION OF *PLANKTOCHLORELLA NUREKIS* EXTRACTS

2.2.1 Ultraviolet-Visible Spectroscopy (UV-VIS) Profile

Spectrophotometric assays were carried out using a SpectraMax 190 Microplate Reader (96-well flat bottom cell culture plate – Kasvi®). The dichloromethane, methanol, and hexane extracts were eluted four times and the absorbance spectra of the samples was measured at a range of wavelengths from 200 to 750 nm. The UV-vis scanning spectra (n = 3) were pre-processed using baseline correction, scatter correction, and standardization.

2.2.2 Nuclear Magnetic Resonance Spectroscopy (NMR)

The three extracts were individually dried in a rotary evaporator and the drying completed by lyophilization for 24 h in a Labconco Freezone 4.5 plus instrument. The methanol sample was then suspended in 500 µL methanol-d4 and the dichloromethane and hexane extracts directly in $CDCl_3$. The NMR experiments were performed on a Fourier 300 Bruker® 9.4 Tesla (300 MHz for hydrogen frequency and 75.48 MHz for carbon frequency) spectrometer equipped with a 5 mm internal diameter BBI probe, with reverse detection and field gradient coils in the coordinate. The chemical shifts were determined relative to TMS as the internal standard and are expressed as δ values (ppm), with coupling constants reported in Hz. The spectra were acquired for the $CDCl_3$ solutions using 5 mm quartz tubes at 303 K. For all analyses, 64 scans were used. NMR data acquisition and processing were performed using Bruker TopSpin™ software.

2.2.3 Total Phenolic Content

One hundred mg of the three extracts were suspended and homogenized in 400 µL ethanol and 100 µL transferred to test tubes containing 75 µL Folin–Ciocalteu reagent. Subsequently, 875 µL of a 2% sodium carbonate solution (w/v) was added. The suspensions were vortexed for 1 min and the samples incubated for 1 h at room temperature in the dark. Then, the mixtures were transferred to the wells of a microplate and the absorbance at 760 nm measured using a SpectraMax 190 Microplate Reader. The stock standard solution for the calibration curve was prepared by appropriate dilution of the compound with methanol to provide a concentration range of 100 to 2500 µg/mL for gallic acid (y = 0.0005x + 0.023, R2 = 0.958). Results are expressed as mg/g of gallic acid equivalents (GAEs) (SAFAFAR et al., 2015).

2.2.4 β-Carotene Content

The extracts of *P. nurekis* were suspended in 400 µL dichloromethane, vortexed for 1 min, and quantified using a SpectraMax 190 Microplate Reader at 470 nm. The stock standard solution was prepared by dilution of β-carotene with dichloromethane to provide a concentration range of 100 to 2500 µg/mL (y = 0.008x + 0.570, R^2 = 0.980). Results are expressed as micrograms per gram of fresh weight of the sample (AONO et al., 2021).

2.2.5 Amino Acids Characterization

Ten grams of *P. nurekis* biomass was subjected to acid hydrolysis with 100 mL 3N HC_l for 24 h. Subsequently, the extract was filtered, rinsed with water, and centrifuged at 3000 rpm for 10 min until the removal of excess HC_l. The filtered samples were lyophilized and 1 mL of the acid hydrolysate and 3 mL of a 0.2% w/v ninhydrin solution in 200 nM sodium phosphate buffer at pH 7.0 were transferred into an assay tube and the samples heated in a water bath for 30 min at 100 °C. Finally, the absorbance of the samples was measured at 570 nm with a spectrophotometer SpectraMax 190 Microplate Reader. The amino-acid concentration was predicted using calibration curves prepared with standard amino-acid solutions; curves were

made to determine the concentration of cysteine, phenylalanine, histidine, isoleucine, proline, serine, threonine, tryptophan, and valine (RAJESHWARI; RAJASHEKHAR, 2011).

2.2.6 Qualitative Analysis of the Extracts by Ultraperformance Liquid Chromatography-Mass Spectrometry (UPLC-MS)

Acetonitrile (ACN), water (LCMS grade, Sigma-Aldrich, St. Louis, MI, USA), and 85% p.a. grade formic acid (Vetec Química Fina, Rio de Janeiro, Brazil) were used for the (UPLC-ESI-MS) analyses. All solutions prepared for the UPLC analyses were filtered through a 0.22-µm hydrophobic membrane made of cellulose. Chromatographic analyses were performed on an Acquity H-Class UPLC-PDA system (Waters Co., Milford, AS, USA). An Acquity UPLC BEH C18 (50 × 2.1 mm i.d., 1.7 µm) column (Waters Co., USA) was used for the analysis. The column was maintained at 40 °C during the analyses. MS data were obtained using a quadrupole orthogonal acceleration time-of-flight (QTOF) mass spectrometer, Xevo GS-2 QTof, with an electrospray ionization (ESI) source, operating in both positive and negative modes, with the mass range between 100 and 1200 Da and a scan time of 1 s. The mobile phase system consisted of a gradient of 0.1% aqueous formic acid (pH 3.0) (A) and ACN (B) at a flow rate of 0.3 mL/min. The gradient consisted of 0–2 min, 90% A/10% B; 2–10 min, 55% A/45% B; 10–15 min, 10% A/90% B; 15–20 min, 90% A/10% B. The injection volume was 2 µL. The instrument settings in positive mode were a capillary voltage of 3.0 kV, sampling cone voltage of 40 V, source offset voltage of 80 V, desolvation temperature of 200 °C, source temperature of 80 °C, cone gas flow of 50 L/h, and desolvation gas flow of 500 L/h. Nitrogen was used as the nebulizer gas and argon as the collision gas. MS and MSE data (in two scan functions) were acquired in the centroid mode and monitored with a scan time of 1 s. The collision energy was 6 eV in function 1 and ramped from 25 to 35 eV in function 2. To assure accurate mass values, data were corrected during acquisition by an external reference (LockSpray^{TM}) named leu-cine-enkephalin solution (1 ng/mL) at a flow rate of 20 µL/min. System control and data processing were performed using MassLynx 4.1 software (Waters Co., USA). All samples were prepared by dissolution of each extract in water: acetonitrile (9:1, v/v) to reach a concentration of 800 µg/mL.

2.3 CYTOTOXICITY ASSAY

Mouse fibroblast cells (L929, ATCC® CCL^{-1}) were maintained in Minimum Essential Medium (MEM; Thermo Fisher Scientific, Warsaw, Poland) supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Poland), seeded into plates (96-well plate format, 2.5 × 104 cells/well), and maintained for 24 h at 37 °C in an atmosphere containing 5% CO_2. Cells were exposed to various concentrations of the samples (48 µg/mL to 500 µg/mL of *P. nurekis* hexane, dichloromethane, and methane extracts) for 48 h under the same conditions of cell culture. Cell viability was assessed by the sulforhodamine B assay, which measures total protein mass (VICHAI; KIRTIKARA, 2006). The percentage of viable cells was plotted against each sample concentration. The CC_{50} values (the concentration of each extract that reduced cell viability by 50%) were calculated based on concentration-response curves using GraphPad Prism 8.0 (Graph Pad Software 8.0.0 version, La Jolla, CA, USA).

2.4 VIRUCIDAL ASSAY

This assay followed EN 14476:2013+A2:2019 for the evaluation of virucidal activity. Infectious virus titres were measured in plaque-forming units (PFU) as described by EN 14476:2013+A2:2019. Various amounts of virus (10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 PFU) were incubated with various dilutions of extracts of *P. nurekis* ranging from 0 to 50 µg/mL (1:2 serial dilutions) for 15 min at 37 °C or 24 °C. Then, the reaction was blocked with 10% fetal bovine serum, and 400 µL of each test dilution added to L929 cells previously prepared in 24-well plates. After 1 h of contact with the cells, the inoculum was removed and the cell monolayer washed with phosphate-buffered saline. The cell cultures were then covered with MEM containing 1.5% carboxymethylcellulose (CMC; Sigma Chemical Co., St Louis, MO, USA) and incubated for 72 h at 37 °C in an atmosphere containing 5% CO_2. The re-duction of the virus was determined by the number of PFU relative to that of the untreated viral controls.

2.5 STATISTICAL ANALYSIS

The average and standard deviation were determined for each variable. The metabolite dataset and virus reduction were analyzed by analysis of variance (ANOVA) and the post hoc

Tukey test. Principal component analysis (PCA) was also performed to investigate sample grouping and similarities and to identify the variables that most strongly influenced the classification of the *P. nurekis* extracts. Algorithms for the statistical analysis were developed using the MATLAB program (version 7.12.0.635) (POMBAL et al., 2017).

3 RESULTS AND DISCUSSION

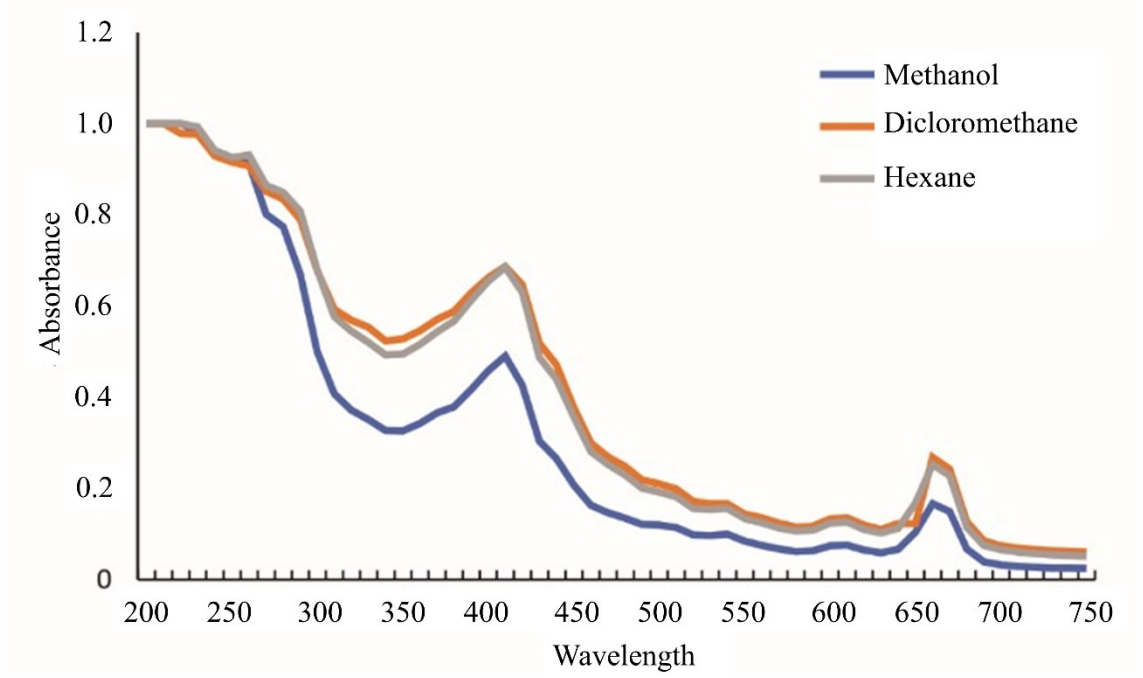
3.1 CHEMICAL CHARACTERIZATION OF *PLANKTOCHLORELLA NUREKIS* EXTRACTS

3.1.1 UV-VIS Profile

The extract of *P. nurekis* showed UV-VIS absorption profiles similar to those of other members of the taxonomic group following the same typical green algae extraction procedures (TEKINER et al., 2018). The absorbance profiles showed different peak intensities for the three organic solvent extracts (Figure 2). The extracts were analyzed by UV-VIS spectroscopy over a range of 200 to 750 nm. No peaks were identified in the UV region, but rather a plateau between 240–260 nm, with a lower intensity peak at 280 nm. Maximum absorbance in the PAR region was detected at wavelengths of 410 nm and 660 nm.

The UV-VIS profile was used to compare the solvents of different polarity to identify possible differences in the spectral pattern. Despite the dichloromethane spectrum showing the highest intensities, it was impossible to identify distinct peaks between the extracts. In the UV spectral range, there was a band, with the absorbance peaks forming a plateau. In the PAR region, the extracts exhibited two significant absorption peaks at 460 nm and 660 nm, indicating the presence of carotenoids and chlorophyll compounds (LICHTENTHALER; BUSCHMANN, 2001; TEKINER et al., 2018; SCHMITZ et al., 2019).

Figure 2 – Spectral profiles of UV-Vis (λ = 200–750 nm) absorbances of methanol, dichloromethane, and hexane extracts (n = 3).



