СОФИЙСКИ УНИВЕРСИТЕТ
„СВ. КЛИМЕНТ ОХРИДСКИ“
БИОЛОГИЧЕСКИ ФАКУЛТЕТ

SOFIA UNIVERSITY
St. KLIMENT OHRIDSKI
FACULTY OF BIOLOGY

PhD Thesis Abstract

for acquiring the educational and science degree “Doctor”
in the professional field 4.3. Biological Sciences,
scientific specialty: Microbiology

MILENA NIKOLOVA PETROVA

Development of Biologically Active Products from New Natural Sources

Supervisors: prof. Petya Hristova

assoc. prof. Ganka Chaneva

Sofia, 2022

The dissertation contains 160 pages of text in A4 format, 41 tables and 26 figures. The appendices are 21 pages containing 12 tables and 28 figures. The references include 477 scientific titles.

The experimental work was carried out in the Department of "General and Industrial Microbiology" at the Faculty of Biology of Sofia University "St. Kliment Ohridski", Laboratory "Experimental Algology" of the Institute of Plant Physiology and Genetics at the Bulgarian Academy of Sciences and Agrobioinstitute at the Agricultural Academy.

The dissertation was discussed at an extended meeting of the Department of "General and Industrial Microbiology" at the Faculty of Biology of Sofia University "St. Kliment Ohridski", held on 28.06.2022 (protocol No. 160). The scheduled final defense by order No. …….. of the Rector of Sofia University "St. Kliment Ohridski" has a scientific jury consisting of:

1. Prof. Dr. Penka Angelova Moncheva - SU "St. Kliment Ohridski"
2. Prof. Svetla Trifonova Danova - Institute of Microbiology, BAS
3. Assoc. Dr. Miroslava Konstantinova Zhiponova - SU "St. Kliment Ohridski"
4. Associate Professor Dr. Adriana Georgieva Gushterova – Institute of Microbiology, BAS
5. Associate Professor Dr. Plamen Stoynev Pilarski, IFRG, BAS

The official reviewers are:

…….

……..

The defense of the dissertation will be held on ……..... at ……. in the building of the Faculty of Biology of Sofia University “St. Kliment Ohridski".

The defense materials are available on the website of the Faculty of Biology and on paper in the Department of "General and Industrial Microbiology".

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INTRODUCTION

Microbial and viral infections lead to both public health problems and economically significant losses (eg when affecting crops and the food industry in general). For that reason, they are significant challenge for the scientific community. According to the Activity Plan of the National Reference Center “Food and Waterborne Infections” in Bulgaria from 2021 "the food and waterborne infectious diseases are a global health problem and it is second in frequency after the respiratory infections". Most of the diseases are in the gastrointestinal tract and are "transmitted orally through the consumption of contaminated food".

Mankind currently has a limited number of drugs for systematic usage. A major problem is the emergence of strains that are resistant to one or more of the limited amounts of synthetic antibiotics and drugs. Dealing with resistant bacterial strains leads to the constant need for new drugs (Newman et al., 2003; Koehn and Carter, 2005). One of the reasons for the emergence of resistant bacteria is the uncontrolled usage of antibiotics not only for the treatment of humans, but also in veterinary practice and the agriculture. Thus, the entry and use of drugs in food has an indirect effect on the human health. Therefore, the scientific community is paying attention not only to human pathogenic bacteria, but also to the food quality issues and the agriculture.

The reduction or the complete loss of agricultural production is due to various reasons. One of them is the lack of sufficiently effective protection of plants from diseases caused by plant phytopathogenic bacteria. Pesticides are one of the main weapons for pest control in agriculture, but the main disadvantage of their massive usage is that they adversely affect the environment - their excessive use can pose a risk to animal and human health. For example, sulfur and serovar solutions are the most commonly used to control apple scab, but they are phytotoxic to plants and, although not carcinogenic, can cause respiratory problems in humans. Some scientists even claim that diseases like Parkinson's have increased as a result of prolonged exposure to such products (Bonetta, 2002; Freire and Koifman, 2012), diabetes and some cancer types (Jungmann, 1966; Roth, 1958), endocrine disorders, neurotoxicity, asthma (Hernandes et al., 2011) and even obesity (Thayer et al., 2012). According to EPA 2010 more than 70 pesticides in use are classified as potentially carcinogenic. The carcinogenic effect of

pesticides has been discussed in detail by Mostaflou and Abdollahi (2013). The environment is also affected by the usage of synthetic pesticides that accumulate in the soil and pollute the groundwater (Rashid et al., 2010; Harizanova-Bartos and Stoyanova, 2019; Terziev and Petkova-Georgieva, 2020; Tamm et al., 2022).

There are many worrying trends around the discussed problems in the recent years - a significant increase in pesticide pollution, on the one hand, and the emergence of increasingly resistant bacterial strains causing infections, on the other. There is also a danger of cross-pathogens - human pathogens that integrate into a plant or animal microbiome (Van Baarlen et al., 2007; Христова, 2020). Bacteria that contaminate food infect humans and cause foodborne illnesses such as diarrhea (Shannon and Abu-Ghannam, 2016). Infected foods include bacteria and bacterial spores, microscopic fungi, viruses, yeast, etc.

It can be argued that the growing resistance of harmful bacteria also increases the need to increase the use of pesticides. This dependence is very worrying and it is quite possible to reach very unpleasant consequences for agriculture in general. For that reason, in the recent years there was a significant increase in the interest on studies of the biological effects of natural compounds against bacterial and / or fungal infections.

MAIN OBJECTIVE AND TASKS

The main objective of the dissertation is the research and the evaluation of the antioxidant and antimicrobial activity of bioactive substances acquired from new natural sources - products of microalgae and invertebrates (hemocyanin).

For the completion of the defined objective the dissertation defines the following **tasks**:

1. Selection, isolation, identification and laboratory cultivation of microalgae strains;
2. Preparation of extracts and exopolysaccharides from selected microalgae strains;
3. Determination of the antioxidant activity of the selected microalgae products;
4. Determination of the antimicrobial activity of the selected microalgae extracts and exopolysaccharides against human and plant pathogens;
5. Determination of the antimicrobial activity of the combination between microalgae extracts;
6. Determination of the antimicrobial activity of natural antibiotics against phytopathogens;
7. Determination of the antimicrobial activity of hemocyanin against some human pathogens.

MATERIALS AND METHODS

1. Materials

The dissertation uses three different types of microalgae – cyanobacteria: *Arthronema africanum* Lukavský 1981/01, *Nostoc commune* Vaucher and *Chlorella*-like microalgae isolate (*CLM-A1*).

***A. africanum* Lukavský 1981/01** was provided by dr. Jaromir Lukavski from the Institute of Botany at the Czech Academy of Sciences. In Bulgaria it is stored in the collection of the Laboratory "Experimental Algology" in Bulgarian Academy of Sciences in Zehnder nutrient medium (Staub, 1961) under constant illumination with fluorescent lamps (at 2000 lx) in 100 ml Erlenmeyer flasks.

***N. commune* Vaucher** was isolated from a natural sample from rock surface in the region of Rupite in Bulgaria. It was collected by assoc. prof. Dr. Plamen Pilarski in April 2019. The procedure for purification of the sample was performed by us together with PhD student Tanya Toshkova-Yotova from the laboratory "Experimental Algology" from Bulgarian Academy of Sciences. The strain was identified taxonomically as *N. commune* Vaucher ex Bornet et Flahault, 1888, by assoc. prof. Detelina Belkinova from Bulgarian Academy of Sciences.

The third strain (*CLM-A1*) belongs to the green microalgae and belongs to the group of *Chlorella*-like microalgae. It was isolated and identified by us with the scientific assistance of assoc. prof. Blagoy Uzunov from the Department of Botany at the Faculty of Biology at Sofia University "St. Kliment Ohridski" and assistant-professor dr. Katya Stefanova from the Agrobioinstitute.

The following strains of test microorganisms were used to determine the antimicrobial activity:

Food isolates: *Escherichia coli*, *Bacillus* sp., *Listeria* sp., *Enterococcus* sp., *Staphylococcus* sp.; Strain isolated from contaminated water: *Pseudomonas aeruginosa*; Strains of 4 species of phytopathogenic bacteria: *Xanthomonas gardneri* 62t, *Xanthomonas gardneri* 64t, *Xanthomonas euvesicatoria* 105d, *Xanthomonas euvesicatoria* 269p, *Xanthomonas vesicatoria* 68t, *Xanthomonas vesicatoria* 60t, *Pseudomonas syringae* pv. *tomato 32f*. All strains are identified and are part from the collection of the Department of General and Industrial Microbiology.

The following collection microorganisms were used as controls:

Human pathogens: Gram-positive bacteria: *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* NBIMCC 3486, *Staphylocuccus epidermidis* NBIMCC 1093, *Enterococcus faecalis* NBIMCC 3915, *Bacillus subtilis* NBIMCC 3562, *Bacillus cereus* NBIMCC 1085, *Listeria innocua* NBIMCC 8755, *Propionibacterium acnes* PA266; Gram-negative bacteria: *Escherichia coli* ATCC 25923, *Esherishia coli* UPEC NBIMCC 8954, *Esherishia coli* EPEC clinical isolate, *Esherishia coli NBIMCC* 3397, *Salmonella typhimurium* NBIMCC 3669, *Salmonella enterica* NBIMCC 8691, *Proteus mirabilis* NBIMCC 8747, *Pseudomonas aeruginosa* NBIMCC 3700, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* NBIMCC 3670; yeast: *Cryptococcus neoformans*, *Candida glabrata* NBIMCC 8656 (ATCC 2001), *Candida albicans* NBIMCC 74 (ATCC 10231).

Phytopathogenic bacteria: Gram-negative - *Xanthomonas gardneri* NBIMCC 8730, *Xanthomonas euvesicatoria* NBIMCC 8731, *Xanthomonas vesicatoria* NBIMCC 2427, *Xanthomonas perforans* NBIMCC 8729, *Pseudomonas syringae* pv. *tomato* NBIMCC 3374, *Burkholderia gladioli* pv. *alliicola* NBIMCC 8616, *Burkholderia gladioli* pv. *g*ladioli NBIMCC 8569, *Erwinia amylovora* NBIMCC 8492*;* Gram-positive: *Clavibacter michiganense* NBIMCC 2425.

For the cultivation of *A. africanum* we used a food medium according to Allen and Arnon (1955) and Zehnder (Staub, 1961), modified by Chaneva et al. (2007). For the preparation of an enriched culture of *Chlorella*-like microalgae and for the cultivation of *N. commune* we used food medium BG11 as defined by Hughes (1958) and modified by Allen (1968).

2. Methods

The cultivation of *A. africanum* was performed under sterile conditions and using the facilities described by Dilov (1985). The purification of the natural sample *N. commune* is made according to the method described by Diao and Yang (2014) in the modified variant by Guillard (2005). The purification of CLM-A1 was made using serial drop dilutions in liquid medium BG11 and after that it was was cultured in a shaker and transferred to fermentation flasks.

Cultural medium (CM) is obtained from the microalgae suspension in a stationary growth phase. It was centrifuged at 5000 rpm for 10 min. The separated supernatant is

then lyophilized and the resulting product is dissolved in sterile distilled water by the required concentrations.

Exopolysaccharide (EPS) was obtained by precipitating the extracellular extract with 96% pure ethanol at a 1:2 ratio for 24 hours. Then the liquid was centrifuged at 5000 rpm for 10 minutes. The EPS fraction was isolated from the supernatant by precipitation with cooled 96% ethanol in a ratio of 1:2 for another 24 hours. The isolated extracellular polysaccharide was dialyzed for 24 hours and lyophilized. The obtained product is dissolved in ethanol in the required concentrations (diluted with distilled water).

High temperature (HT) aqueous extract is obtained by heating at high temperature, using methodology developed in the Laboratory "Experimental Algology" at Bulgarian Academy of Sciences.