3.1.2 β-Carotene, Phenols, and Amino Acids Content

The results of the tests for β-carotene, phenols, and amino acids of the *P. nurekis* extracts are presented in Table 2. The dichloromethane extracts showed a higher concentration of carotenoids (16.6 µg/g) than the hexane (10.1 µg/g) and methanol (7.14 µg/g) extracts. The polarity of the solvent had a positive effect on the concentration of total polyphenols. The most polar solvent, methanol, extracted more than twice the amount than the most nonpolar solvent, hexane. The concentration of polyphenols in the methanol, dichloromethane, and hexane extracts was 84 mg/g, 40 mg/g, and 29 mg/g, respectively. The amino acid that showed the greatest difference between the solvents was valine, with a concentration of 119 mg/g in the hexane extracts, which dropped to 54 mg/g in methanol. The other quantified amino acids did not show significant differences between the solvents. In a previous study using *P. nurekis*, the most abundant amino acids found were glutamic acid (56 mg/g), aspartic acid (50 mg/g), alanine (45 mg/g), leucine (45 mg/g), valine (32 mg/g), and arginine (30 mg/g) (SZPYRKA et al., 2020). However, quantified amino acids were not an appropriate biochemical marker in this study because our data were very different from the pattern found in the literature, a factor that

may have resulted from the culturing of this microalgae in swine wastewater, as it contains a high concentration of essential components that promote algal growth, such as carbon, nitrogen, and phosphorus (KUMAR et al., 2018).

Quantitative data on carotenoids showed a tendency towards greater extraction of these pigments by dichloromethane. This effect is influenced by several variables, such as the solubility of the molecules in the extractor solvent, the polar character of the extracted molecules and the permeability of the cell membrane, and the transport of this analyte in the cytoplasm (HEJAZI; KLEINEGRIS; WIJFFELS, 2004). The prominent carotenoids are lutein (polar) and β-carotene (relatively nonpolar) (DEL CAMPO et al., 2004). The solubility of these two carotenoids is higher in dichloromethane, which explains the tendency towards greater extraction of these analytes by this solvent (CRAFT; SOARES, 1992). Lutein showed low solubility in hexane (approximately 20 mg/L) and β-carotene low solubility in methanol (approximately 10 mg/g). Thus, a solvent of intermediate polarity that solubilizes the molecules is best suited for this purpose.

Table 2 – Cytotoxic evaluation of hexane, dichloromethane, and methanol extracts of *Planktochlorella nurekis*.

Biomarkers	Dichloromethane	Hexane	Methanol
Carotenoids (µg/g)	16.65 ± 3.04 [a]	10.10 ± 0.91 [b]	7.14 ± 0.49 [c]
Polyphenols (mg/g)	40.96 ± 3.58 [a]	29.17 ± 3.20 [b]	84.91 ± 4.13 [c]
Cysteine (mg/g)	15.94 ± 0.89 [a]	16.21 ± 0.68 [a]	15.67 ± 0.88 [a]
Phenylalanine (mg/g)	30.06 ± 1.54 [a]	30.52 ± 1.50 [a]	29.59 ± 1.51 [a]
Histidine (mg/g)	14.63 ± 1.23 [a]	15.00 ± 0.94 [a]	14.25 ± 1.21 [a]
Isoleucine (mg/g)	164.54 ± 9.80 [a]	167.48 ± 7.46 [a]	161.58 ± 9.64 [a]
Leucine (mg/g)	9.18 ± 2.05 [a]	9.79 ± 1.56 [a]	8.56 ± 2.02 [a]
Proline (mg/g)	31.57 ± 3.08 [a]	32.49 ± 2.34 [a]	30.63 ± 3.03 [a]
Serine (mg/g)	73.13 ± 6.15 [a]	74.98 ± 6.15 [a]	71.27 ± 6.05 [a]
Threonine (mg/g)	99.13 ± 6.15 [a]	100.98 ± 6.15 [a]	97.27 ± 6.05 [a]
Tryptophan (mg/g)	44.24 ± 2.05 [a]	44.86 ± 1.56 [a]	43.62 ± 2.02 [a]
Valine (mg/g)	94.56 ± 6.94 [a]	119.40 ± 5.29 [b]	54.96 ± 6.83 [c]

Values with similar letters do not present significant differences according to Tukey's test ($p < 0.05$).

3.1.3 NMR Profile

Results of the NMR analysis are presented in Supplementary Material Figure S1 and the identified metabolites are listed in Table 3. The NMR spectra shown here are the first such results for the microalgae *P. nurekis* to be reported in the literature. The data were compared

with previous reports in the literature and online databases, such as the Human Metabolome Database (HMDB), the Biological Magnetic Resonance (BMR) database, and PubChem.

Table 3 – Concentration of the *Planktochlorella nurekis* bioactives in NMR.

Compound Name	**Concentration (µM)**	
	Hexane Extract	**Dichloromethane extract**
2-Hydroxybutyrate	0.3	11.1
3-Hydroxyisobutyrate	4.2	15.2
Acetic acid	0.2	0.6
Citric acid	0.8	10.2
Ethanol	0.7	0.7
Glycerol	0.1	N/A
Formic acid	0.9	11.4
L-Glutamic acid	1.0	1.1
Hypoxanthine	0.6	4.0
L-Tyrosine	0.1	1.1
L-Alanine	4.3	N/A
L-Threonine	1.6	12.1
L-Lactic acid	1.1	12.8
L-Aspartic acid	1.5	8.5
Pyruvic acid	N/A	1.2
Succinic acid	N/A	12.2
Pyroglutamic acid	N/A	6.7
3-Hydroxybutyric acid	0.6	2.0
Creatinine	3.7	N/A
L-Glutamine	N/A	13.3
L-Leucine	0.7	7.5
L-Methionine	N/A	1.9
L-Valine	9.6	14.3
Acetone	30.3	100.2
Isobutyric acid	19.0	17.8
1,5-Anhydrosorbitol	1.0	0.1
Dimethylsulfone	0.2	1.0
EDTA	0.1	N/A

The NMR profile showed the dichloromethane extract to have the highest metabolite concentration, followed by the hexane extract; the number of peaks in methanol was lower than for other solvents and were also less intense. The largest peaks in the methanol extract were located at 1.23 ppm and 3.16 ppm, which have been linked to fatty acids, such as linolenic acid. The other distinctive peaks in the sample, such as those located between 0.83 ppm and 0.95 ppm, indicate the presence of palmitic acid and isoleucine. Other peaks found at 1.90 ppm and 5.32 ppm confirm that the *P. nurekis* methanol extracts contained a significant amount of canthaxanthin and linoleic acid. However, these peaks were small relative to the those of the other main metabolites in the sample, confirming that the samples contained only low concentrations of these compounds (PANTAMI et al., 2020). The metabolites of the extract of *P. nurekis* in methanol were not quantified because they were below the limit of detection (LOD).

Both the dichloromethane and hexane extracts shared peaks at several frequencies, such as those found at 0.98 ppm, which signals the presence of vitamin B5, 1.26 ppm, which belongs to zeaxanthin, 3.50 ppm, which is associated with valine and violaxanthin, and 5.37 ppm, indicating common metabolite or bioactive groups found in the two extracts. The hexane sample had a distinctive peak at 2.32 ppm, which signals the presence of vitamin B2; whereas the dichloromethane extract had distinct peaks between 1.64 ppm, indicating the presence of lutein, 2.05 ppm, associated with the allylic protons on unsaturated fatty acids, 2.81 ppm, belonging to oleic acid, and 2.83 ppm, which indicates the presence of asparagine (NUZZO et al., 2013; HUSSEIN et al., 2022).

One of the main differences found between the dichloromethane and hexane extracts concerned the organic acids present in the two samples. First, the dichloromethane extract had a higher concentration and higher amounts of the already mentioned metabolites; only EDTA and isobutyric acid were present in higher concentrations in the hexane extract than in the dichloromethane extract. Isobutyric acid was the predominant acid in the hexane extract and the only secondary metabolite present at a higher concentration than in the dichloromethane extract. The hexane extract also contained organic substances, such as citric, acetic, formic, L-lactic, and 3-hydroxybutiric acid, but the dichloromethane extract contained a much higher concentration of these compounds. In addition, the bioactive compounds pyruvic, pyroglutamic, and succinic acid were found in the dichloromethane extract but not in the hexane extract.

On the other hand, both extracts contained alcohols, such as ethanol, glycerol, and 1,5-anhydrosorbitol. The hexane extract contained a higher concentration of these low molecular weight metabolites. Alcohols have a highly negative effect on metabolism, especially glycerol in microalgae. It has been reported that this compound inhibits the production of three metabolites, such as glucose-6-phosphate, fructose-6-phosphate, and glyceric acid-3-phosphate, key intermediates in carbon metabolism (NZAYISENGA; SELLSTEDT, 2021). In addition to alcohols, the hexane extract contained higher concentrations of dimethyl sulfone and creatinine than the other extracts. These metabolites are less polar than the carboxylic compounds, which were predominant in the dichloromethane extract, and nonpolar solvents have a higher affinity for both metabolites. Nonpolar solvents, such as dichloromethane, elute nonpolar substances in a more significant proportion but elute medium polarity substances in smaller proportions. The virucidal efficiency of extracts with a more polar character is associated with the action of polar substances that bind to viral capsids, blocking the binding to proteins of the host cells (DEYAB et al., 2019). Similar results were observed in a previous study in which dichloromethane extracts from green algae showed excellent antiviral activity against equine rhinopneumonitis, highlighting the potential of dichloromethane extracts as a source of molecules from green algae with virucidal and antiviral activity (MARINHO et al., 2016).

The signals for the amino acids were seen in the NMR spectra and helped us to reinforce the data obtained by amino-acid characterization. The peaks at $\delta = 0.99–1.04$ ppm are related to valine and the multiplet between $\delta = 1.24$ and 1.40 ppm indicates the presence of proline, isoleucine, and leucine protons. Except for alanine, which was not found in the dichloromethane extract, all the amino acids identified in NMR analysis were found in higher concentrations in dichloromethane extract. For example, amino acids such as L-threonine, L-tyrosine, L-leucine and L-methionine were present in a much higher proportion in the dichloromethane extract than the hexane extract, whereas L-glutamic acid was found at a similar concentration in both nonpolar solvents. These results indicate that *P. nurekis* is rich in amino acids, as already reported for other *Chlorella* sp., such as *Chlorella vulgaris*, for which leucine, phenylalanine, and tryptophan have been found (PANTAMI et al., 2020). Other amino acids, such as L-glutamic acid and alanine, have been found in *Chlorella* sp. (ZHANG et al., 2015). One amino acid that has not yet been reported as a biomarker in microalgae is L-glutamine. Due to its high polarity, it was found at a higher concentration in dichloromethane

and at a higher value than glutamate, indicating low N-stress, which has an impact on the concentration of carotenoids and isoprene derivates. A higher proportion of glutamate indicates higher nitrogen stress, resulting in a higher concentration of fatty acids, terpenic compounds, and carotenoids (MORALES et al., 2019). According to the literature, glutamine is used by microalgae to produce nucleosides and deoxy/ribonucleotides by the pyrimidine metabolism pathway (CHEN et al., 2017). Nonetheless, it will be necessary to analyze more compounds to draw a more solid conclusion.

Another indicator of the pyrimidine metabolism pathway found in the NMR spectra aside from glutamine was the presence of hypoxanthine. This metabolite is important due to its role in microalgae metabolism as a precursor of xanthine, which can be used to synthesize bioactive substances, such as astaxanthin (CARBONE et al., 2021), theobromine, and other methylxanthines, which have been reported to be antiviral agents against numerous pathogens, including SARS-CoV-2 (ROMERO-MARTÍNEZ et al., 2021).

In vitro studies on the activity of siphonaxanthin against SARS-CoV-2 in HEK293 cells overexpressing angiotensin-converting enzyme 2 (ACE2) showed the carotenoid to be the main bioactive compound with an antiviral action (YIM et al., 2021). The authors suggested that the compound interferes with the interaction between the virus and the cells by binding to the S-glycoprotein (GHASEMNEJAD-BERENJI, 2021). Crocin has been shown to induce the downexpression of ACE2 expression and can reduce virally induced oxidative stress (AL-HORANI; KAR, 2020). Finally, there is little information related to hypoxanthine and its activity against SARS-CoV-2, but it has been documented that hypoxanthine is a counterpart of the antiviral Favipavir, which inhibits the viral RNA polymerase and is effective against several strains of influenza viruses. Furthermore, it has recently been tested with other drugs, such as tocilizumab and oseltamivir, for the treatment of COVID-19 (AFREEN et al., 2021).

Butanol derivatives, such as 3-hydroxybutyrate, and isobutyric acid, were present in both extracts. These compounds can be produced by various microalgae from glucose and xylose. The polyhydroxyalkanoates (PHAs) in microalgae are obtained by the conversion of carbohydrate to 3-phosphoglycerate (PGA), followed by three possible pathways: the Entner–Doudoroff (ED) pathway, glycolysis, or the pentose phosphate pathway. These three pathways lead to the formation of pyruvate, which is transformed into acetyl-CoA, leading to the synthesis of butyrates by the PHA synthetic pathway (AZIZAN et al., 2018).

Our samples were rich in fatty acids, such as oleic acid, linoleic acid, and alpha-linolenic acid. Those three lipids have also been found in microorganisms such as *Chaetoceros calcitrans* (AZIZAN et al., 2018). The peak at 2.05 ppm that appeared in the nonpolar extracts has also been found in the extracts of microalgae such as *Thalassiosira weissflogii*, *Cyclotella cryptica,* and *Nannochloropsis salina*. This peak is associated with palmitic acid and n-3 omega polyunsaturated fatty acids (PUFAs), such as alpha-linolenic acid. The presence of these metabolites will need to be confirmed by further tests (SARPAL et al., 2015). Our results indicate a relationship between the amount of lipidic compounds extracted and the solvent used. The solvent with intermediate polarity allowed the best isolation of lipids due to the existence of an aliphatic and oxygenated region in the studied metabolite.

The peaks at $\delta = 0.50–2$ ppm that were useful in identifying aliphatic compounds have also been observed for microorganisms such as *Chlorella* MAT-2008 (DAVEY et al., 2012) and *Chlorella* sp. (ZHANG et al., 2015). Thus, the protonic NMR technique allows the identification of various metabolites and provides an initial approach for the quantification of specific bioactive lipids, such as fatty acids and polar lipids.

The NMR data showed the *P. nurekis* extracts to be rich in polar lipids, such as acylglycerols, xanthophylls, glycolipids, and phospholipids. In addition, there was a significant presence of carbohydrates and polyphenols. The signals for cholesterol are seen at $\delta = 0.83$ ppm −0.95 ppm and 1.90 ppm, suggesting the presence of compounds such as zeaxanthin, lutein, and violaxanthin, which are commonly found in green plants and green microalgae (AZIZAN et al., 2018).

Peaks between 3.16 and 5.32 ppm suggest the presence of carbohydrates, but more data are needed for confirmation. It will be necessary to carry out FT-IR and, in particular, MS analysis in which the main objective is the targeted study of the profile of polyphenols, carbohydrates, and lipids, focusing on the presumed metabolites that have been reported in the literature to have a virucidal effect. In the literature, both metabolites show similar peaks and despite the high concentration of phenolic derivatives, there appears to be a significant quantity of bioactive saccharides between 2.0 ppm and 4.0 ppm (NUZZO et al., 2013).

3.2 ANALYSIS OF THE EXTRACTS BY UPLC-MS

The fractions from the hexane and dichloromethane extractions displayed almost the same UPLC-ESIMS profile (Supplementary Material—Figures S2 and S3). Hexane showed the presence of 11 secondary metabolites and dichloromethane 18 in their chromatogram spectra (Supplementary Material—Table S1).

The base peak ion (BPI) chromatogram of both extracts showed a peak at tR 4.53 min, with mass values of *m*/*z* 531.2817 $[C_{25}H_{42}O_9 + HCO_2]^-$ and *m*/*z* 509.2745 $[C_{25}H_{42}O_9 + Na]^+$ in the negative and positive ionization modes, respectively. No fragmentation was observed in the negative ionization mode, whereas fragment ions of *m*/*z* 469.2790, *m*/*z* 325.2374, and *m*/*z* 233.1918 were obtained in the positive mode analysis. The precursor *m*/*z* 509.2745 produced *m*/*z* 469.2790 after losing NaOH (40 Da). The ion *m*/*z* 325.2374 appeared to be an in-source fragment formed by the of loss of a sugar moiety (162 Da) from $[M+H]^+$. Cleavage of the ester function provided the fragment ion *m*/*z* 233.1918, corresponding to the fatty acyl cation. Based on these data, the structure of this metabolite was assigned to be (7Z,10Z,13Z)-2-hydroxy-3-(β-D-galactopyranosyl)oxy)propyl hexadeca-7,10,13-trienoate. The fragmentation pattern of compound 1 was consistent with that reported by Guella, Frassanito, and Mancini (2003).

Compounds 2 and 3 were identified as 13-hydroxyoctadecatrienoic acid and vernolic acid, respectively, with a *m*/*z* 293.2130 $[C_{18}H_{30}O_3\text{-}H]^-$ and 295.2289 $[C_{18}H_{32}O_3\text{-}H]^-$. Compound 2 represents a group of metabolites called oxylipins, which are widespread in algae (BARBOSA; VALENTÃO; ANDRADE, 2016).

Compounds 4 to 8 detected at tR 6.29, 7.39, 8.42, 9.59, and 9.89 min, respectively, yielded no fragments. However, their mass values of *m*/*z* 249.1852 $[C_{16}H_{26}O_2\text{-}H]^-$, 277.2180 $[C_{18}H_{30}O_2\text{-}H]^-$, 279.2330 $[C_{18}H_{32}O_2\text{-}H]^-$, 255.2332 $[C_{16}H_{32}O_2\text{-}H]^-$, and 281.2485 $[C_{18}H_{34}O_2\text{-}H]^-$ correspond to the structures of hexadecatrienoic acid, linolenic acid, linoleic acid, palmitic acid, and oleic acid, respectively. These fatty acid derivatives have been previously reported to be present in microalgae species from the genera *Aphanizomenon, Botryococcus, Chaetoceros, Chlorella, Cylindrotheca, Crypthecodinium, Isochrysis, Haematococcus, Dunaliella, Neochloris, Nostoc, Nannochloropsis, Pavlova, Phaeodactylum, Porphyridium, Arthrospira, Schizochytrium,* and *Thalassiosira* (MALTSEV; MALTSEVA, 2021). The dichloromethane extract contained all these fatty acids, whereas hexadecatrienoic acid and palmitic acid were not found in the hexane extracts. A study that used a mixture of vegetable oils composed mostly

of fatty acids demonstrated the effectiveness of these compounds against SARS-CoV-2 and influenza viruses in an *in vitro* study (KRISTJÁNSSON; ROLFSSON, 2021). The authors suggested that the antiviral action was related to the fatty-acid molecules, hypothesizing that the compounds penetrate and break down the lipid coat that covers enveloped viruses, thus deactivating the viruses at their point of entry.

Compound 8 was found at tR 3.72 min in the positive ionization mode and present only in the dichloromethane extract. Its mass value of *m*/*z* 181.1233 corresponds to the molecular formula $[C_{11}H_{16}O_2{}^{+}H]^{+}$. This precursor was different from its fragment ion *m*/*z* 163.1130 by 18 Da, suggesting the loss of H_2O. A literature search on this molecular formula led to a structure related to 4,4,7a-trimethyl-5,6,7,7a-tetrahydrobenzofuran-2(4H)-one, previously identified in seaweed (MOFEED et al., 2022).

The structure of a fatty acid found in the dichloromethane extract, namely stearidonic acid, was assigned to compound 10 detected at tR 4.97 min with a *m*/*z* 277.2166 $[C_{18}H_{28}O_2{}^{+}H]^{+}$. Compound 11, detected at tR 5.48 min with a *m*/*z* 577.1348 $[C_{30}H_{24}O_{12}{}^{+}H]^{+}$ produced a fragment ion of *m*/*z* 385.0938. A literature search showed compound 11 to be related to protocyanidin A1. The formation of the ion of *m*/*z* 385.0938 occurs after the cleavage of the CarO-Cacetal bond with hydrogen migration followed by RDA opening of ring C and the loss of ketene (42 Da). Thus far, only flavonoids and phenolic derivatives have been identified and reported in algae and microalgae (FERDOUS; YUSOF, 2021). Therefore, compound 11, which was found only in the hexane extract, is an unprecedented finding in microalgae metabolites. Compound 12 was detected in both the hexane and dichloromethane extracts at tR 6 min, with a mass value of *m*/*z* 301.1416, corresponding to $[C_{16}H_{22}O_4{+}Na]^{+}$. In its tandem mass spectrum, this metabolite showed two fragment ions of *m*/*z* 205.0866 and 149.0244 resulting from the elimination of sodium butanolate (96 Da) and sodium butanolate and but-1-ene (56 Da), respectively. Compound 13, found in dichloromethane at tR 8.35 min with a *m*/*z* 637.3063 $[C_{29}H_{48}O_{15}{}^{+}H]^{+}$, produced fragment ions of *m*/*z* 581.2450, 525.1830, 495.26262, and 469.1125 in its tandem mass spectrum; these ions correspond to the sequential loss of four units of prop-1-en-1-one (56 Da). Based on these data, the structure of compound 13 was assigned to be related to O-tetrapropanoyloctanoate of sucrose.

Compound 14, observed at tR 8.35 min with a *m*/*z* 331.2852, produced two fragment ions in its tandem mass spectrum of *m*/*z* 313.2727 and 239.2368. The precursor is different from

the first fragment by 18 Da, suggesting the loss of H_2O, whereas the lighter fragment corresponds to the cation hexadecylidyneoxonium formed after glycerol (92 Da) elimination.

Compound 15, obtained at tR 9.01 min showed the precursor *m*/*z* 593.2747 $[C_{34}H_{40}O_9{}^{+}H]^{+}$ and the fragment ion *m*/*z* 533.2573 in its mass spectra. Both ions differed by 60 Da, suggesting the loss of acetic acid. The structure was not identified because of the lack of diagnostic fragments.

Compound 16 was also identified as another monoglyceride, namely glyceryl stearate. It was detected at tR 10.47 min with a *m*/*z* 359.3179, corresponding to the molecular formula $[C_{21}H_{42}O_4{}^{+}H]^{+}$. This precursor ion lost a molecule of H_2O (18 Da) and glycerol (96 Da) to produce ions of *m*/*z* 341.3074 and 267.2713 (fatty acid moiety), respectively. Monoglycerides represent one of the valuable chemical constituents of microalgae used to produce bioenergy and their identification in this biomass has been well-documented (FERREIRA et al., 2021). The metabolite (17) at tR 11.13 min with a *m*/*z* 551.4241 $[C_{40}H_{54}O{+}H]^{+}$ did not produce fragment ions in its MS/MS spectrum. However, a literature search using its molecular formula led to the structure of echinenone, a carotenoid previously identified in the microalgae *Scenedesmus obliquus* (NASCIMENTO et al., 2020). Compound 18 showed the same fragmentation pattern as compound 15 by losing only 60 Da, corresponding to acetic acid. Nevertheless, its structure could not be assigned because of low fragmentation. Compound 19 was identified as dioctyl phthalate. It was found at tR 11.94 min with a *m*/*z* 419.2663, corresponding to $[C_{24}H_{38}O_4{+}Na]^{+}$. This precursor gave the daughter ions *m*/*z* 301.1410 and 149.0244 in its MS/MS spectrum after losing oct-1-ene (112 Da) and sodium octanolate (272 Da). The methanol extract was more sensitive to the LC-MS analysis performed in the negative ionization mode (Supplementary Material—Figure S4).

Compound 20 was detected at tR 7.98 min with a *m*/*z* 693.3342 $[C_{31}H_{52}O_{14}{}^{+}HCO_2]^{-}$ and gave the ion *m*/*z* 647.3315 $[M\text{-}H]^{-}$ and the fragment *m*/*z* 249.1852 in its MS/MS spectrum, corresponding to hexadecatrienoic acid. This information led assignment of 3-hydroxy-2-[[(7Z,10Z,13Z)-1-oxo-7,10,13-hexadecatrienyl]oxy]propyl 6-O-β-D-galactopyranosyl-β-D-Galactopyranoside. Compound 21 was different from compound 20 by 164 Da, corresponding to the absence of a hexose (162 Da) and the presence of a double bond equivalent (2 Da). The tandem mass of compound 21, identified as 3-hydroxy-2-[[-1-oxo-4,7,10,13-hexadecatetraenyl]oxy]propyl β-D-Galactopyranoside, produced the ion *m*/*z* 247.1671, assigned as hexadecatetraenoic acid. This fragmentation pattern was consistent with those

reported in the literature (GUELLA; FRASSANITO; MANCINI, 2003). This ion was formed after the precursor lost the hexose and glycerol moieties. Two isomeric metabolites (22 and 23) with a structure related to 3-hydroxy-2-[[-1-oxo-7,10,13-hexadecatrienyl]oxy]propyl β-D-galactopyranoside were obtained at 8.49 and 8.71 min with the same mass value of *m*/*z* 531.2817 $[C_{25}H_{42}O_9+HCO_2]^-$. These compounds differ from compound 20 by a hexose unit (162 Da). After losing the sugar and glycerol, an ion of *m*/*z* 249.1852, corresponding to the fatty acid chain, was formed. Compound 24, identified as 2-hydroxy-3-[[(9Z,12Z,15Z)-1-oxo-9,12,15-octadecatrien-1-yl]oxy]propyl6-O-β-D-galactopyranosyl-β-D-Galactopyranoside, at tR 8.93 with a mass value of *m*/*z* 721.3671 $[C_{33}H_{56}O_{14}+HCO_2]^-$, produced a fragment ion of *m*/*z* 277.2180 after losing two hexoses and glycerol. Compound 25, identified as (2S)-2-hydroxy-3-[[(7Z,10Z)-1-oxo-7,10-hexadecadien-1-yl]oxy]propyl β-D-galactopyranoside, was heavier than compound 23 by 2 Da, suggesting the presence of less unsaturation. Their fragmentation pattern was identical, with the production of an ion corresponding to the fatty acid with a mass of *m*/*z* 251.2029. As found in the literature, such metabolites have been previously reported in the microalga *Chlorella sorokiniana* (BANSKOTA et al., 2013). Another glycosylated monoglyceride was identified at tR 10.03 with a mass of *m*/*z* 699.3804 $[C_{31}H_{58}O_{14}+HCO_2]^-$. A fragment ion was observed in its MS/MS spectrum of *m*/*z* 255.2332, corresponding to the ion palmitate. This information led to the assignment of 1-O-palmitoyl-3-O-[α-D-galactopyranosyl-(1→6)-β-D-galactopyranosyl]-sn-glycerol, previously reported in microalgae.