Low temperature (LT) aqueous extract is obtained by lyophilized biomass. It was poured with boiling sterile distilled water in a 1:10 ratio and cooled to 40°C. Then the mixture is placed in a refrigerator (4°C) for 48 hours (with periodical stirring). Following is the separation of cell mass by centrifugation at 5000 rpm for 10 min. Finaly the aqueous extract was lyophilized and the resulting lyophilisate was diluted with distilled water in the required concentrations.

Alcohol extract (AE) is obtained by extraction with 96% ethanol and heating at 50°C. The extract is filtered and evaporated to dryness on a rotary evaporator at 60°C and the dried product is dissolved in DMSO at the desired concentrations.

Isolation of **endo-polysaccharide** – we add H_2O to the biomass and blend for 5 minutes. The substance is centrifuged on 3000 rpm for 10 minutes. The endo-polysaccharide fraction is extracted from the supernatant by precipitation with 96% ethanol in a ratio of 1:2 for 24 hours. The isolated endo-polysaccharide was dialyzed for 24 hours and freeze-dried.

For the hemocyanin the hemolymph is extracted from sea crabs *Eriphia verrucosa*, which are collected in the region of Kamchia, Black Sea. It was provided by the Laboratory of Structural Organic Analysis at the Institute of Organic Chemistry with the Center of Phytochemistry in the Bulgarian Academy of Sciences. The samples include native hemocyanin CpH and its subunits SU1, SU2, SU3, SU4 and SU5.

The determination of absolute dry matter is performed by its weight after removal of salts from the medium by 11.5% CH_3COOH and centrifugation at 5500 rpm.

Subsequently the supernatant is separated and the biomass is dried at 105°C until it reached constant weight. For the total protein content, we used colorimetric method by Lowry (1951). The total carbohydrate content of the algal biomass is determined by the phenol-sulfur method of Dubois et al. (1956). The lipid content was determined by an adapted method of Petkov (1990).

The content of plastid pigments (chlorophyll *a*, chlorophyll *b* and carotenoids) in the microalgae samples were determined spectrophotometrically by two methods - extraction with boiling methanol and extraction with acetone.

The total phenol content was determined by the method of Singleton et al. (1999).

The total flavonoid content was determined by the method of Chang et al. (2002) by reaction with $AlCl_3$.

The DPPH scavenging activity of the extracts was determined by the method of Brand-Williams et al. (1995).

The total antioxidant activity was determined by the method of Prieto et al. (1999).

The antimicrobial activity is determined by different methods: the agar wells by Rose and Miller (1939), the disk-diffusion method of Bayer and Kirby (Bauer et. al., 1966), minimum inhibitory concentration (MIC) by the method of micro-dilution in a liquid medium (Hendriksen and Larsen, 2003), and maximum bactericidal concentration (MBC) method as described by Sampaio et al. (2009).

DNA isolation for CLM-A1 was performed with a DNA isolation kit – Blood - Animal-Plant DNA by the company Jana Bioscience. The following universal primer pairs were used to amplify 18S rDNA: ITS1+ ITS2, ITS1+ ITS4. For chloroplast DNA we used rbcL-F+ rbcL-R, tuf A-F+ tuf A-R. PCR products were purified by The thermo Scintific GenJetGel Extraction Kit K#0692. The purified PCR products were sent for sequencing in concentration 53,4 ng/µl. The sequencing was performed according to a protocol of the company Macrogen (Netherlands). The resulting sequences were analyzed with the programs vector NT1 v.10 (Life Technologies), BLASTn (Camacho et al., 2009) and Clustal W.

Regarding the processing of the results, all experiments were performed in a minimum of 3 replicates. They are presented as means and standard deviations (±SD). Statistically significant difference between individual experiments was determined by one-way analysis of variance (ANOVA). Values of $p \leq 0.05$ were considered significant.

RESULTS AND DISCUSSION

1. Isolation and cultivation of strains

A. africanum is a strain provided by the collection of the Institute of Botany, Trebon, Czech Academy of Sciences. Nostoc, which is used in the present dissertation, is isolated from the Rupite region in Bulgaria and is natural, i.e., not completely purified to algologically pure culture. The cultivation of both strains was performed in an intensive cultivation unit, under conditions suitable for the respective strain.

FIG. 1. Cultivation of microalgae strain (*CLM-A1*)

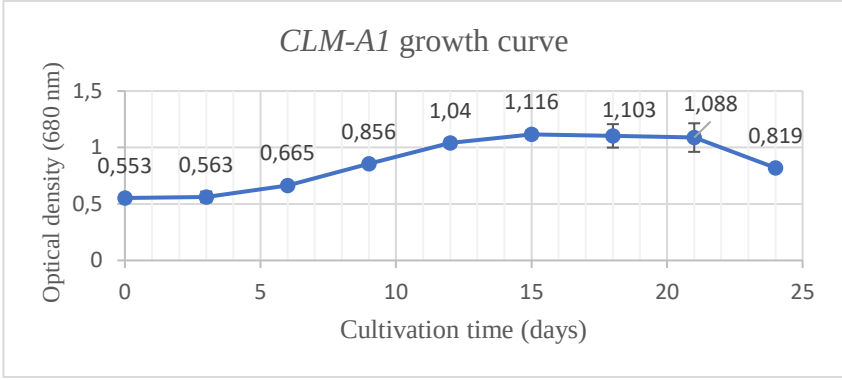


FIG. 2. Growth curve of *CLM-A1*

For the isolation of *CLM-A1*, the classical procedure was used, in which an enriched culture of microalgae was first obtained and then diluted in liquid nutrient medium BG11. Cultivation was performed in Erlenmeiler flasks (FIG. 1). The growth curve is shown in FIG. 2.

2. Strains identification

The identification of *Nostoc* is based on phenotypic characteristics and was determined by Assoc. Prof. Dr. Detelina Belkinova from the Bulgarian Academy of Sciences. The strain is taxonomically defined as *N. commune* Vaucher.

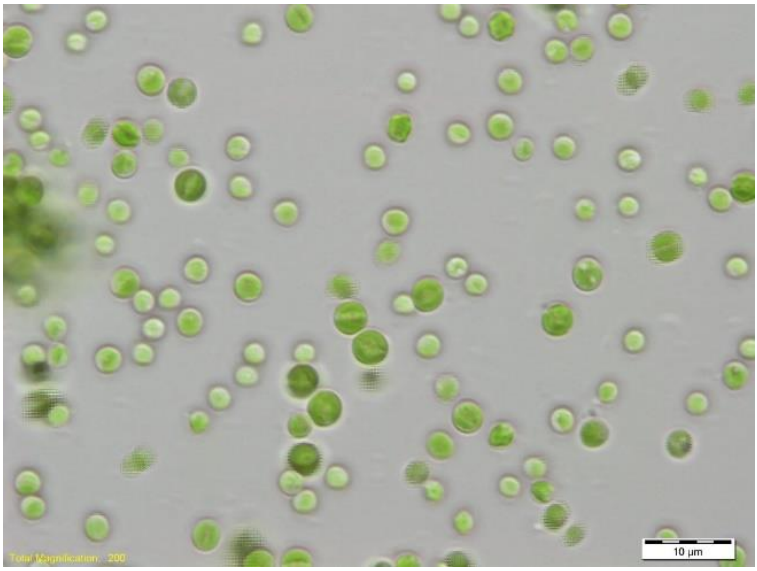


FIG. 3. Microscopic image of *CLM-A1* cells, 40 x (Olympus DP72)

CLM-A1 was phenotypically studied with the support of Assoc. Prof. Dr. Blagoy Uzunov from the Department of Botany at Sofia University "St. Kliment Ohridski". Based on its morphological characteristics, the lack of pyrenoid in the cell and with the help of a determinant, it was concluded that the observed microorganisms are close to members of the genus *Chlorella*, family *Chlorophyceae*. A microscopic image is shown on FIG. 3. Genetic-molecular studies have been performed to determine the species. PCR amplification was performed, the results of which are shown on FIG. 4. The results of the comparative analysis of the ITS and rbcL sequences and the phylogenetic tree show that the three fragments sequenced by us can be associated with one family *Chlorellaceae* and genus *Muriella*. The ITS1_2 fragment is grouped with an identity of 97% with 6 strains of the genus *Muriella*, representatives of *M. terrestris*, and the ITS1_4 sequence has a higher identity of 98.28% and can also be associated with 6 strains of the same genus and species. The rbcL gene showed on the one hand that the

studied gene showed the best match with 99.57% identity with only two sequences of *Muriella sp*., but the rbcL sequence was separated from both strains *Chlorella sorokiniana* and *Chlorella* sp. present in the NCBI database and tree. Additional molecular genetic analyzes are needed to determine the isolate by species. The results of the phylogenetic studies are presented On FIG. 5 A), B) and C).

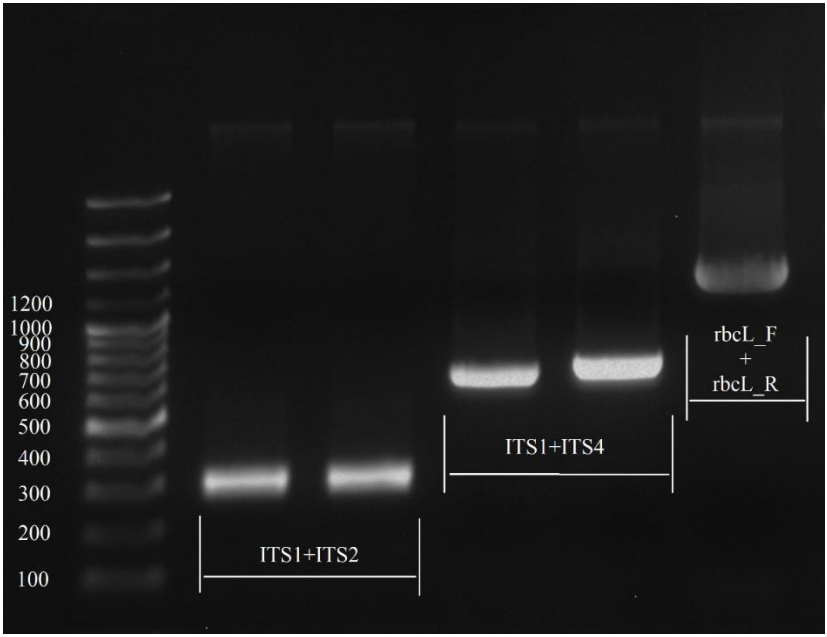


FIG. 4. PCR amplification of DNA from isolated microalgae with universal primers (ITS1, ITS2 and ITS4) and specific primers (rbcL_F and rbcL_R).