Compounds 27 and 28 gave ions of *m*/*z* 481.2598 $[C_{29}H_{38}O_6{}^-H]^-$ and 483.2742 $[C_{29}H_{40}O_6{}^-H]^-$, respectively. A literature search led to the structures of steroids related to poly-oxygenated ergosterol. Their structures could not be elucidated because of their low fragmentation. Compounds 29–32 were identified as derivatives of linolenic, linoleic, palmitic, and oleic acids.

Compound 33 with a tR at 14.73 min and *m*/*z* 953.5460 $[C_{49}H_{80}O_{15}{}^+HCO_2]^-$ was identified as disaccharide diglyceride. It produced two fragment ions in its tandem mass spectrum, assigned as the ions hexadecatrienoate (*m*/*z* 249.1852) and octadecatrienoate (*m*/*z* 277.2180). On the basis of this information, the structure of compound 33 was assigned as [[-1-oxo-9-hexadecatrien-1-yl]oxy]-3-[[-1-oxo-octadecatrien-1-yl]oxy]propyl 6-O-α-D-galactopyranosyl-β-D-galactopyranoside. Hexosyl mono- and diglycerides have been

previously reported to be present in microalgae, supporting our finding (SOLINSKI et al., 2017).

The compounds identified in the UPLC-MS analysis were mostly associated with the lipid class, in which palmitic and stearidonic acid stood out as being present only in the dichloromethane extracts, which presented greater virucidal activity. Our results show that there is a relationship between the number of lipidic compounds extracted and the solvent used. The solvent with intermediate polarity was the one that allowed the best isolation of lipids, due to the existence of an aliphatic and oxygenated region in the metabolite.

3.3 CYTOTOXICITY OF *PLANKTOCHLORELLA NUREKIS*

For the cytotoxic evaluation, all samples were tested on L929 cell line for 48 h and stained with SRB that binds stoichiometrically to cell proteins and then can be extrapolated to measure cell viability. The CC_{50} values were calculated for each sample and are summarized in Table 4. All the samples showed low cytotoxicity, and the dichloromethane extract presented an essential cytotoxic effect on L929 cells with a CC_{50} value of 53.19 µg/mL.

Table 4 – Cytotoxic evaluation of hexane, dichloromethane, and methanol extracts of *P. nurekis* using L929 cells. Mean value and standard deviation (SD).

Extracts	**CC_{50}[a] µg/mL**	**CI_{95}[b] µg/mL**
Hexane	330.4	270.1 to 404.3
Dichloromethane	53.19	30.94 to 91.45
Methanol	73.30	58.74 to 91.46

[a] CC_{50}—50% cytotoxic concentration; [b] CI_{95}—95% confidence interval.

3.4 VIRUCIDAL ACTIVITY OF *PLANKTOCHLORELLA NUREKIS*

The viricidal activity of the *P. nurekis* extracts is summarized in Table 5. Treatment with the microalgae methanol extract resulted in a reduction of coronavirus MHV-3 infection of 6 Log10 PFU and 8 Log10 PFU at 24 ± 2 °C and 35 ± 2 °C, respectively, regardless of the extract concentration (Table 5). Treatment with the hexane extract resulted in a reduction of 7 Log10 PFU (12.5 to 50 µg/mL) at 24 °C, but there was no reduction in coronavirus MHV-3

infection at 35 ± 2 °C (Table 5). Finally, the dichloromethane extracts led to a reduction from 6 Log10 PFU to 8 Log10 PFU (3.1 to 50 µg/mL) at 24 °C. The dichloromethane extract had the highest virucidal activity. Dichloromethane possesses an intermediate polar character between that of methanol and hexane: eluting forces of 0.26, 0.01, and 0.70, respectively.

Table 5 – Coronavirus – MHV-3 – reduction (Log10) after treatment with hexane, dichloromethane, and methanol extracts of *P. nurekis*.

		Conc (µg/mL)				
Extract		**3.12**	**6.25**	**12.5**	**25.00**	**50.00**
Hexane	24 ± 2 °C	NR	NR	−7.0 ± 1.2	−7.0 ± 1.4	−7.0 ± 1.2
Dichloromethane		−6.0 ± 0.5	−6.0 ± 0.3	−6.0 ± 0.2	−8.0 ± 0.3	−8.0 ± 0.4
Methanol		−6.0 ± 1.5	−6.0 ± 0.5	−6.0 ± 0.8	−6.0 ± 0.3	−6.0 ± 0.4
Hexane	35 ± 2 °C	NR	NR	NR	NR	NR
Dichloromethane		NR	NR	−8.0 ± 0.3	−8.0 ± 0.3	−8.0 ± 0.4
Methanol		−6.0 ± 1.5	−6.0 ± 0.5	−6.0 ± 0.8	−6.0 ± 0.3	−6.0 ± 0.4

NR: no reduction.

3.5 PCA ANALYSIS OF THE *P. NUREKIS* EXTRACTS

The results of the multivariate analysis are presented in the Supplementary Material Figure S5. There was a correlation between the carotenoid and polyphenol concentrations and the virucidal action of each extract, allowing differentiation between the extracts. The compounds with the greatest impact on antiviral activity were isobutyric acid and ethanol, followed by amino acids, such as L-aspartic acid, L-methionine, and glutamine. There was also a large impact of carotenoids, hypoxanthine, and organic acids, such as L-lactic, formic, pyruvic pyroglutamic, and succinic acid in the bioactivity of the samples against murine Coronavirus (Figure S5A, B). Amino acids other than those already mentioned, polyphenols, and alcohols other than ethanol had less of an impact on the antiviral activity against murine coronavirus.

The influence of carotenoids and phenols on the antiviral activity against herpesvirus (HSV-1) has already been reported for two green microalgae *Haematococcus pluvialis* and *Dunaliella salina*. The ethanol extract of *H. pluvialis*, a freshwater microalga, showed that such antiviral activity may be partially related to the presence of short-chain fatty acids. However, other compounds also contribute to this activity, such as those found in the ethanol extract of *D. salina*, a marine microalga. Further compounds may also be involved, such as β-ionone, neophytadiene, phytol, palmitic acid, and α-linolenic acid (SANTOYO et al., 2012). Ishikawa et al. (2008) reported that β-carotene and astaxanthin, fucoxanthin, and the deacetylated

metabolite fucoxanthinol had mild inhibitory effects on human T-cell leukaemia virus type 1-infected T-cell lines.

Our extracts could be differentiated by biomarkers, and it was possible to correlate the chemical composition with the antiviral activity. Previous studies have suggested that the antiviral activity against Coronavidae members can be attributed to metabolites such as phytol and polysaccharides (REYNOLDS et al., 2021), which was not found in this research. Other researchers have reported a prominent role of organic acids in the viricidal effect, with one of the most prominent metabolites being formic acid, which can inhibit the replication of the porcine epidemic diarrhea virus (PEDV) (GÓMEZ-GARCÍA et al., 2021). According to the PCA, isobutyric acid was the metabolite with the highest impact on viricidal activity. It has already been reported that isobutyric acid and esters, such as 3-hydroxyisobutyrate, in combination with a low pH, generate an acidic environment that damages the structure of the virus and leads to viral inactivation. This mechanism has been observed for other compounds, such as acetic acid, but its effect is much weaker, which could explain why it showed a lower contribution to the antiviral activity against MHV-3 in the PCA. This mechanism is used by drugs, such as propylamylatin, against SARS-CoV-2, highlighting the potential application of *P. nurekis* extracts for the development of compounds against pathogens such as SARS-CoV-2 (BROWN et al., 2021).

4 CONCLUSIONS

With the recent COVID-19 pandemic, research on coronavirus-inactivation mechanisms is urgently needed to find potential therapeutic agents against SARS-CoV-2. In this context, algae biotechnology has much to offer in the fight against coronaviruses. The use of algae is extremely promising due to its proven effectiveness against the virus when applied to animal cells and its low cost of production. Because it is a natural organism, it is not harmful to human beings or the environment. In this study, extracts of *P. nurekis* obtained using three different solvents were evaluated against MHV-3, a surrogate model for SARS-CoV-2. All the extracts efficiently inactivated MHV-3 (over 6 Log reductions), regardless of the solvent or concentration used. However, the dichloromethane extract showed the highest virucidal activity, demonstrating that this organic solvent of intermediate polarity is the most effective in extracting compounds with this bioactivity. As more metabolites become known, an

increasingly detailed chemical profile will also become available, translating into a stronger correlation between the biological activity against SARS-CoV-2 and the compounds isolated from *P. nurekis* extracts. Based on multivariate analysis, polyphenols, carbohydrates, and isoprene derivatives, such as terpenic compounds and carotenoids, have a more significant impact on virucidal potential. More specific results are still needed to determine the precise correlation between the elucidated metabolites and the virucidal action of microalgae. Therefore, new tests on the L929 strain are recommended to evaluate the respiratory burst (NBT) and possible mechanisms of cellular damage, at the genetic level, as well as *in vivo* tests on MHV-3 in order to evaluate the activity of the extracts in the organism.

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CAPÍTULO III

CYTOTOXIC ASSAY AND ACCELERATION OF INACTIVATION OF MURINE CORONAVIRUS IN HUMAN SEWAGE BY THE APPLICATION OF *PLANKTOCHLORELLA NUREKIS* EXTRACT

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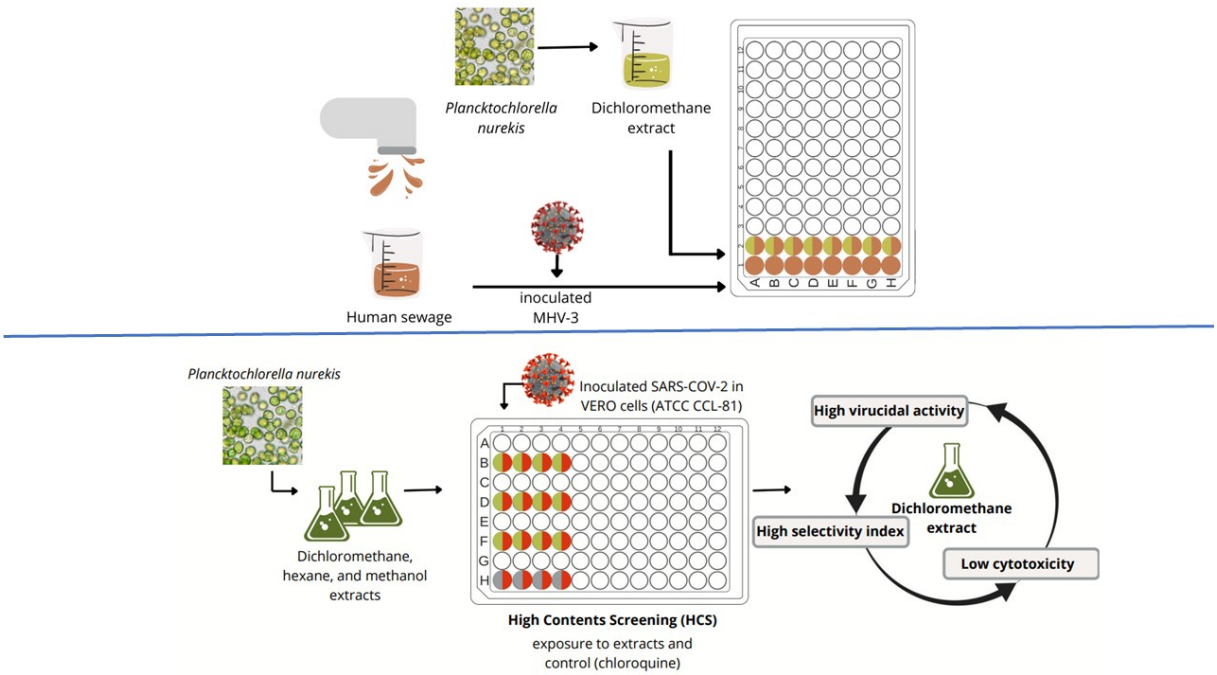
ABSTRACT

The new coronavirus has spread across all continents, causing severe respiratory illness and other symptoms. Viruses have a high replicative capacity and can generate new strains in different environments. SARS-CoV-2 particles are excreted in patients' feces and can survive for more than 30 days in human sewage. In this sense, the prospection of compounds of biological origin as alternative ways to obtain new virucides and antivirals has become even more critical. Microalgae have a range of compounds with antimicrobial activity, but several species are still poorly studied, such as *Planktochlorella nurekis*. Thus, this research aimed to test the cytotoxicity in different extracts (hexane, dichloromethane, and methanol) from *P. nurekis* on cell lines L929 and Vero. The extract of dichloromethane from *P. nurekis* (PNDE) with showed lower toxicity and a higher selectivity index against the virus (high content screening - HCS), therefore, it was selected to test the promotion of viral inactivation in human sewage using MHV-3 as a model. MHV-3 was inoculated into human sewage and treated with 10 µg/mL of PNDE for 5, 30, 45, and 60 minutes at 25 ± 2 ºC. Human sewage treated with PNDE promoted a reduction of 4, 5, and 6 Log of MHV-3 after 30, 45, and 60 min of exposure, respectively. The results indicated the possibility of an environmentally friendly, non-cytotoxic, and highly effective microalgae-based product against infectious coronavirus in human sewage.

Keywords: microalgae; cytotoxicity; SARS-CoV-2; MHV-3; antiviral.



1 INTRODUCTION

The year 2019 was marked by the outbreak of SARS-CoV-2 (COVID-19), originating in the city of Wuhan, the capital of the Chinese province of Hubei. This new virus is directly linked to the SARS virus, being able to cause more severe respiratory diseases than the others (ACTER et al., 2020). Its infection occurs mainly by inhalation, however, potential routes of secondary transmission are also observed, such as example, on infected surfaces and through human sewage (BEDROSIAN et al., 2021). These viruses are excreted in patient feces and, patients with diarrhea can even eliminate about $1x10^6$ virus/gram of feces (AMICO et al., 2020; LOU et al., 2021).

Several studies around the world have characterized the presence of RNA fragments of SARS-CoV-2 in sewage (BIVINS et al., 2020). Thus, viral RNA fragments have been detected in untreated wastewater and sewage in countries such as Spain (RANDAZZO et al., 2020), Australia (AHMED et al., 2020), Italy (LA ROSA et al., 2020), Netherlands (MEDEMA et al., 2020), USA (SHERCHAN et al., 2020), Brazil (FONGARO et al., 2020), Chile (AMPUERO et al., 2020) and Japan (HARAMOTO et al., 2020) suggesting the importance of studies with virus control in the environment. Sewage may be a factor contributing to these viruses' persistence in the environment due to organic matter particles. One of the significant challenges facing COVID-19 is the lack of effective treatments against SARS-CoV-2 and the control of viral particles and their environmental aerosolization (GUNDY et al., 2009).

Knowing the risks linked to human health of the new coronavirus, the prospection of compounds of biological origin from alternative sources is an essential approach to obtaining new virucides and antivirals. In this context, microalgae are a promising source of growing interest when seek the biosynthesis of bioactive compounds (WANG; WANG; GUAN, 2012; DIAS; URBAN; ROESSNER, 2012). The microalgae group is phylogenetically diverse; therefore, their cellular matrix differs between clades, making them biochemically diverse. Microalgae are present in fresh and marine water environments. Their compounds are highly relevant for health, such as vitamins, proteins with essential amino acids, fatty acids, polysaccharides, minerals, enzymes, fibers, and photosynthetic pigments, such as carotenoids and chlorophylls (SILVA et al., 2018; MONTALVÃO et al., 2016).

Extracts with natural bioactive have shown effective potential against several viruses (REIS et al., 2022a). For example, the microalgae extract of *Spirulina platensis* demonstrated an antiviral effect against the non-enveloped *Adenovirus* type 40 virus (ABDO et al., 2012). Furthermore, the same specie has proven antiviral effects against *Adenovirus* type 7, *Astrovirus* type 1, *Coxsackievirus* B4, and *Rotavirus* Wa, indicating that it is a suitable type of development as an antiviral treatment (EL-BAZ et al., 2013). Some compounds obtained from algae have already been shown to be effective against several members of the coronavirus family, including SARS-CoV (O'KEEFE et al., 2010). Thus, this study aimed to test the cytotoxicity of hexane, dichloromethane, and methanol extracts from the microalgae *Planktochlorella nurekis* on cell lines L929 and Vero. After obtaining the best result regarding cellular cytotoxicity, sought to promote the acceleration of the inactivation of the murine coronavirus (using mouse hepatitis virus strain 3 - MHV-3 as a model) in human sewage.

2 MATERIAL AND METHODS

2.1 MICROALGAE EXTRACT

Planktochlorella nurekis grown in 10 L photobioreactors, exposed to light (99 μmol m^{-2} s^{-1}), mixotrophic conditions (12h:12h, light:dark), continuous agitation using a mechanical recirculation pump 1,200 $L.h^{-1}$ (Sarlobetter brand), and room temperature (23 °C). Photobioreactors were inoculated with 70 ± 0.6 mg.DW microalgae L^{-1}. After 11 days following inoculation, the biomass reached a fresh weight of 0.4 ± 0.1 g L^{-1}. Photobioreactors were operated in fed-batch mode using effluents from an anaerobic treatment system, which was diluted by adding 1.0 L into 6.0 L of chlorine-free tap water.

This biomass was then harvested by centrifugation at 3,000 × g (Eppendorf, 5810/5810R), frozen immediately (-20 °C), and then lyophilized for further analysis and assays. A total of 20 g of *P. nurekis* biomass were exhaustively extracted with methanol, dichloromethane, and hexane, in a ratio of 1:5 (w:v; g:mL). These extracts were dried on a rotary evaporator maintained under vacuum and at 50 °C, later stored in a freezer (-20 °C). The extracts were then resuspended in dimethyl sulphoxide (DMSO) (≥ 99.7%; Sigma-Aldrich, USA) at 50 mg/mL.

2.2 CYTOTOXICITY ASSAY

2.2.1 L929 Cell line

Cell viability was assessed by the Sulforhodamine B assay, which measures total protein mass (VICHAI; KIRTIKARA, 2006). Mouse fibroblast cells (L929, ATCC® CCL-1) were maintained in Minimum Essential Medium (MEM; Thermo Fisher Scientific, Poland) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Poland), then seeded in plates (96-well plates format, 2.5 x 104 cells/well) maintained during 24 h at 37 °C in an atmosphere containing 5% of CO_2. The cells were exposed at concentrations of 48 μg/mL to 500 μg/mL with hexane, dichloromethane, and methanol extracts of the microalgae *P. nurekis* for 48 h. The percentage of viable cells was plotted against each sample concentration. CC_{50} values were calculated based on concentration-response curves using GraphPad Prism 8.0 software (Graph Pad Software version 8.0.

2.2.2 Vero cell line: High Content Screening (HCS)

Compounds were tested in dose response. The compound collection was reformatted into a 384-well plate and the compounds were diluted to a concentration of 20 mg/mL in dimethyl sulphoxide (DMSO) (≥ 99.7%; Sigma-Aldrich, USA). Before performing cell treatment, compounds were diluted 33.33x in PBS, and 10 μL of each dilution was transferred to assay plates, thus having a final dilution factor of 200x. As a control, chloroquine was used.

Tests with African Green Monkey Kidneys Cells (Vero cells, ATCC CCL-81) cells were performed in 384-well plates. After 24h, the cells received the compounds as indicated above, and then the virus was added at an assortment of assay (MOI) of 0.1 viral particles per cell. After 33h of SARS-CoV-2 assay, the plates were fixed, immunofluorescence was performed with sera from COVID-19 patients and the images were acquired and analyzed by the HCS Operetta equipment. The final concentration of DMSO in the assay plates was 0.5% (v/v).

The parameters measured in each of the wells were: the total number of cells and the number of infected cells. From the infected and uninfected controls, the activity of each

compound was normalized, as well as the cellular survival rate. The reduction in the number of infected cells indicates the percentage in antiviral activity of the samples.

2.3 CORONAVIRUS INACTIVATION IN HUMAN SEWAGE

From the results obtained in cytotoxicity tests with cell lines L929 and Vero, only the dichloromethane extract of *P. nurekis* (PNDE) was tested for viral inactivation in human sewage. Human sewage used in this study was obtained from a sewage treatment plant in Florianópolis, Santa Catarina, southern Brazil, and was known to be harmful to SARS-CoV-2. Human sewage was inoculated with 1×10^8 plate forming unity/mL (PFU/mL) fluid of MHV-3 and treated with 10 μg/mL of *P. nurekis* extract with dichloromethane during 5-, 15-, 30-, 45-, and 60-min. Results were compared with untreated human sewage under the same conditions as a control. All tests were performed in independent triplicates.

Concentrations of PNDE that reduced cell viability by 50% (CC_{50}) were calculated based on concentration-response curves using linear regression. Two-way ANOVA was used to assess differences in viral inactivation rate between treated and untreated control, followed by a t-test, and 1 Log10 or 90% coronavirus reduction (T90) times were calculated by linear regression. These analyses were considered statistically significant when $p \leq 0.05$. All tests were performed on GraphPad Prism 8.2.1 (Graph Pad Software, La Jolla, CA).

3 RESULTS AND DISCUSSION

3.1 CYTOTOXICITY ASSAY

In the L929 cell line, the mean value of CC_{50} (50% cytotoxic concentration) for the dichloromethane extract of *P. nurekis* was the one that showed a low cytotoxic effect, with 53.19 μg/mL (95% confidence interval of 30, 94 μg/mL to 91.45 μg/mL) (Table 6).

Table 6 – Cytotoxic evaluation of hexane, dichloromethane, and methanol extracts of *P. nurekis* using L929 cells. Mean value and standard deviation (SD).

Extracts	CC_{50}[a] µg/mL	CI_{95}[b] µg/mL
Hexane	330.4	270.1 to 404.3
Dichloromethane	53.19	30.94 to 91.45
Methanol	73.30	58.74 to 91.46

[a] CC_{50}—50% cytotoxic concentration; [b] CI_{95}—95% confidence interval.

In the HCS evaluating the antiviral effect of methanol, dichloromethane, and hexane fractions on the survival of Vero CCL-81 cells there were different results for the half-maximal effective concentration (EC_{50}), and for 50% cytotoxic concentration (CC_{50}) compared to the control infected. When using the methanolic fraction in the cells, EC_{50} and CC_{50} above 100 µg/mL and maximum activity for inhibition of 42% were observed. In the dichloromethane fraction of EC_{50}, CC_{50} and maximum activity of 57.50 µg/mL, 100 µg/mL, and 64% were observed, respectively. The hexane fraction results show EC_{50}, CC_{50}, and maximum activity of 41.27 µg/mL, 51.92 µg/mL, and 89%, respectively. Chloroquine, approved by the Food and Drug Administration (FDA) with anti-SARS-CoV-2 activity in *in vitro* tests in 2020, was used as a control, with an EC_{50} of 2.18 µM and a CC_{50} of 50 µM, with a maximum activity of 104% (Table 7).

Table 7 – High Content Screening (HCS) of *Planktochlorella nurekis* extracts (methanol, dichloromethane, and hexane).

Compound	EC_{50}	CC_{50}	SI	Max. Activity (%)
Chloroquine as positive control (µM)	2,18	> 50,00	22,9	104
Methanol (µg/mL)	> 100,00	> 100,00	ND	42
Dichloromethane (µg/mL)	57,50	> 100,00	1,7	64
Hexane (µg/mL)	41,27	51,92	1,3	89

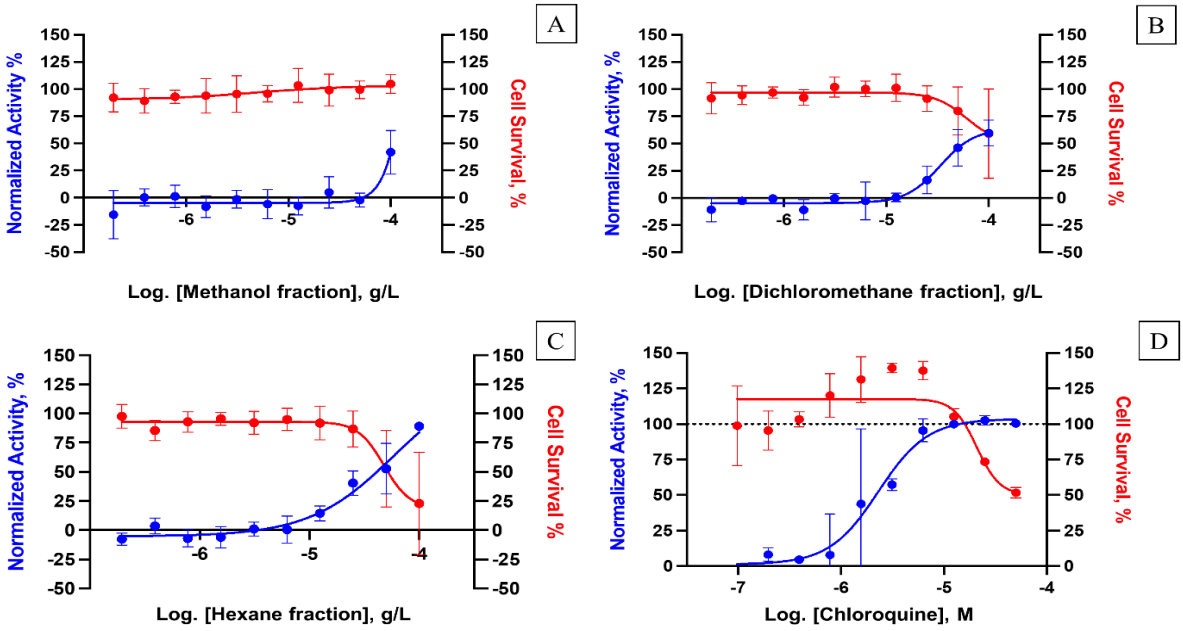
It was observed in the *in vitro* data that the hexane extract and dichloromethane showed superior virucidal potential to the methanol extract. The EC_{50} data at the concentrations tested, rule out the use of methanolic extract aiming at virucidal action, despite having low cellular toxicity (CC_{50}). Among the two extracts with virucidal potential (EC_{50}), the extract of *P. nurekis* with dichloromethane showed less toxicity than hexane. Despite the hexane extract being higher

in virucidal activity, the dichloromethane extract showed a higher selectivity index against the virus.