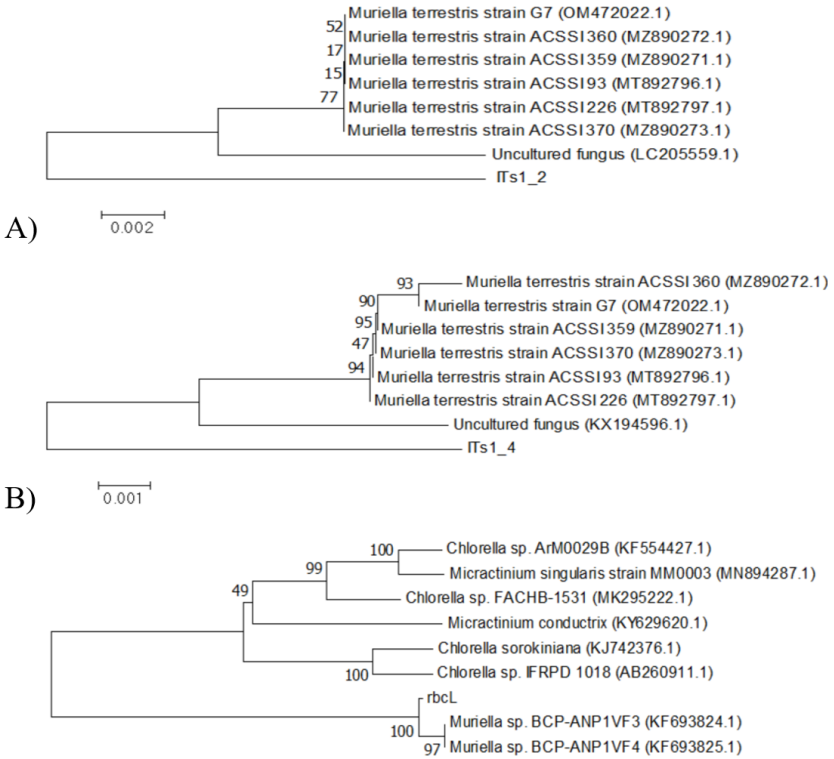
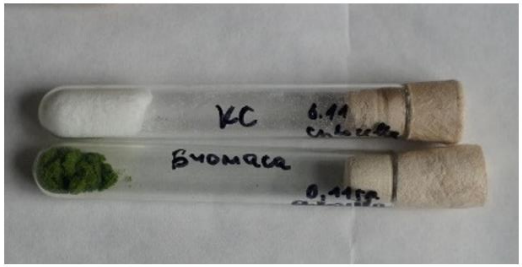


FIG. 5. Neighbor-joining phylogenetic trees constructed using nucleotide sequences of PCR fragments Its 1_2, ITs1_4, rbcL and the nearest sequences obtained after Blast search in NCBI database with their specified accession number in NCBI. Bootstrap values are shown at branch points (percentage of 1000 tests).

A) phylogenetic tree and connections of the studied Its 1_2 fragment,

B) of ITs1_4 and the gene

C) on rbcL, based on remote analysis by the Neighbor-joining method. The Uncultured fungus branch was used as an external branch in the phylogenetic tree.



A) *A. africanum* B) *N. commune* C) *XIIB-A1*

FIG. 6. Lyophilized extracts

A) *A. Africanum* B) *N. commune* C) *XIIB-A1*

FIG. 7. Alcoholic extracts

3. Preparation of microalgae extracts

The microalgae extracts were dried by vacuum-freeze-drying and by rotary vacuum evaporator at the Experimental Algology Laboratory in the Bulgarian Academy of Sciences and a laboratory of the Department of Biotechnology at Sofia University "St. Kliment Ohridski". A total of 14 lyophilized products from the aqueous extracts were obtained. The alcoholic extracts are 3 and are obtained by rotary evaporation. Pictures of some of the extracts are shown in FIG. 6. A), B) and C) and FIG. 7. A), B) and C).

4. Biochemical composition of microalgae

The research was conducted in the Laboratory of Experimental Algology in Bulgarian Academy of Sciences and the Department of Plant Physiology, Sofia University "St. Kliment Ohridski". The analysis includes quantification of proteins, carbohydrates, lipids and pigments. The results are presented in Table. 1.

Microalgae	Biochemical composition of biomass				
	Proteins %DW	Carbohydrates %DW	Lipids %DW	Pigments %DW	Others %DW
A. africanum	51,5%	31,0%	5,2%	1,5%	10,8%
N. commune	30,9%	15,8%	5,2%	0,6%	47,5%

Table 1. Biochemical composition of biomass

5. Determination of biochemical parameters characterizing the antioxidant activity of the microalgae extracts

The results of the antioxidant activity of the studied strains of *A. africanum* and *N. commune* were published by Petrova et al. (2020). Antioxidant activity is determined by the following indicators: total phenol content, total flavonoid content and total antioxidant activity (TAA). It is highest in alcoholic extracts, followed by low-temperature extracts. The lowest TAA was measured in the exopolysaccharide fraction. The cultural medium shows slightly increased activity compared to the exopolysaccharide fraction. These results can be explained by the fact that algae cells actively secrete and excrete a large number of metabolites and enzymes in the

cultural medium, which may exhibit different biological activity. The highest level of total antioxidant activity was measured in the alcoholic extract of the *A. africanum* biomass (362.1 mM g^{-1} DW), followed by the LT extract (53.4 mM g^{-1} DW). Lower results were reported for the exopolysaccharide fraction, the cultural medium and the HT extract. In general, the content of total phenols and flavonoids is higher in products derived from the *A. africanum* biomass. The largest difference was found in the TAA, which is 3 times higher in the alcoholic extract of *A. africanum* compared to *N. commune*.

Maximum number of flavonoids was reported in the alcoholic extract of *A. africanum*'s biomass (84.9 ± 2.5 mg QE / g DW). The content of flavonoids in the aqueous extracts is the lowest in the exopolysaccharide (0.62 ± 0.02 mg QE/g DW). The results for total flavonoids are again the highest in the alcoholic extract of *N. commune*'s biomass (70.6 ± 2.8 mg QE/g DW). The lowest values are in the exopolysaccharide fractions and the cultural medium: in the range of 0.35-1-45 followed by the low-temperature and high-temperature extract.

A series of concentrations of methanol extracts from lyophilized cultural medium (10, 200 and 400 mg / ml) and biomass (10 and 150 mg / ml) were made for *CLM-A1*. The highest content of total phenols is found in the biomass of *CLM-A1*, while in the cultural medium it is about 8 times lower. At a concentration of 10 mg / ml, the number of phenols extracted is the highest, while with increasing the dry matter, the extraction efficiency decreases. In the biomass of *CLM-A1* we found relatively high antioxidant activity by the DPPH method. No DPPH activity was measured in the cultural medium.

The total antioxidant activity of aqueous extracts (HT and LT) from the biomass of *CLM-A1* shows similar values: 3.9 mM g^{-1} LW and 3.82 mM g^{-1} LW. No TAA was found in the cultural medium.

In conclusion, the alcoholic extracts obtained from the biomass of the studied microalgae have the most pronounced antioxidant activity – high amount of phenolic and flavonoid compounds. The antioxidant properties of the microalgae are still poorly understood, so the study of these properties in promising strains may play a significant role in elucidating their ecophysiology.

6. Determination of antimicrobial activity of the microalgae extracts

In the Thesis we used 44 strains of test microorganisms. Total of 28 are pathogenic for humans and 16 are phytopathogens which cause diseases in fruit and agricultural crops. The sensitivity of the selected test microorganisms was determined by the Bauer-Kirby disk diffusion method, then some strains are tested for Minimum Inhibitory Concentration (MIC) by the micro-dilution in broth method. The results were compared with the action of antibiotic (gentamicin) for human pathogens and copper sulfate for the phytopathogens.

6.1. A new method for manual measurements of inhibition zones with the Bauer-Kirby disk susceptibility test

We used lots of photographic material during the study (mainly to verify results). The imperfections of photographs and especially the frequent non-perpendicular viewing angle, were a common problem. For this reason, a new method for measuring the zone of inhibition by the Bauer-Kirby disk susceptibility test was developed using photographic material and a specialized software product was developed for it. The results are published in (Petrova and Petrov, 2021).

6.2. Determination of the microbial susceptibility of extracts from *A. africanum*

The microbial susceptibility of *A. africanum* against human pathogens for culture and biomass is shown on FIG. 16 and FIG. 17, and against phytopathogens on FIG. 18. A comparison between the inhibitory activity of cultural medium and an alcoholic extract of *A. africanum*, and gentamicin against human pathogens is presented on FIG. 19.

Aqueous extracts of *A. africanum* have a broad spectrum of action against most of the tested microorganisms. Both bactericidal and bacteriostatic action of the extract was observed, with bactericidal zones ranging from 7 to 25 mm and bacteriostatic zones ranging from 7 to 26 mm. From all 41 tested strains (human and phytopathogenic), two strains (*S. typhimurium* NBIMCC 3669 and *C. michiganesis* NBIMCC 2425) show resistance against all extracts. Three strains (*P. aeruginosa* NBIMCC 3700, *P. mirabilis* NBIMCC 8747 and *C. albicans* NBIMCC 74) are

sensitive to all extracts. The largest bactericidal zones are from *P. mirabilis* NBIMCC 8747 for all extracts, the largest reported area being for the HT extract (25 mm). Next is *P. aeruginosa* NBIMCC 3700 HT extract (15 mm) and *B. cereus* NBIMCC 1085 HT (13 mm). Of the bacteriostatic zones, Gram-positive ones have been reported with the highest effect *L. innocua* NBIMCC 8755 (24 mm), *E. faecalis* NBIMCC 3915 (20 mm), *S. epidermidis* NBIMCC 3486 (15 mm). The significant Gram-negative are *P. mirabilis* NBIMCC 8747 (26 mm) и *P. aeruginosa* ATCC 27853 (16 mm).

The cultural medium is effective against 92.6% of all tested strains. 26 Gram-negative, 10 Gram-positive and 2 fungal pathogens were inhibited. Both bactericidal and bacteriostatic zones are reported. Therefore, the effect of the extract is broad-spectrum. Some of the results are presented on FIG. 8.

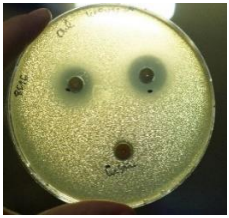
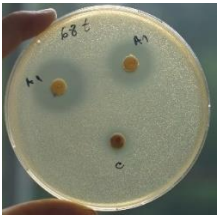
Staphylococcus sp. *P. acnes* *C. glabrata*

FIG. 8. Antimicrobial activity of *A. africanum* cultural medium against selected pathogens

The exopolysaccharide is active against fewer strains than the cell-free supernatant. Inhibition was observed in 8 of 21 tested strains, most of which were Gram-negative bacteria (*E. coli* ATCC 25922 with zone 8 mm, *P. aeruginosa* NBIMCC 3700 with zone 10 mm, *Pseudomonas – изолат, P. mirabilis* NBIMCC 8747 with zone 12 mm, *K. pneumoniae* NBIMCC 3670 with zone 8 mm). The Gram-positive are three (*B. cereus* NBIMCC 1085 with zone 11 mm, *S. aureus* ATCC 25923 with zone 10 mm and *C. albicans* NBIMCC 74 with zone 8 mm).

The high-temperature extract is effective against 16% of the tested strains, while the extract obtained by the low-temperature method shows activity against 21%. The least active is the alcoholic extract inhibiting only 3 of 38 tested strains, or 7.9% of all tested strains.

There is no susceptibility of phytopathogenic bacteria (both type crops and isolates) with respect to products derived from biomass (HT, LT and AE) and from the exopolysaccharide fraction separated from the cultural medium. It can be concluded that the only active extract against phytopathogenic bacteria obtained from the cell-free supernatant affects 94.1% of the tested bacteria. Some of the results are presented in FIG. 9.

E. emylovora *B. gladioli* *X. vesicatoria*

FIG. 9. Antimicrobial activity of *A. africanum* CM against phytopathogens

6.3. Determination of the microbial susceptibility of extracts from *N. commune* Vaucher

The microbial susceptibility of *N. commune* to human pathogens for culture and biomass is presented on FIG. 20 and FIG. 21.

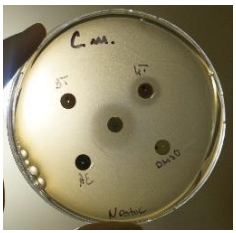


FIG. 10. Antibacterial activity of *N. commune* against *C. michiganesis*

The analysis was performed over 29 strains against 6 extracts. LT extract has the strongest inhibitory activity in 44.4% of the tested strains. Its action is broad-spectrum, as it covers two large groups of bacteria and one strain of yeast *C. albicans* NBIMCC 74. Most are Gram-positive bacteria: *B. cereus* NBIMCC 1085, *E. faecalis* NBIMCC 3915, *L. innocua* NBIMCC 8755, *S. aureus* ATCC 6538, *S. epidermidis* NBIMCC 3486, *Bacillus sp.- food isolate* and the phytopathogen *C. michiganesis* NBIMCC 2425 (FIG. 10).