Selectivity index (SI) data were calculated for the control and treatment with the three extracts (Table 7), being estimative of compound selectivity toward the viral activity and representing the ratio between CC_{50} and EC_{50} (CC_{50}/EC_{50}). The control presented SI values of 22.9, while the hexane and dichloromethane extracts presented values of 1.3 and 1.7, respectively. The methanolic extract showed no activity against the virus.

The maximum activity (%) was higher for the hexanic extract when compared to the other extracts. However, the SI values were higher for dichloromethane. The factor linked to this can be related to the lower values found in CC_{50} for the hexanic extract, indicating a reduction in cell viability (Figure 5).

Figure 3 – Virucidal effect of methanol (A), dichloromethane (B), and hexane (C) extract from *Planktochlorella nurekis* against SARS-CoV-2.



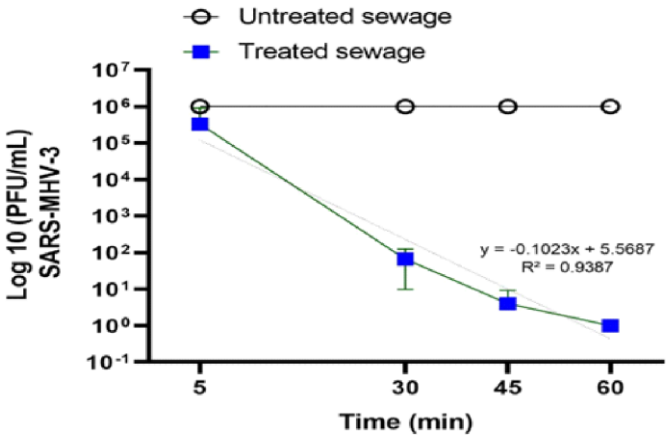
The solvents used in the present work follow an increasing order of polarity, hexane>dichloromethane>methanol, so the hexane extract will elute nonpolar compounds to a greater extent, while methanol will elute polar compounds (HEJAZI; KLEINEGRIS; WIJFFELS, 2004; REIS et al., 2022a). Dichloromethane is a non-polar solvent, eluting non-polar substances in a more significant proportion and substances of medium polarity in smaller proportions. Biochemical characterization of this dichloromethane extract demonstrated that carotenoids and organic acids are the main compounds with bioactive effects against

coronavirus strains (MICHELON et al., 2022; REIS et al., 2022a). Considering the results obtained in the cytotoxicity tests on the L929 and Vero cell lines, the dichloromethane extract with *P. nurekis* (PNDE) was used in the MHV-3 inactivation tests in human sewage.

3.2 CORONAVIRUS INACTIVATION IN HUMAN SEWAGE

The inactivation of MHV-3 in PNDE-treated human sewage and untreated human sewage was compared in the function of the contact time. The PNDE promotes significant inactivation ($p = 0.0016$) of MHV-3 in human sewage, reducing 4, 5, and 6 Log10 after 30-, 45-, and 60-min exposure, respectively (Figure 4). The MHV-3 is a coronavirus used as a common surrogate for SARS-CoV, as in phylogenetic analysis, they have already been reported as close relatives (LIÒ; GOLDMAN, 2004); their survival in water was 25 °C for 17 days and at 4 °C for 14 days, in sewage at 25 °C for 21 days and at 4 °C for 35 days (CASANOVA et al., 2009), showing its survival ability. Promoting the acceleration of viral inactivation in human sewage can improve sanitary quality and control viral pathogens transmitted via fecal-oral route and aerosolization.

Figure 4 – Reduction of MHV-3 in human sewage after PNDE treatment.



As for the probability of MHV-3 inactivation in treated and untreated human sewage as a time function (min), according to Figure 4, there is no inactivation of MHV-3 during the first 60 min without treatment. However, in the presence of PNDE, there was accelerated inactivation of MHV-3, obtaining an inactivation coefficient (k) of 0.1023 PFU/min,

prospective by linear regression (R^2 = 0.93), which implies that 90% of virus reduction (T90) at 9.7 min after human sewage treatment with PNDE (T90 min = 1/k min^{-1}).

Inactivation of SARS-CoV was investigated in wastewater by Wang et al. (2005) using sodium hypochlorite and chlorine dioxide. Experiments suggested that the virus was wholly inactivated with 10 mg/L for 10 minutes or more using chlorine. Another study demonstrated that inactivation of SARS-CoV-2 viral RNA is possible with sodium hypochlorite (NaOCl) after 1.5 h of contact at a dosage of 6700 g/m^3 (ZHANG et al., 2020). However, the effects of sodium hypochlorite are harmful to the environment, as effluents from chemical reactions with organic matter give rise to organic chlorine compounds, such as adsorbable halogenated organic compounds on activated carbon (AOX), which are toxic to aquatic organisms and contaminants that persist in the environment (EMMANUEL et al., 2004). Furthermore, chlorinated products can be carcinogenic, harmful to human and animal health (COURI et al., 1982; KUMARI et al., 2015; AL-GHEETHI et al., 2020).

An alternative for the non-toxic removal of coronaviruses is using compounds of plant/algae origin. Seaweeds are bioresources with a broad spectrum of metabolites with high potential to be explored. Studies have indicated using micro and macroalgae with virucidal action against several microorganisms (REIS et al., 2022b). Cirne-Santos et al. (2018) demonstrated that when using ethanolic extract of the red algae *Osmundaria obtusiloba* against the *Zika virus*, there was significant inhibition of viral replication when treating Vero cells with different concentrations of the extract, generating an EC_{50} of 1.82 µg/mL. Furthermore, it is noteworthy that there was low cytotoxicity of the extract in the cells (CC_{50} of 525 µg/mL). Likewise, Sumayya, Lubaina and Murugan (2020) point out the virucidal effect of the purified terpenoid extract of the red algae *Gracillaria dura* against the *Chikungunya virus*, presenting an EC_{50} of 1.25 µg/mL, with inhibition of around 90% with a dosage of 5 µg/mL.

Currently, few studies are related to *Planktochlorella nurekis*, as it was defined in a new genus and species belonging to the Parachlorella clade in Chlorellaceae in 2014, being commonly identified as *Chlorella* sp. (ŠKALOUD et al., 2014). *P. nurekis* is a unicellular, autotrophic green alga with uninuclear, spherical, and planktonic vegetative cells (ČERMÁK et al., 2015).

In the biochemical characterization study of the dichloromethane extract of *P. nurekis* carried out previously (REIS et al., 2022a), it was verified that it mainly presented carotenoid compounds (mainly β-carotene and lutein), polyphenols, and organic acids. In addition, with

the aid of nuclear magnetic resonance (NMR), it was found that the dichloromethane extract had the highest concentration and more diversity of metabolites. From the analysis of Liquid Chromatography-Ultraperformance Mass Spectrometry (UPLC-MS) it was verified that 18 secondary metabolites were found in the dichloromethane extracts of *P. nurekis*, higher than that found in other extracts. It was also found that palmitic and stearidonic acids present in the lipid class were only found in this type of extract (REIS et al., 2022a), demonstrating its potential source of compounds with virucidal and antiviral action.

These compounds have attracted attention for their antitumor, antiviral, anticoagulant, and antioxidant activities (WANG; WANG; GUAN, 2012; WANG et al., 2018; BESEDNOVA et al., 2019). In addition to their broad-spectrum antiviral activities, they also have low cytotoxicity *in vitro* and *in vivo* (BESEDNOVA et al, 2019). However, its potential lacks further supporting studies to promote its application in the pharmaceutical industry (KUMAR JHA; ZI-RONG, 2004). One of the major bottlenecks in using these compounds is the lack of studies *in vivo* and in the environment, which prove the antiviral effects of algae are carried out only *in vitro* studies (ROSA et al., 2019). There is an urgent need for further investigations to advance this application-oriented area.

4 CONCLUSION

Planktochlorella nurekis extracts showed low cytotoxicity in L929 and Vero cell lines. Considering all the parameters analyzed in the *in vitro* test, the extract of *P. nurekis* with dichloromethane showed less cellular toxicity and a higher selectivity index against the virus, therefore, the viral inactivation test was performed in human sewage. The results pointed to the possibility of a product with high efficacy against the murine coronavirus, and low toxicity to animal cells for a better environmental context. These results reinforce the role of microalgae as essential sources of natural compounds applied as accelerators for the inactivation of the coronavirus in contaminated human sewage. Thus, this research could serve as a basis for the development of SARS-CoV-2 inactivation products in sewage, as well as strategies for practical projects to combat the survival and possible transmission of the virus in sewage and its aerosolization.

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4 CONSIDERAÇÕES FINAIS

Os compostos presentes nas algas apresentam diversas atividades biológicas, entre essas, virucida e antiviral. Os bioativos encontrados nesses organismos são explorados para utilização biotecnológica, visando o desenvolvimento de bioprodutos para o mercado. Com o avanço das epidemias e pandemias, como a SARS-CoV-2 que atingiu todo o mundo no ano de 2019, compostos naturais que apresentam atividades virucidas e antivirais tem sido cada vez investigados, por não serem prejudiciais aos seres humanos e ao meio ambiente. Assim, a intensificação de pesquisas com microalgas tem se mostrado efetiva para a demanda existente.

O extrato da microalga *Planktochlorella nurekis* exibiu atividade virucida, devido ao perfil bioquímico apresentado. Os polifenóis, carboidratos e derivados de isopreno, como compostos terpênicos e carotenoides, apontaram maior correlação com o potencial virucida quando testado frente ao SARS-CoV-2. Além disso, resultados apontam que os extratos de *P. nurekis* são mais efetivos quando utilizados extratores de polaridade intermediária, como o diclorometano. Uma das hipóteses para explicar essa atividade se dá pela baixa citotoxicidade e maior índice de seletividade desses extratos em linhagem celular L929 e Vero.

Assim como a disseminação primária do COVID-19 por meio de aerossolização, esse vírus também foi detectado em esgoto humano em diversos estudos ao redor do mundo. Dessa maneira, bioprodutos que almejam o contexto ambiental são de extrema importância como estratégia para combate à sobrevivência e transmissão do vírus. O extrato de *P. nurekis* demonstrou efetividade na aceleração da inativação de coronavírus murino em esgoto humano contaminado, o que pode servir para que novos estudos sejam desenvolvidos considerando o SARS-CoV-2.

Os resultados encontrados na presente tese doutoral apontam que os extratos da microalga *P. nurekis* são importantes fontes de compostos naturais, que podem ser empregados como aceleradores na inativação do coronavírus em esgoto humano contaminado e para fins clínicos, apresentando baixa toxicidade celular e alto índice de seletividade. Neste sentido, ressalta-se a importância de novos estudos *in vivo* e pré-clínicos, de modo a estabelecer a efetividade antiviral e virucida dos extratos da microalga *P. nurekis*. Além disso, destaca-se que os extratos dessa microalga apresentam metabólitos de relevância que poderiam ser empregados em pesquisas antitumorais e que precisam ser mais bem estudados.

APÊNDICE (APPENDIX)

Figure S1 – Representative 1D ^{1}H NMR spectra of the *Planktochlorella nurekis* extract in hexane (A), dichlorometane (B), and methanol (C).

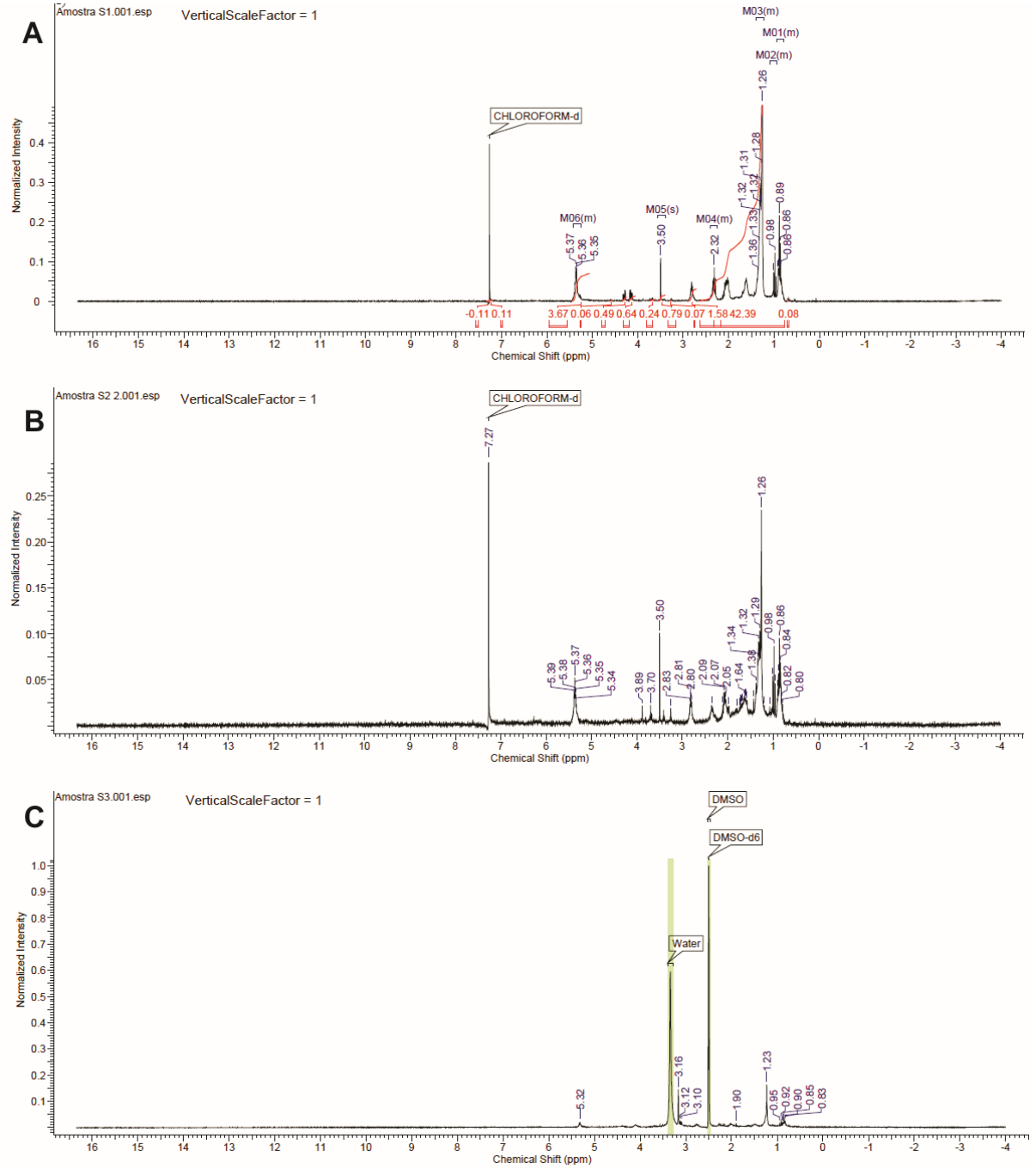


Figure S2 – UPLC-ESIMS profile of the hexane extract.

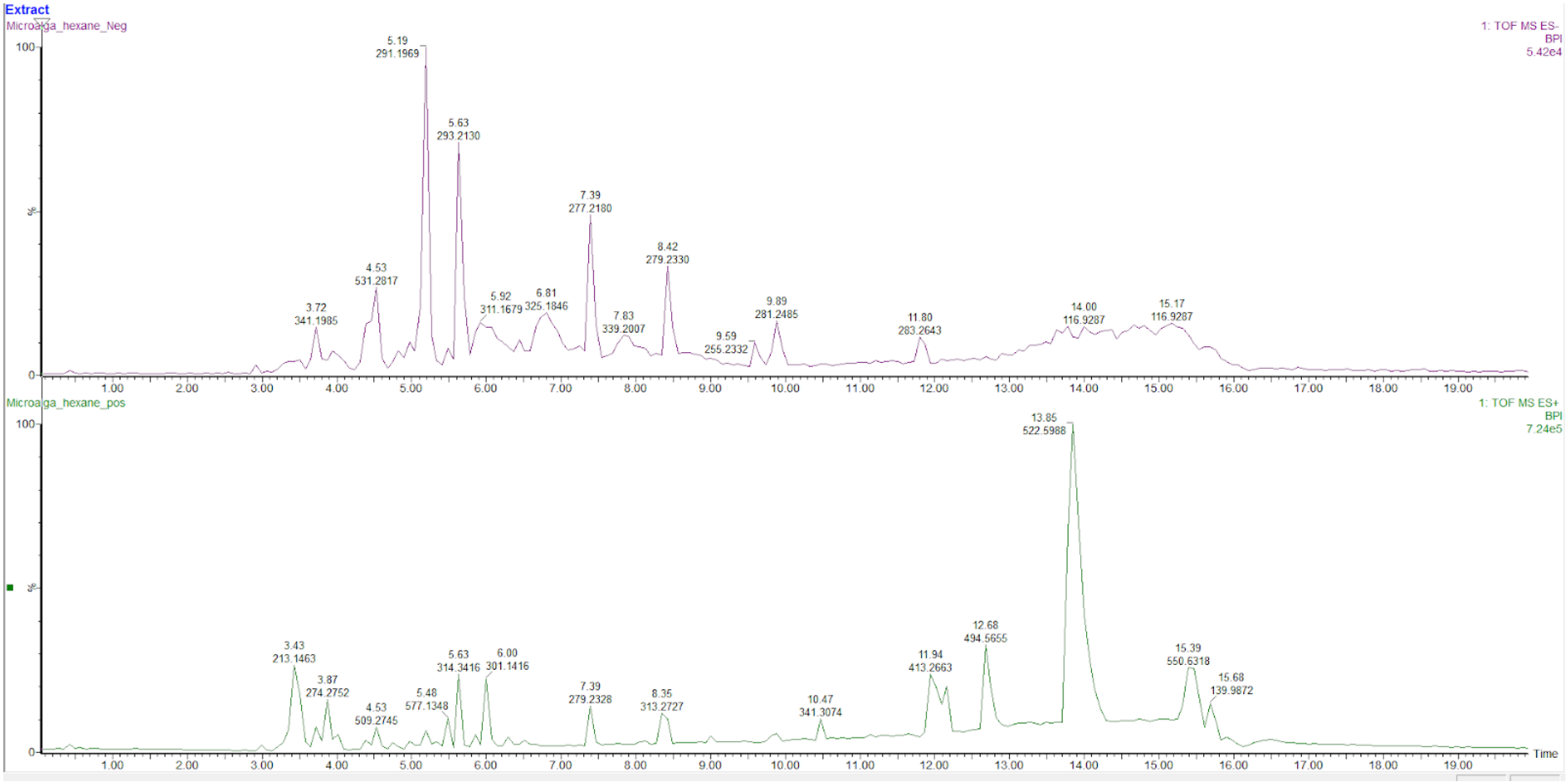


Figure S3 – UPLC-ESIMS profile of the DCM extract.

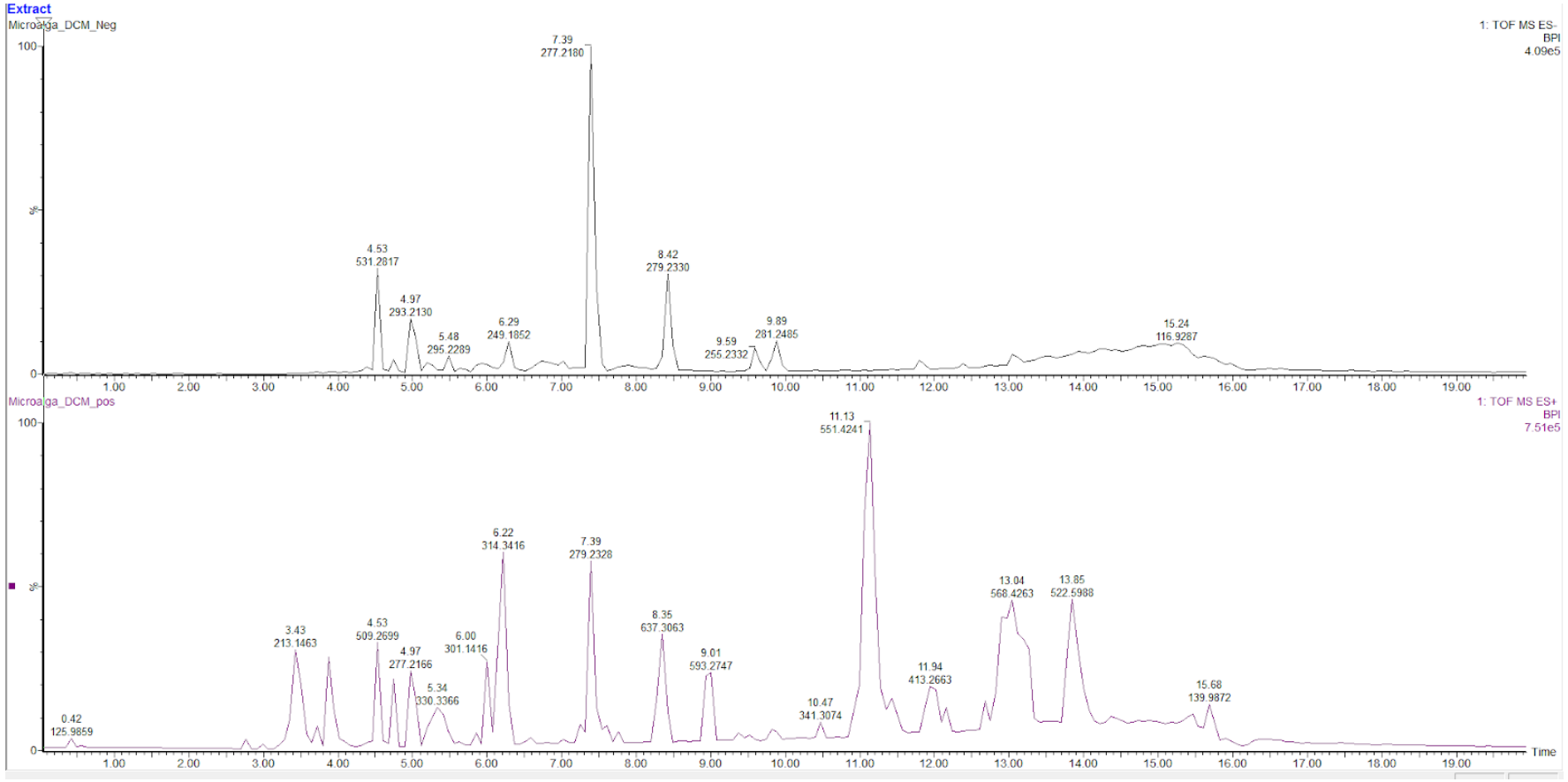


Figure S4 – UPLC-ESIMS profile of the methanol extract.