The obtained results are supported by Shaieb et al. (2014) and are explained by the fact that the compound Noscomin 57 which is contained in *N. commune* has antibacterial activity. Such a study against *B. cereus*, *S. epidermidis* and *E. coli* is presented by Jaki et al. (1999). Jaki et al. (2000) additionally reported ingredients with antibacterial activity from *N. commune* – the diterpenoids Comnostin A, Comnostin B, Comnostin C, Comnostin D, Comnostin E, and the Noscomin which is reported to have antibacterial and antifungal effect. Two alcaloids are reported to have antifungal agent in *N. commune* - Nostodion A from Bhadury and Wright (2004) and the lipopeptide Nostofungicidine by Kajiyama et al. (1998).

AE inhibits only 4 strains out of 29 tested: *P. mirabilis* NBIMCC 8747 with inhibitory zone 9 mm, *S. epidermidis* NBIMCC 3486 with bacteriostatic zone of 7 mm, *Bacillus sp.* with bacteriostatic zone of 11 mm and *C. albicans* NBIMCC 74 with bactericide zone of 8 mm. According to Najdenski et al. (2013) ethanol extracts from *Nostoc sp.* should have antibacterial activity against *S. aureus*. The thesis research does not confirm that *N. commune*; however, there are results against *S. epidermidis* NBIMCC 3486. Our results show activity of the ethanol extract against two Gram-positive, one Gram-negative and one fungal pathogen. Most results are supported by Hameed et al. (2013) – there is only difference with *P. mirabilis*.

The cell-free supernatant show activity on 6 out of 29 cultures and isolates. They are: *P. mirabilis* NBIMCC 8747, *S. typhimurium* NBIMCC 3669, *B. cereus* NBIMCC 1085, *E. faecalis* NBIMCC 3915, *L. innocua NBIMCC* 8755 and *C. albicans* NBIMCC 74**.**

6.4. Determination of the microbial susceptibility of extracts from *CLM-A1*

The microbial susceptibility of *CLM-A1* to human pathogens for culture and biomass is presented on FIG. 22 and FIG. 23. The microbial susceptibility of *CLM-A1* to phytopathogens is presented on FIG. 24.

Total of 32 strains were studied. They include human pathogens, phytopathogens and fungi against. We tested 5 extracts obtained from the microalgae *CLM-A1*. Most effective is the cultural medium which inhibits more than half of the tested strains - 60,6%. Its action is mostly against Gram-negative bacteria

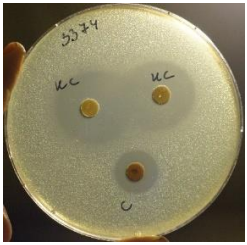
(75% of all tested) and lower against Gram-positive bacteria (50% of all tested). It is noteworthy that only phytopathogenic bacteria are inhibited from Gram-negative ones - the other strains are resistant. From the Gram-positive bacteria the following are inhubited: *B. cereus* NBIMCC 1085, *Bacillus sp. isolate, E. faecalis* NBIMCC 3915, *Enterococus – isolate* (FIG. 11) *and L. innocua* NBIMCC 8755. The only Gram-positive phytopathogen is resistant. *C. albicans* NBIMCC74 and *C. glabrata* ATCC 2001 are also resistant.

FIG. 11. Antimicrobial activity of *CLM-A1* against *Enterococcus – isolate*

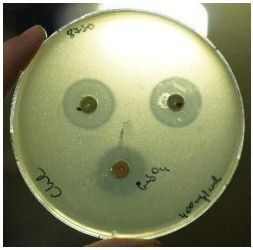
The AE in concentration of 50 mg/ml inhibits only 2 out of 25 strains. They are *S. aureus* ATCC 6538 and *P. aeruginosa* ATCC 27853 with small bactericidal zones between 7 and 8 mm. Weak activities are also observed against the other extracts, mostly against Gram-positive strains: *B. cereus* NBIMCC 1085, *Bacillus sp. изолат, E. faecalis* NBIMCC 3915, *Enterococus – isolate, L. innocua* NBIMCC 8755 and *S. aureus* ATCC 6538. Regarding the low activity from LT, HT and AE, the reasons could be the choice of the extractant or because of unsuccessful destruction of the cell wall. Maregesi et al. (2008) and Ghasemi et al. (2010) discuss that the extracts concentrations may be important. Ghasemi et al. (2010) also argue that the resistance may be associated with an enzyme which is present in the periplasmic space – it can possibly degrade the molecules introduced from the outside.

The *CLM-A1* results were compared by some other *Chlorella* strains. Studies on *Chlorella* against phytopathogens are very few in the scientific literature. One such research has been published by Ranglová et al. (2021). There, an aqueous extract of the biomass of 10 different *Chlorella* strains were tested against the following phytopathogens: *C. michiganensis* subsp. *michiganensis, X. campestris*

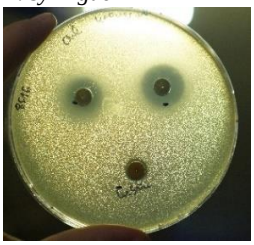
pv. *vesicatoria*, *P. syringae* pv. *tomato*. Only two are reported to be active against *C. michiganesis*, 7 against *X. campestris* and 1 against *P. syringae.* Our results show activity of the *CLM-A1* cultural medium against all *Xanthomonas* and *Pseudomonas* strains; however, there was no activity against *C. michiganesis* NBIMCC 2425 which is the opposite of what Ranglová et al. (2021) reported. The difference can be explained by the fact that they tested biomass extract. Other reason can be some unknown species-specific characteristic. Some results are presented on FIG. 12



P. syringae



X. gardneri



B. gladioli

E. emylovora

FIG. 12. Antibacterial activity of *CLM-A1* against selected phytopathogens

The cell-free supernatant is active only against Gram-positive bacteria, which are also fewer number: *B. cereus* NBIMCC 1085, *Bacillus sp. isolate, E. faecalis* NBIMCC 3915, *Enterococus – isolate, L. innocua* NBIMCC 8755. We obtained results for the cell-free supernatant of *CLM-A1* in concentration 400 mg/ml.

6.5. Determination of MIC and MBC of *A. africanum* cultural medium

We determined the MIC of 16 strains against the cultural medium. The obtained results show that the highest MIC value is equal to 100 mg / ml. These are the results obtained against *S. epidermidis* NBIMCC 1085 MIC=100 mg/ml and

MBC=200mg/ml, *E. faecalis* NBIMCC 3915 MIC=100 mg/ml and MBC= 200mg/ml and *L. innocua* NBIMCC 8755 MIC=100 mg/ml and MBC=200 mg/ml. It is the lowest with *Pseudomonas - isolate* MIC=25 mg/ml and MBC=50 mg/ml. For comparison, the MIC of an antibiotic (gentamicin) was calculated. The effect of the extract was about a thousand times lower.

According to Kitonde et al. (2013) the extracts with the lowest MIC value have the highest activity. In our analisys the extract from *A. africanum* it is the MICs in rage between 3,12 and 6,25 mg/ml. They are registered for *X. vesicatoria* 60t MIC=3,12 mg/ml and MBC=6,25 mg/ml, *X. gardneri* 62t *u X. vesicatoria* 68t MIC=6,25 mg/ml and MBC=12,5 mg/ml. From the type cultures they are *B. gladioli* pv. *alliicola* NBIMCC 8616 and *B. gladioli* pv. *gladioli* NBIMCC 8569 MIC=6,25 mg/ml and MBC=12,5 mg/ml, then *X. vesicatoria* NBIMCC 2427 MIC=12,5 mg/ml and MBC=50 mg/ml and *E. amylovora* NBIMCC 8492 MIC=25 mg/ml and MBC=50 mg/ml. Moderately sensitive is *P. acnes* NBIMCC MIC=50 mg/ml and MBC=100 mg/ml. The lowest sensitivity is with *Pseudomonas - isolate* MIC=100 mg/ml and MBC=200 mg/ml. Compared to the positive control copper sulfate, the action of the extract is between 7 and 8 times weaker.

We skipped calculating the MIC of *X. perforans* NBIMCC 8729 because it showed bacteriostatic effect. *X. gardneri* NBIMCC 8730, *X. euvesicatoria* NBIMCC 8731, *P. syringae* pv. *tomato* NBIMCC3374, *X. gardneri* 64t, *X. euvesicatoria* 105t, *X. euvesicatoria* 269 and *P. syringae* pv. *tomato* 32f are also not tested as they show small zones of less than 13 mm.

6.6. Determination of MIC and MBC of extracts from *N. commune*

No antibacterial activity with a bactericidal zone greater than or equal to 13 mm was reported. For that reasons, MICs and MBC were not calculated.

6.7. Determination of MIC and MBC of extracts from *CLM-A1*

We calculated MICs of CM, HT and LT extracts against 22 strains, including phytopathogens and human pathogens. The results show lowest concentration with: *X. gardneri* NBIMCC 8730, *X. vesicatoria* NBIMCC 2427, *X. perforans* NBIMCC 8729, *B. gladioli pv. gladioli* NBIMCC 8569, *E. amylovora* NBIMCC 8492. They

have MIC=12,5 mg/ml and MBC=25 mg/ml. This is much weaker than synthetic antibiotics, but it is comparable to other studies on microalgae. Next is *P. syringae* pv. *tomato* NBIMCC 3374 with MIC=25 mg/ml and MBC=50 mg/ml, *X. euvesicatoria* NBIMCC 8731 with MIC=50 mg/ml and MBC=100 mg/ml and *B. gladioli pv. alliicola* NBIMCC 8616 with MIC=100 mg/ml and MBC=200 mg/ml. As for the HT and LT extracts, with concentration of 100 mg/ml we were not able to register MIC. For comparison we calculated the MIC of $CuSO_4$. No extract was equal or higher than it.

The MIC index of 2 shows that the activity of all strains is bactericidal, except the ones which are resistant.

The CM is most effective against the following human pathogens: *Enterococcus - isolate* with MIC=100 mg/ml and MBC=200 mg/ml, *E. faecalis* NBIMCC 3915 with MIC=200 mg/ml and MBC=400mg/ml. For *Pseudomonas – изолат, B. cereus* NBIMCC 1085, *Bacillus sp. isolate, L. innocua* NBIMCC 8755 and *Listeria – isolate* we calculated MIC=400 mg/ml which is the initial extract concentration. MIC for HT and LT in concentration 100 mg/ml was not found with exception for *L. innocua* NBIMCC 8755 with MIC_{HT} = 100 mg/ml and *Enterococcus*-isolate with MIC_{LT} = 50 mg/ml. The MIC of the antibiotic gentamicin was used as a control.

The method for determining the MIC in a liquid medium is better than diffusion in agar, especially when it comes to testing extracts. This is due to the fact that the extracts contain a set of biologically active molecules of different nature (polar and non-polar). Most antimicrobial compounds are non-polar in nature and do not diffuse well into agar like polar ones would.

Serial dilutions of the extract were performed in a liquid medium in microplates. The same concentration of test microorganism was used in all wells. As an indicator of the growth of the microorganism is judged by its turbidity. During the experiment, when the plant extract was added to the cultural medium, in some of the extracts we observed precipitation. Other extracts were too dark and this makes it difficult to analyze the results after reading ELISA Reader. This necessitated the use of another method to account for the presence or absence of growth in the wells.

An indicator for microbial growth is used - tetrazolium salt TTC (2,3,5, - triphenyltetrazolium chloride). It acts as a receptor for electrons in the electron transport chains of microorganisms and during the process of reduction it changes from colorless to colored insoluble compound - formazan. The color changes within 10-60 minutes, and the reaction is light sensitive (Eloff, 1998).

It is very difficult to assess the degree of activity on a four-point scale "high-medium-low-inactive". If, for example, a comparison with synthetic antibiotics is to be made, microalgae extracts would turn out to be inactive. There is also a strong difference between different types of plant extracts. That is why very often the authors evaluate their results without quoting specifically which scale they accepted to use. For example, Kitonde et al. (2013) concentrations of extract of *Vernonia glabra* in the range from 0.2 to 15,625 mg/ml records as high activity, from 31,25 to 62,5 mg/ml medium activity and from 125 mg/ml and lower as low activity (the intervals are not connected as it was specific to the research results). Holetz et al. (2002) studied on medicinal plants and accepted that concentrations lower than 0,1 mg/ml are high activity, from 0,1 to 0,5 mg/ml are medium, from 0,5 to 1 mg/ml are weak, and above 1 mg/ml are inactive. It is clear that the difference is huge and it is due to the specifics of the studied plants – some are so stronger than others that they are incomparable on such scale. It would probably be good to accept different standardized scales for different types of extracts, but at this stage no such were found. For that reason, we decided to refrain from using such assessments, but instead to present only the raw results and to leave the possibility for researchers to compare and evaluate against their own.

As a result of the MIC results, phytopathogenic bacteria are most strongly inhibited by the cultural medium extract from *A. africanum*, with exception for *E. amylovora NBIMCC 8492*, which is more sensitive to the cultural medium from *CLM-A1*. *C. michiganens* is resistant to both microalgae.

6.8. Our study on the effect of the combined action of microalgae extracts against phytopathogenic bacteria

The MIC of cultural medium of A. africanum against human pathogens and phytopathogens alone is shown on FIG. 26. For CLM-A1 is shown on FIG. 27. We

therefore performed examination on the eventual synergetic effect between the two microalgae. We used the extracts from *A. africanum* and *CLM-A1* against those pathogens against which they have both bactericidal effects. We mixed them in different proportions and the resulting new antibacterial agent is applied against the tested bacteria. The optimal concentration and the ideal ratio corresponding to synergistic activity is then determined. We found no synergy between the tested microalgae extracts, but it is not antagonistic either. This means that the amount of their action is comparable to their individual effects alone.

No studies have been found on the use of combination therapy in combination with microalgae and the obtained results cannot be compared with other research. In such circumstances we made a conclusion with a hypothesis that *CLM-A1* and *A. africanum* do not interact each other.

On FIG. 13 and FIG. 14 we show two example microtiter plates with study of the combined action of the two microalgae strains.

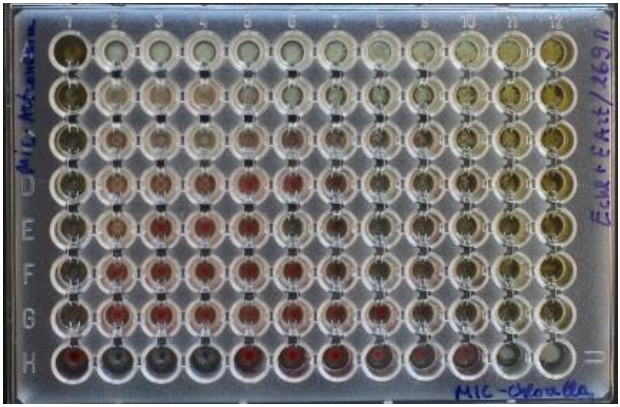


FIG. 13. Determination of FIC of cultural medium from *A. africanum* and *CLM-A1* against *X. euvesicatoria* 269p.

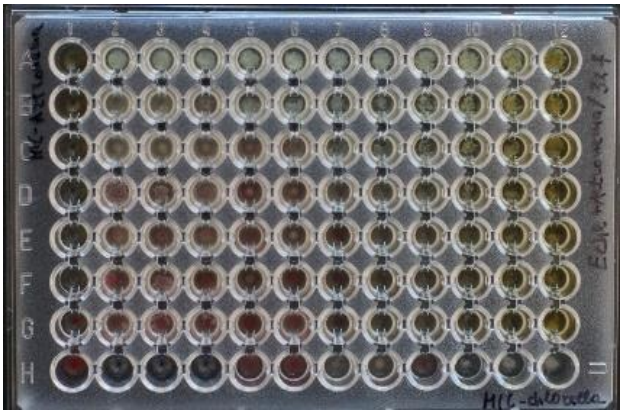


FIG. 14. Determination of FIC of cultural medium from *A. africanum* and *CLM-A1* against *Pseudomonas* 32f

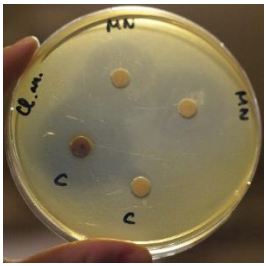
7. Determination of susceptibility of phytopathogenic bacteria to natural antibiotics salinomycin and monensin

The study for the antibiotics salinomycin and monensin is presented on FIG. 25. The two antibiotics are isolated from actinobacteria by Biovet, Peshtera. Pure alcohol was used to dissolve the commercial products. The final products were prepared in the required concentrations.

The disk diffusion method on a solid medium was used to determine the antimicrobial activity of the natural antibiotics. The same procedure for determining the antimicrobial activity of salinomycin is described by Antoszczak et al. (2014) where the authors made the dilutions of the antibiotic in ethanol and soaked them on sterile disks with a certain concentration of antimicrobial agent.

Total of 16 phytopathogenic bacteria were tested for antibiotic sensitivity to two natural antibiotics. They are isolated from actinomycetes from *Streptomyces* - the monensin from *S. cinnamonensis* and salinomycin from *S. albus.* Salinomycin is effective against 13 out of 16 tested phytopathogen strains. Sensitive are mainly representatives of *Xanthomonas*, *Pseudomonas* and *C. michiganesis*; however, there is resistance to three Gram-positive bacteria: *B. gladioli* pv. *gladioli*, *B. gladioli* pv. *alliicola* and *E. amylovora*. Monensin shows resistance to all but one - *C.*

michiganesis, which shows large zone with diameter around 50 mm. Some results are shown on FIG. 15.


X. euvesicatoria *C. michiganesis* *C. michiganesis*

FIG. 15. MIC of salinomycin against phytopathogens

There is no information in the scientific literature on the sensitivity of the two natural antibiotics to phytopathogenic bacteria. Antoszczak, et. al., (2014) studied the antimicrobial activity of Gram-positive, Gram-negative microorganisms and yeast, receiving sensitivity in Gram-positive cocci and some yeasts, and a lack of sensitivity in the following Gram-negative bacteria: *E.coli*, *P. vulgaris*, *P. aeruginosa*, *Bordetella bronchiseptica*. The authors explain their results with the structure of the cell wall of the Gram-negative bacteria, which does not allow the passage of hydrophobic molecules of larger size and thus are resistant to salinomycin. In contrast, our research shows susceptibility to salinomycin in almost all Gram-negative phytopathogenic bacteria, as well as the one tested Gram-positive strain.

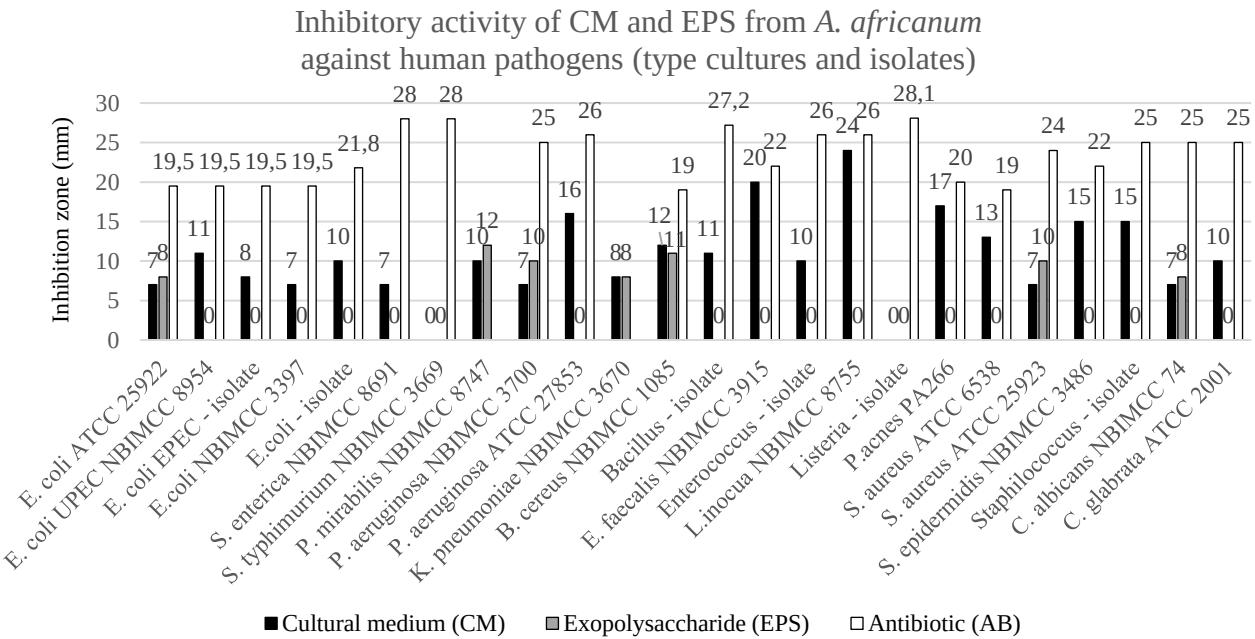


FIG. 16. Inhibitory activity of products from *A. africanum* against human pathogens and the antibiotic gentamicin

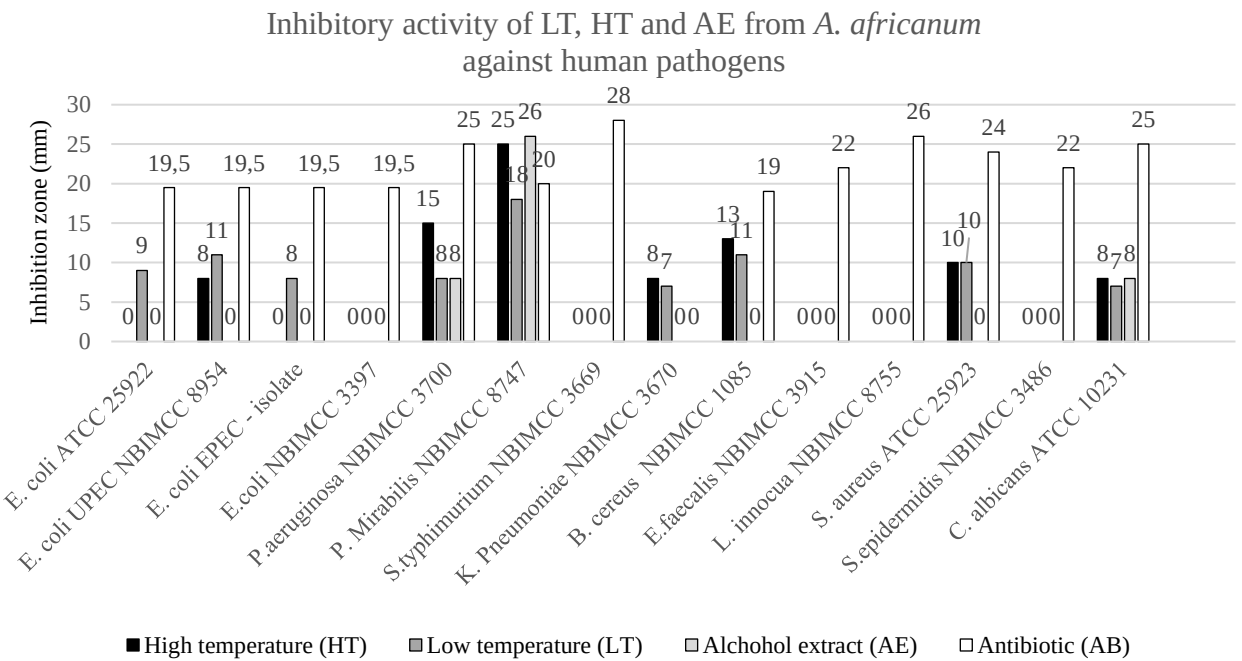


FIG. 17. Inhibitory activity of biomass products from *A. africanum* against human pathogens and the antibiotic gentamicin