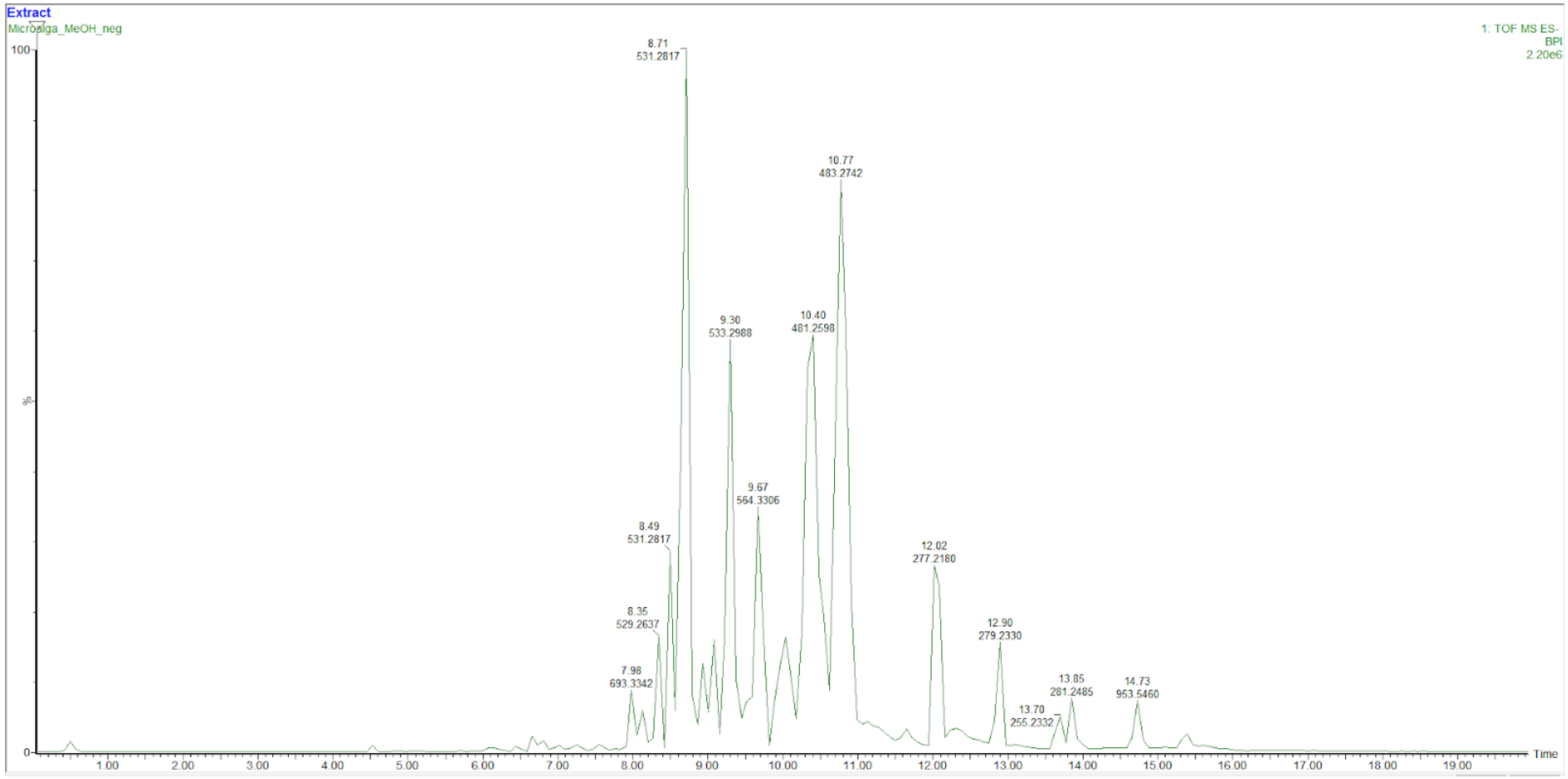
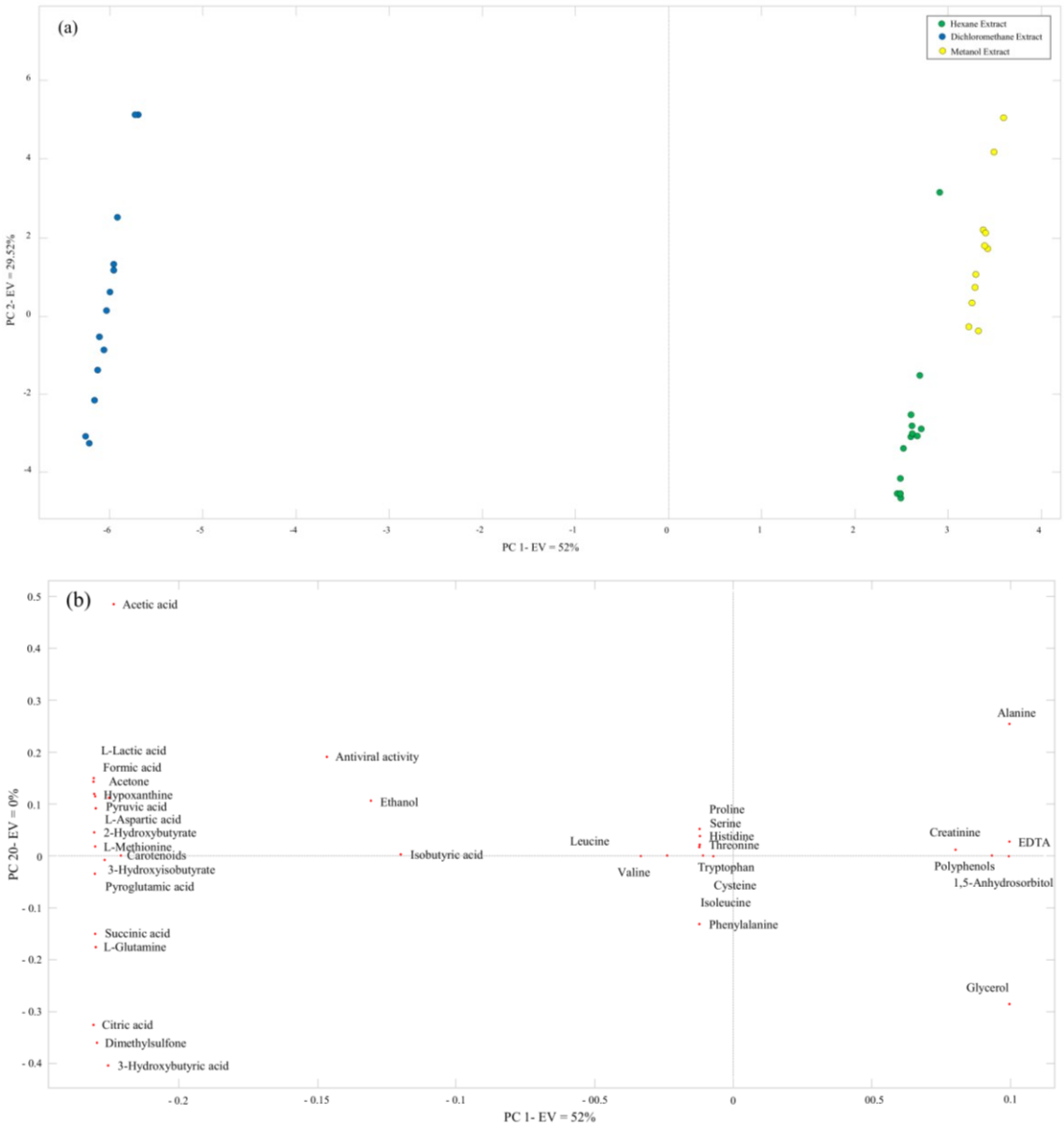


Table 1 – UPLC-ESIMS chemical composition of hexane, DCM, and methanol fractions of the algae.

				ESI-MS-(-)		Extract	
	tR	Peak (m/z) and MF	Error	Fragment ions (m/z)	Proposed structures	Hexane	DCM
1	4.53	531.2817 $[C_{25}H_{42}O_9+HCO_2]^-$ 485.2748 $[C_{25}H_{42}O_9-H]^-$	2.19 -0.53	-	(7Z,10Z,13Z)-2-hydroxy-3-(β-D-galactopyranosyl)oxy)propyl hexadeca-7,10,13-trienoate	X	X
2	4.97	293.2130 $[C_{18}H_{30}O_3-H]^-$	4.54	275.2035	13-hydroxyoctadecatrienoic acid	X	X
3	5.48	295.2289 $[C_{18}H_{32}O_3-H]^-$	5.34	-	Vernolic acid	-	X
4	6.29	249.1852 $[C_{16}H_{26}O_2-H]^-$	-1.02	-	Hexadecatrienoic acid	-	X
5	7.39	277.2180 $[C_{18}H_{30}O_2-H]^-$	4.49	-	Linolenic acid	X	X
6	8.42	279.2330 $[C_{18}H_{32}O_2-H]^-$	2.13	-	Linoleic acid	X	X
7	9.59	255.2332 $[C_{16}H_{32}O_2-H]^-$	3.11	-	Palmitic acid	-	X
8	9.89	281.2485 $[C_{18}H_{34}O_2-H]^-$	1.58		Oleic acid	X	X
				ESI-MS-(+)			
9	3.72	181.1233 $[C_{11}H_{16}O_2+H]^+$	2.46	163.1130	4,4,7a-trimethyl-5,6,7,7a-tetrahydrobenzofuran-2(4H)-one	-	X
1	4.53	509.2745 $[C_{25}H_{42}O_9+Na]^+$	3.63	469.2790, 325.2374, 233.1918	(7Z,10Z,13Z)-2-hydroxy-3-(β-D-galactopyranosyl)oxy)propyl hexadeca-7,10,13-trienoate	X	X
10	4.97	277.2166 $[C_{18}H_{28}O_2+H]^+$	-0,56		Stearidonic acid	-	X
11	5.48	577.1348 $[C_{30}H_{24}O_{12}+H]^+$	0.34	385.0938	Related to proanthocyanidin A1	X	-
12	6.00	301.1416 $[C_{16}H_{22}O_4.Na]^+$	0.07	205.0866, 149.0244	Dibutyl phthalate	X	X
5	7.39	279.2328 $[C_{18}H_{30}O_2+H]^+$	1.41	-	Linolenic acid	X	X
13	8.35	637.3063 $[C_{29}H_{48}O_{15}+H]^+$	-1.33	581.2450, 525.1830, 495.26262, 469.1125	Derivative of O-tetrapropanoyloctanoate of sucrose	-	X
14	8.35	331.2852 $[C_{19}H_{38}O_4+H]^+$	1.10	313.2727, 239.2368	monopalmitin	X	-
15	9.01	593.2747 $[C_{34}H_{40}O_9+H]^+$	-0.6	533.2573	unidentified	-	X
16	10.47	359.3179 $[C_{21}H_{42}O_4+H]^+$	4.91	341.3074, 267.2713	monostearin	X	X
17	11.13	551.4241 $[C_{40}H_{54}O+H]^+$	-2.16	-	Echinenone	-	X
18	11.43	607.2911 $[C_{33}H_{44}O_9+Na]^+$	4.61	547.2725	unidentified	-	X
19	11.94	413.2663 $[C_{24}H_{38}O_4+Na]^+$	-1.16	301.1410, 149.0244	Dioctyl phthalate	X	X
				Methanol Extract - ESI-MS-(-)			
20	7.98	693.3342 $[C_{31}H_{50}O_{14}+HCO_2]^-$	1.21	647.3315[M-H], 249.1852	3-hydroxy-2-[[(7Z,10Z,13Z)-1-oxo-7,10,13-hexadecatrienyl]oxy]propyl 6-O-β-D-galactopyranosyl-β-D-Galactopyranoside		
21	8.35	529.2637 $[C_{25}H_{40}O_9+HCO_2]^-$	-2.24	483.2607 [M-H], 247.1671	3-hydroxy-2-[[-1-oxo-4,7,10,13-hexadecatetraenyl]oxy]propyl β-D-Galactopyranoside		
22	8.49	531.2817 $[C_{25}H_{42}O_9+HCO_2]^-$	2.19	485.2748, 249.1852	3-hydroxy-2-[[-1-oxo-7,10,13-hexadecatrienyl]oxy]propyl β-D-Galactopyranoside		
23	8.71	531.2817 $[C_{25}H_{42}O_9+HCO_2]^-$	2.19	485.2748, 249.1852	Isomer of compound 22		
24	8.93	721.3671 $[C_{33}H_{54}O_{14}+HCO_2]^-$	3.38	675.3586, 277.2180	2-hydroxy-3-[[(9Z,12Z,15Z)-1-oxo-9,12,15-octadecatrien-1-yl]oxy]propyl 6-O-β-D-galactopyranosyl-β-D-Galactopyranoside,		
25	9.30	533.2988 $[C_{25}H_{44}O_9+HCO_2]^-$	4.90	487.2931, 251.2029	(2S)-2-hydroxy-3-[[(7Z,10Z)-1-oxo-7,10-hexadecadien-1-yl]oxy]propyl β-D-Galactopyranoside		

26	10.03	699.3804 $[C_{31}H_{58}O_{14}+HCO_2]^-$	0.13	255.2332			1−O−palmitoyl−3−O−[α−D−galactopyranosyl (1→6)−β−D−galactopyranosyl]−sn−glycerol	
27	10.40	481.2598 $[C_{29}H_{38}O_6-H]^-$	1.63	281.2485, 255.2332			Steroid	
28	10.77	483.2742 $[C_{29}H_{40}O_6-H]^-$	-0.96	255.2332			Steroid	
29	12.02	277.2180 $[C_{18}H_{30}O_2-H]^-$	4.49	-			Linolenic acid derivative	
30	12.90	279.2330 $[C_{18}H_{32}O_2-H]^-$	2.13	-			Linoleic acid derivative	
31	13.70	255.2332 $[C_{16}H_{32}O_2-H]^-$	3.11	-			Palmitic acid derivative	
32	13.85	281.2485 $[C_{18}H_{34}O_2-H]^-$	1.58				Oleic acid derivative	
33	14.73	953.5460 $[C_{49}H_{90}O_{15}+HCO_2]^-$	-1.44	907.5478 277.2180	[M-H]-,	249.1852,	[[-1-oxo-9-hexadecatrien-1-yl]oxy]-3-[[-1-oxo-octadecatrien-1-yl]oxy]propyl galactopyranosyl-β-D-Galactopyranoside	6-O-α-D-

Figure S5 – PCA analysis of the *Planktochlorella nurekis* metabolites action against murine Coronavirus (a) Score plot *Planktochlorella nurekis* extracts (b). Loading plot of the and biomarkers and bioactivities.



ANEXO 1 – PEDIDO DE PATENTE

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

03/12/2021 870210112693 17:04

29409161939519550

Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

Número do Processo: BR 10 2021 024541 7

Dados do Depositante (71)

Depositante 1 de 1

Nome ou Razão Social: UNIVERSIDADE FEDERAL DE SANTA CATARINA

Tipo de Pessoa: Pessoa Jurídica

CPF/CNPJ: 83899526000182

Nacionalidade: Brasileira

Qualificação Jurídica: Instituição de Ensino e Pesquisa

Endereço: CAMPUS UNIVERSITÁRIO REITOR JOÃO DAVID FERREIRA LIMA, BAIRRO TRINDADE

Cidade: Florianópolis

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PETICIONAMENTO ELETRÔNICO

Esta solicitação foi enviada pelo sistema Peticionamento Eletrônico em 03/12/2021 às 17:04, Petição 870210112693