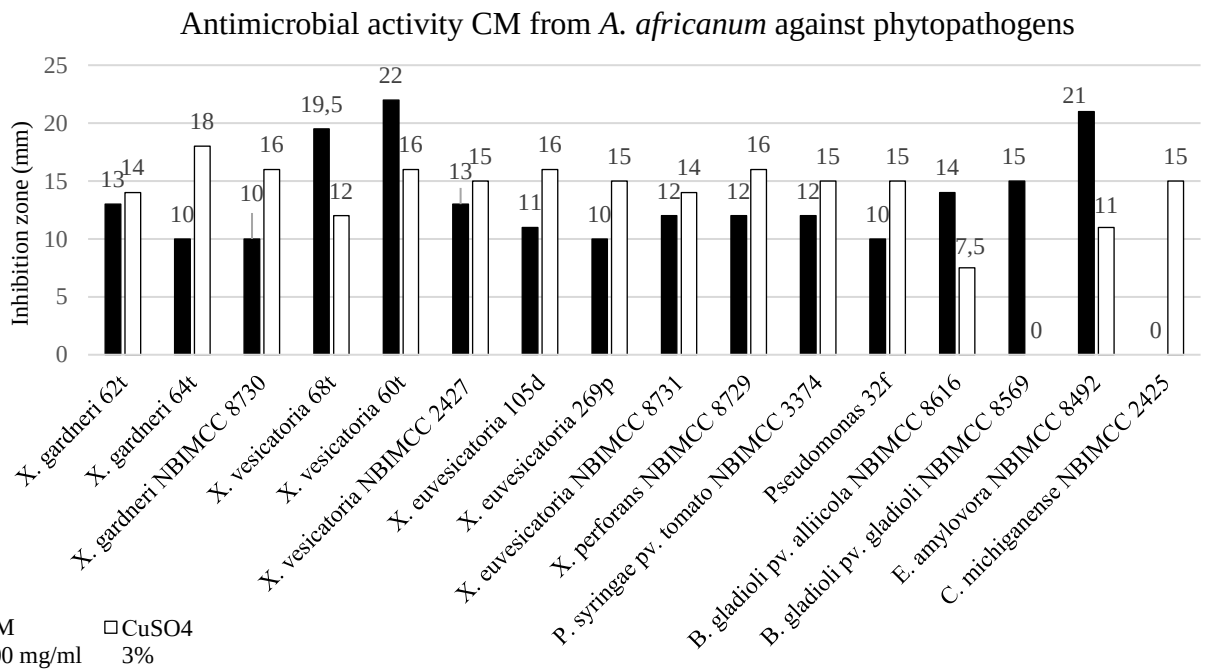


FIG. 18. Antimicrobial activity of cultural medium from *A. africanum* against phytopathogens with control of copper sulphate

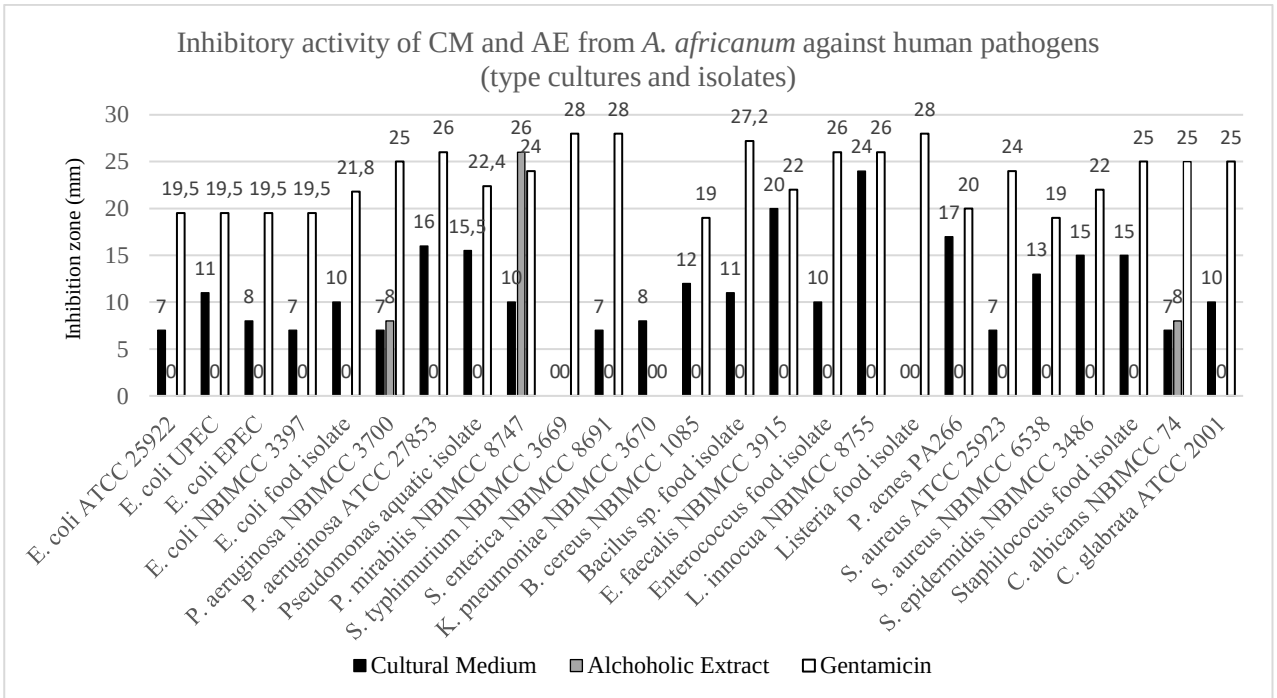


FIG. 19. Inhibitory activity of CM and AE from *A. africanum* against human pathogens with control of gentamicin

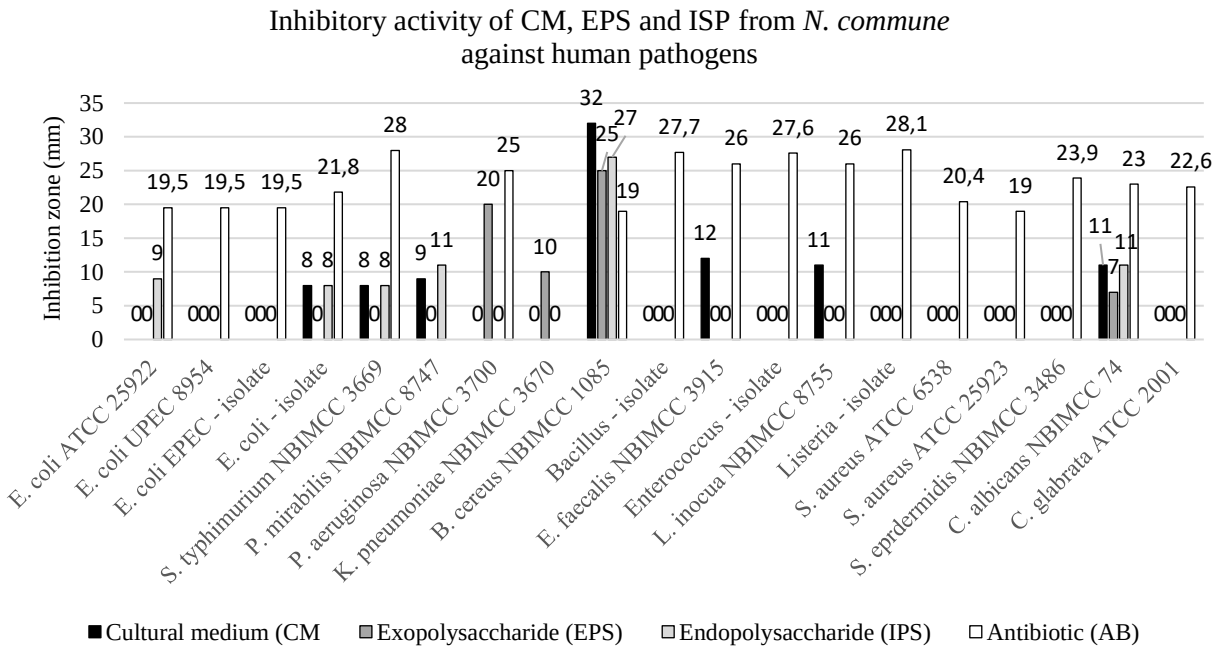


FIG. 20. Inhibitory activity of CM products from *N. commune* against human pathogens with control of gentamicin

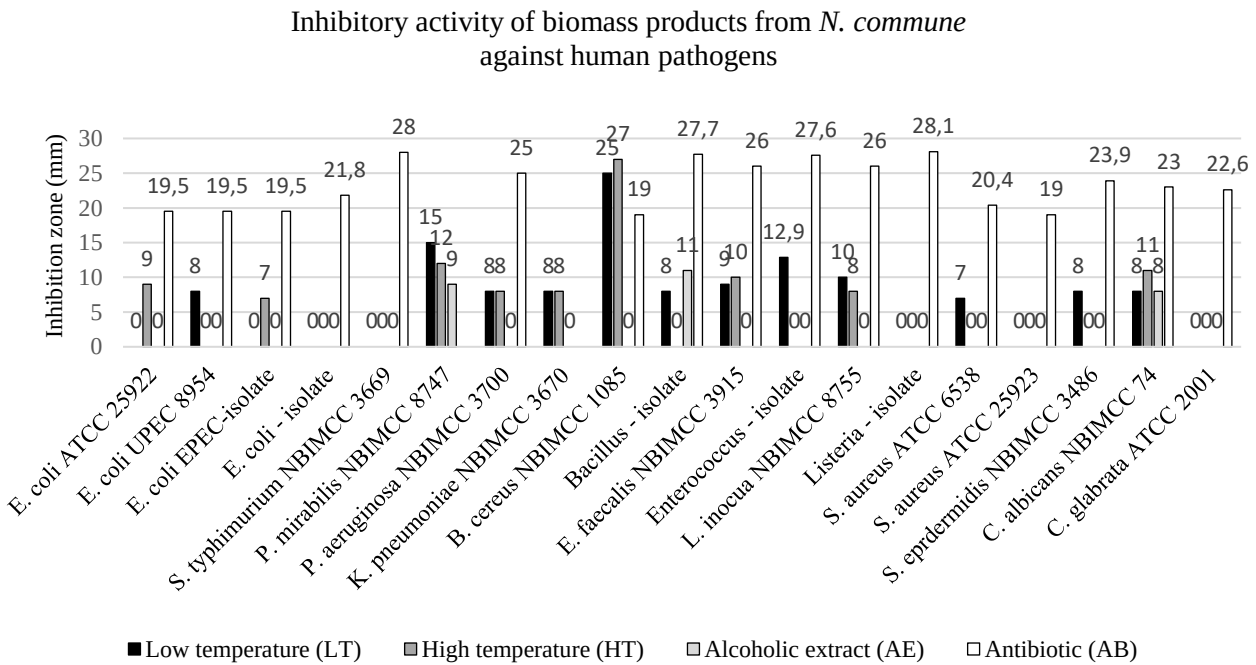


FIG. 21. Inhibitory activity of biomass products from *N. commune* against human pathogens

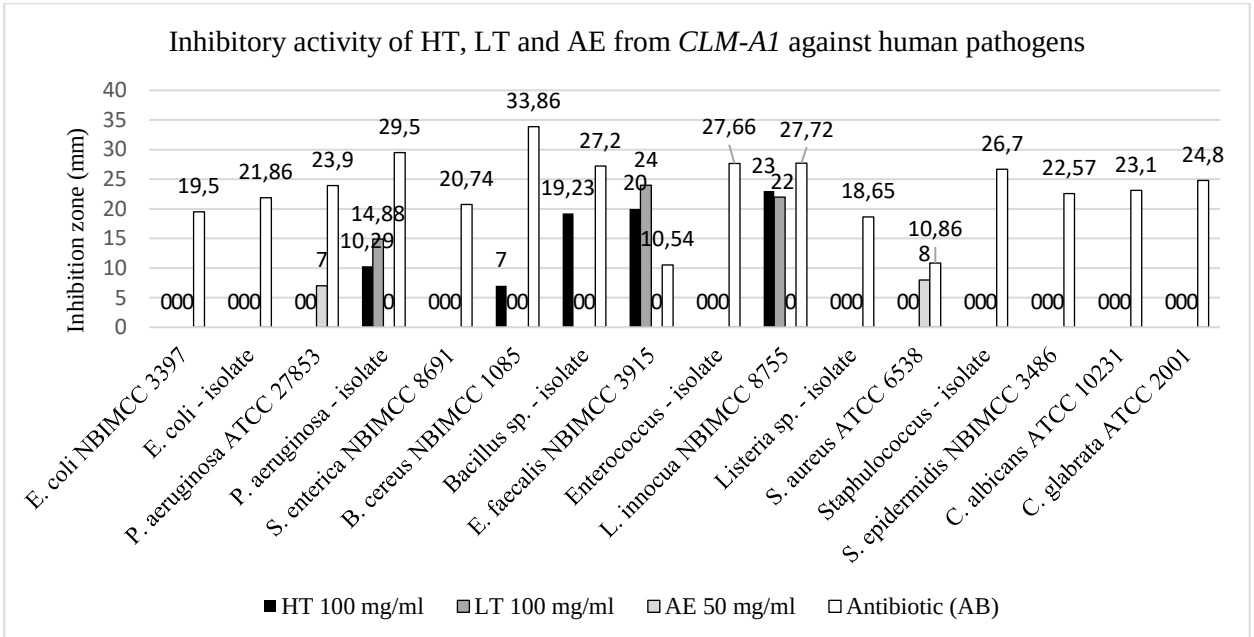


FIG. 22. Inhibitory activity of biomass products from *CLM-A1* against human pathogens with control of gentamicin

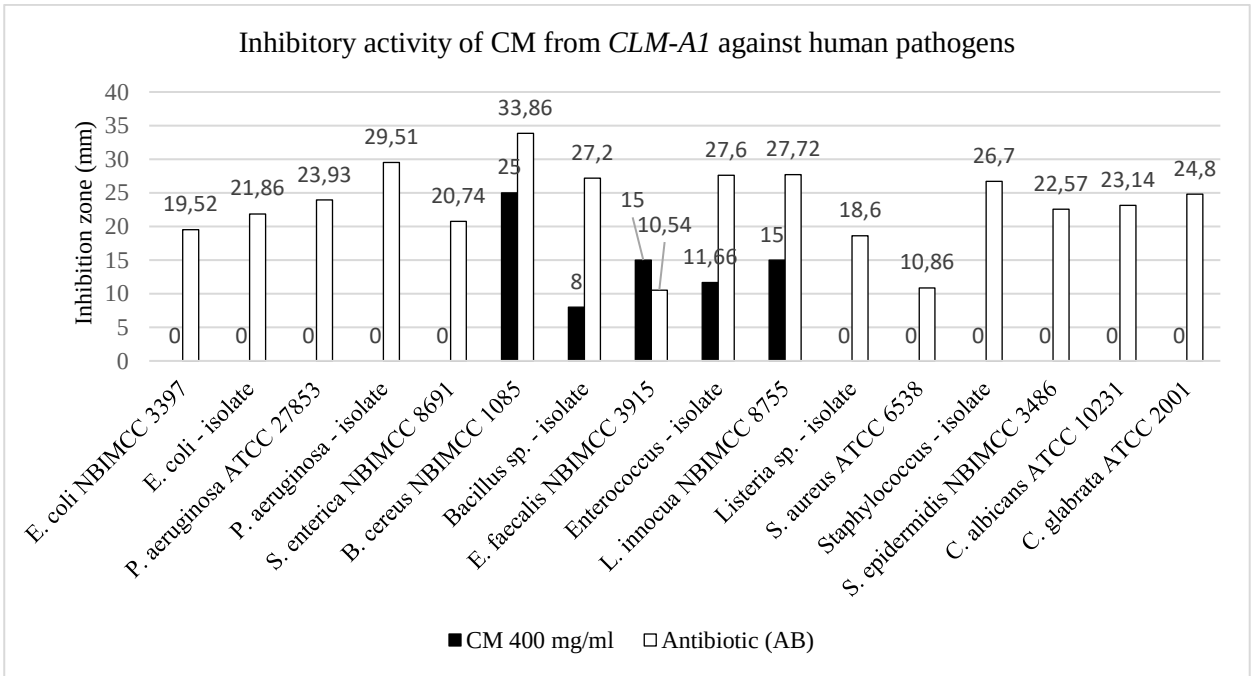


FIG. 23. Inhibitory activity of CM from *CLM-A1* against human pathogens with control of gentamicin

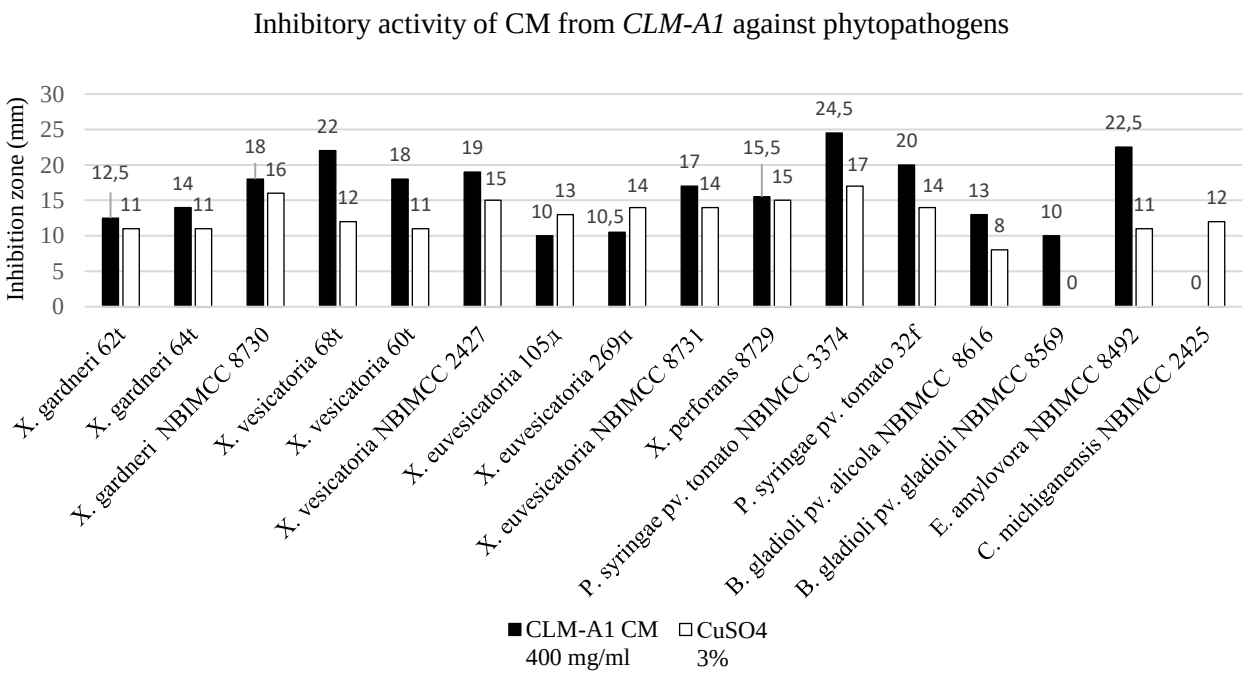


FIG. 24. Antimicrobial activity of cultural medium from *CLM-A1* against phytopathogens with control of copper sulphate

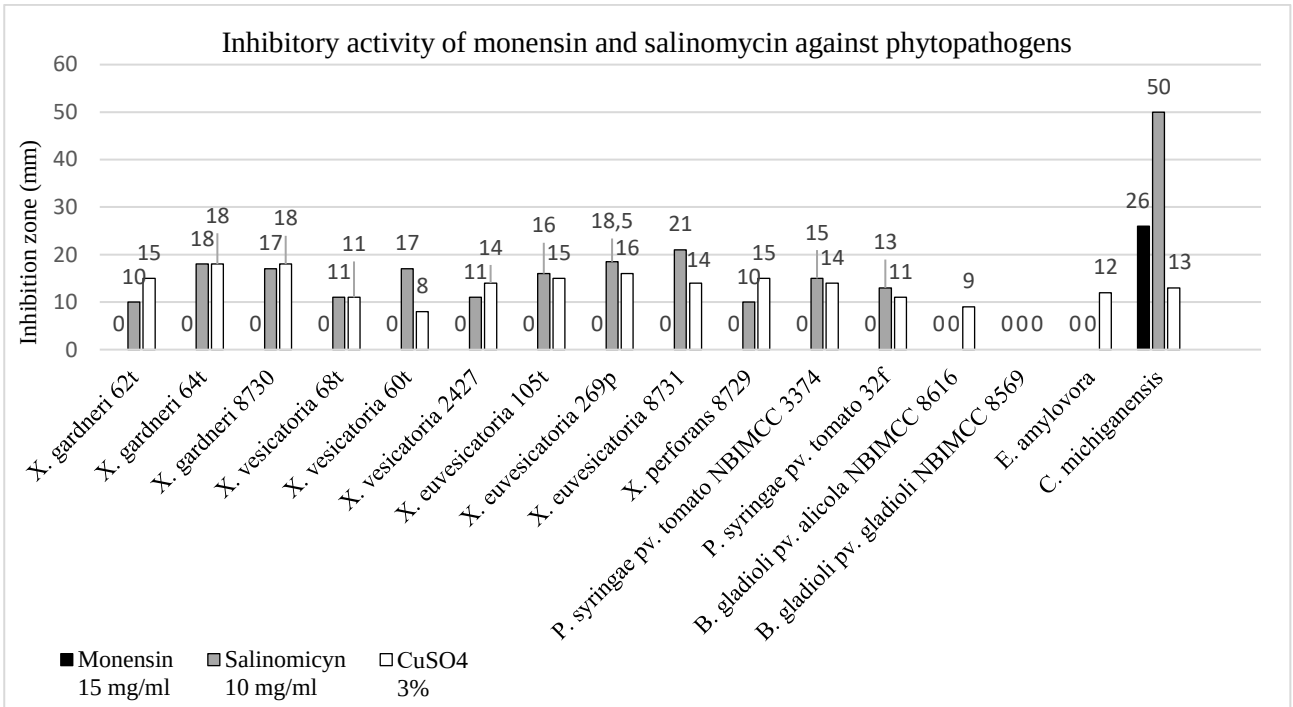


FIG. 25. Inhibitory activity of monensin and salinomycin against phytopathogens with control of copper sulphate

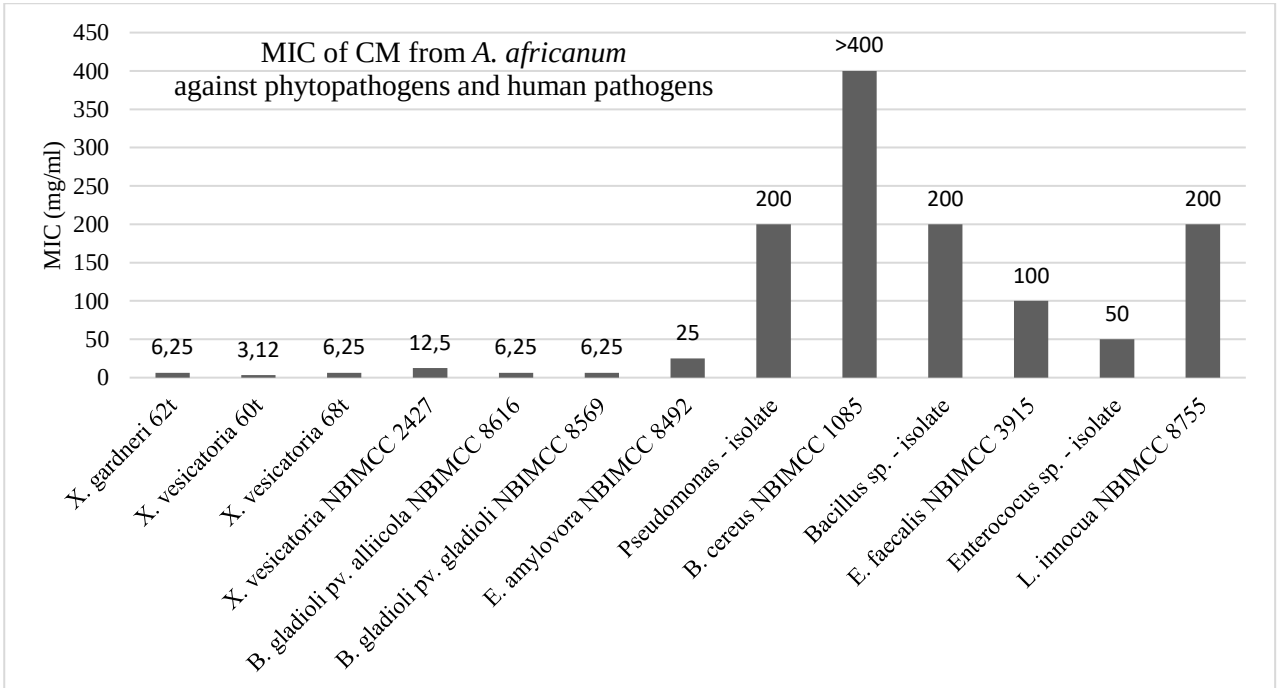


FIG. 26. Comparison of MIC of cultural medium from *A. africanum* against human pathogens and phytopathogens

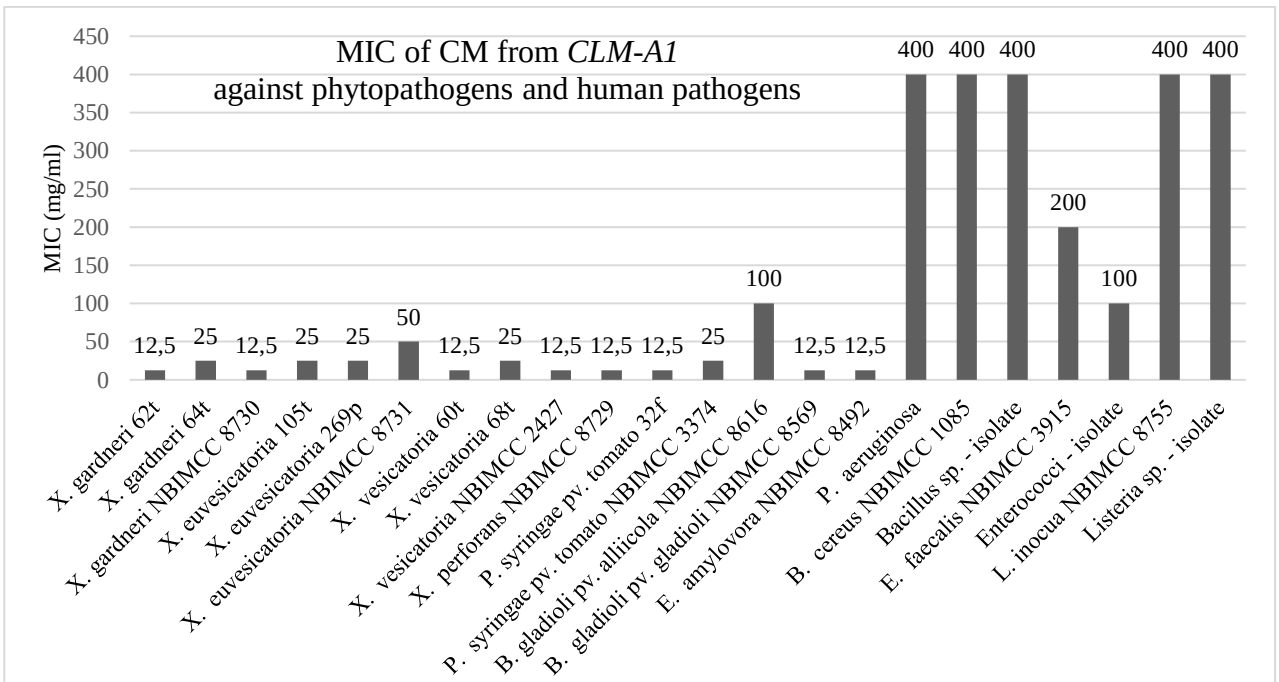


FIG. 27. Comparison of MIC of cultural medium from *CLM-A1* against human pathogens and phytopathogens

8. Sensitivity of the tested strains to hemocyanin

On the antimicrobial activity of the native hemocyanin (EvH) and its fractions (SU1-SU5), the largest zones of inhibition were reported against *B. subtilis* (27.5 mm) and *E. coli* (22.5 mm), medium in size for *S. epidermidis* (14-16.5 мм.) and *S. enterica* (14-17 mm) and small zones for *S. aureus* (9,5 mm) и *P. aeruginosa* (9,9 mm). The largest inhibition and therefore the most effective fraction is SU1. Its action against *B. subtilis* is comparable to the positive control effect of erythromycin and the action against *E. coli* is even larger than the antibiotic control. The weakest fraction is SU3. Results are published in Kizheva et al. (2019).

The minimum inhibitory concentration of all subunits is determined for *E. verrucosa* by the agar well method. Most strongly affected by the hemocyanin fractions (SU1-SU5) are *B. subtilis* and *E. coli*. Their MIC=3,12 μg/ml and MBC=6,25 μg/ml. The less responsive are *S. aureus* and *P. aeruginosa* to the SU3 and SU4 fractions where the MIC=25,0 μg/ml and MBC=50 μg/ml. Our conclusion is that the hemocyanin fractions do not show specificity in terms of cell wall type. On the other side the SU1 fraction is with highest amount of carbohydrates and, with the exception of yeast, inhibits the growth of the tested bacteria to the greatest extent. The weakest glycosylated fractions SU3 and SU4 report lower antibacterial activity. These results indicate that the degree of glycosylation of hemocyanin is related to its antibacterial activity. Lack of activity against native hemocyanin is not an expected result, as many studies confirm its inhibitory activity.

Table 2. Inhibition zones (in mm) of native hemocyanin (EvH) by E. verrucosa and its subunits (SU1-SU5) against human pathogens

Human pathogens	Samples						Positive control	Negative control
	SU1 0,76 mg/ml	SU2 0,93 mg/ml	SU3 1,30 mg/ml	SU4 4,64 mg/ml	SU5 0,39 mg/ml	EvH 3,0 mg/ml	AB	DMSO
B. subtilis NBIMCC 3562	27,5	26,5	24,0	24,1	25,2	0	27,9	0
S. epidermidis NBIMCC 1093	16,5	16,2	14,1	14,4	15,1	0	20,4	0
S. aureus ATCC 6538	14,2	13,4	9,5	9,8	11,3	0	19,8	0
S. enterica NBIMCC 8691	17,3	17,2	14,5	14,0	16,5	0	22,3	0
E. coli NBIMCC 3397	22,5	22,1	20,5	21,0	22,2	0	15,8	0
P. aeruginosa ATCC 27853	13,6	17,7	10,0	9,9	11,8	0	24,0	0
C. albicans ATCC 10231	0	0	0	0	0	0	14,2	0
C. neoformans	0	0	0	0	0	0	12,8	0

Table 3. MIC (µg / ml) of hemocyanin subunits of E. verrucosa against human pathogens

Test microorganisms	Samples									
	SU1		SU2		SU3		SU4		SU5	
	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)
B. subtilis NBIMCC 3562	6,25	3,12	6,25	3,12	6,25	3,12	6,25	3,12	6,25	3,12
S. epidermidis NBIMCC 1093	12,5	6,25	12,5	6,25	25,0	12,5	25,0	12,5	25,0	12,5
S. aureus ATCC 6538	25,0	12,5	25,0	12,5	50,0	25,0	50,0	25,0	25,0	12,5
S. enterica NBIMCC 8691	12,5	6,25	12,5	6,25	25,0	12,5	25,0	12,5	12,5	6,25
E. coli NBIMCC 3397	6,25	3,12	6,25	3,12	6,25	3,12	6,25	3,12	6,25	3,12
P. aeruginosa ATCC 27853	25,0	12,5	25,0	12,5	50,0	25,0	50,0	25,0	25,0	12,5

CONCLUSIONS

1. The newly isolated *HPV-1* strain was phenotypically identified as a representative of the family *Chlorellaceae* and after sequencing of amplified regions of 18S DNA, showed close homology to the genus *Muriella*. Further molecular analyzes are required for complete identification;
2. Water extracts of *Arthronema africanum* (CM, EPS, HY and LT) have a wide spectrum of antimicrobial action. Maximum efficiency is with the cultural medium;
3. Extracts obtained from *Nostoc commune* exhibit antimicrobial activity against human pathogens. The most active is LT extract, which inhibits *Bacillus cereus* and *Proteus mirabilis* the most;
4. The culture medium of the *HPV-A1* isolate inhibits 62.5% of all tested strains. The action is being primarily directed to Gram-negative phytopathogenic bacteria;
5. Alcoholic extracts obtained from the three strains of microalgae show the weakest antimicrobial activity;
6. The alcoholic extracts from the biomass of *A. africanum* and *N. commune* have a highly pronounced antioxidant activity - maximum amounts of phenols, flavonoids and TAA were found;
7. According to the received information for the sensitivity of phytopathogenic bacteria against natural antibiotics, salinomycin exhibits greater activity than monensin;
8. Hemocyanin fractions possess antibacterial activity, which increases with the degree of glycolysis;
9. The culture medium obtained from *A. africanum* and from *HPV-A1* can be used for biological control of phytopathogenic bacteria.

CONTRIBUTIONS

- Detailed information on the antimicrobial activity of various microalgae extracts against a wide range of bacteria and fungi was obtained during the experimental work;
- For the first time in Bulgaria, data is obtained for the antimicrobial activity of *Arthronema africanum* and a newly isolated strain of green microalgae *HPV-A1* against human pathogens and phytopathogens;
- The obtained microalgae extracts have a potential to develop new products for biocontrol against phytopathogenic bacteria;
- New information was obtained on the antioxidant activity of microalgae extracts;
- Software was developed to facilitate the measurement of zones of inhibition by the Bauer-Kirby method from photographic images.

PUBLICATIONS

Scientific publications in specialized peer-reviewed journals (SJR) and in journals with impact factor *(IF):*

1. Kizheva, Y. K., Rasheva, I. K., Petrova, M. N., Milosheva-Ivanova, A. V., Velkova, L. G., Dolashka, P. A., Dolashki, A. K. & Hristova, P. K. (2019). Antibacterial activity of crab haemocyanin against clinical pathogens. *Biotechnology & Biotechnological Equipment*, *33*(1), 873-880.

2. Petrova D., Yocheva L., Petrova M., Karcheva Z., Georgieva Zh., Toshkova-Yotova T., Pilarski P., & Chaneva G. (2020). Antimicrobial and antioxidant activities of microalgal extracts, Oxidation Communications, vol:43, issue:1, 2020, pages:103-116, ISSN (print): ISSN 0209-4541.

Scientific publications in non-refereed journals (SJR) and without impact-factor (IF)

3. Petrova M., & Petrov Ph. (2021). "A new method for manual measurements of inhibition zones with the Bauer-Kirby disk susceptibility test", Proceedings of the Fiftieth Jubilee Spring Conference of the Union of Bulgarian Mathematicians, ISSN 1313-3330, pages 185-190.

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FIGURES AND TABLES

TABLES

FIGURES