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Original Article

Kinetic characterization, antioxidant and *in vitro* toxicity potential evaluation of the extract M116 from *Bacillus amyloliquefaciens*, a Cuban southern coast marine microorganism

[Caracterización cinética y evaluación del potencial antioxidante y de toxicidad *in vitro* del extracto M116 obtenido de *Bacillus amyloliquefaciens,* un microorganismo marino aislado de la costa sur de Cuba]

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Abstract

Context: Marine ecosystems are sources of bioactive compounds. Thirty-eight microorganism strains from the Cuban platform were screened, which allowed us to identify an extract from *Bacillus amyloliquefaciens*, strain CBM-116, as a source for obtaining bioproducts with biomedical applications.

Aims: To physiologically characterize the culture of *Bacillus amyloliquefaciens* (CBM-116 strain) and to evaluate the antioxidant and toxic potentialities in vitro of the M116 extract obtained from CBM-116.

Methods: The growth and metabolite production of the culture were evaluated at a sieve scale. The chemical composition of the M116 extract obtained from the fermented CBM-116 culture was qualitatively characterized. The extract antioxidant activity was measured by DPPH• and FRAP assays, while cytotoxicity was evaluated in MDCK, J774, CT26, 4T1, MCF-7, A549 cell lines and in *Caulobacter crescentus*, as well as the effects on genetic material by SOS colorimetric and Rifampicin Resistance, in the last model.

Results: Grow kinetic parameters of CBM-116 showed the formation of protein metabolites, while the extract revealed antioxidant capacity, which was evidenced by its iron-reducing capacity. M116 was not cytotoxic up to 2000 µg/mL in *C. crescentus*; however, it induced mutagenicity and primary damage to the DNA of the bacteria. The extract significantly inhibited cell viability of CT26, 4T1, MCF-7, A549 cells after 48 hours' exposure. Mean inhibitory concentration (IC_{50}) was calculated for CT26 and 4T1 cells with values of 384 and 488 µg/mL, respectively, in the MTT assay. In the neutral red assay, the values were 478.6 and 398 µg/mL, respectively. Meanwhile, the selectivity index showed values above 2 for both assays. MDCK and J774 cells were not affected.

Conclusions: The M116 extract obtained from *B. amyloliquefaciens* showed bioactive properties with potential application for developing new anti-tumor agents.

Keywords: antioxidant; cytotoxicity; genotoxicity; marine microorganisms.

Resumen

Contexto: Los ecosistemas marinos son fuentes de compuestos bioactivos. Un tamizaje de 38 cepas de microorganismos de la plataforma cubana permitió seleccionar un extracto de *Bacillus amyloliquefaciens*, cepa CBM-116, como posible candidato para obtener bioproductos con aplicaciones biomédicas.

Objetivos: Caracterizar fisiológicamente el cultivo de *Bacillus amyloliquefaciens* (cepa CBM-116) y evaluar las potencialidades antioxidantes y tóxicas *in vitro* del extracto M116 obtenido a partir de CBM-116.

Métodos: Se evaluó el crecimiento y la producción de metabolitos del cultivo a escala de zaranda. Se caracterizó cualitativamente la composición química del extracto M116 obtenido del cultivo fermentado CBM-116. La actividad antioxidante se determinó mediante los ensayos DPPH• y FRAP, mientras que la citotoxicidad se evaluó en líneas celulares MDCK, J774, CT26, 4T1, MCF-7, A549 y en *Caulobacter crescentus*, así como los efectos sobre el material genético mediante SOS colorimétrico y Resistencia a Rifampicina, en este último modelo.

Resultados: Los parámetros cinéticos de CBM-116 evidenciaron la formación de metabolitos con potencial bioactivo, mientras que el extracto mostró actividad antioxidante, lo que se evidenció por su capacidad ferroreductora. M116 no resultó citotóxico hasta 2000 µg/mL en *C. crescentus*, sin embargo, indujo mutagenicidad y daño primario al ADN de la bacteria. El extracto inhibió significativamente la viabilidad de las células CT26, 4T1, MCF-7, A549 después de 48 horas de exposición. Se calculó la concentración inhibitoria media (IC_{50}) para las células CT26 y 4T1 con valores de 384 y 488 µg/mL, respectivamente, en el ensayo de MTT. En el ensayo de rojo neutro los valores fueron de 478.6 y 398 µg/mL, respectivamente. Mientras que el cálculo del índice de selectividad mostró valores por encima de 2 para ambos ensayos. Las células MDCK y J774 no resultaron afectadas.

Conclusiones: El extracto M116 obtenido de *B. amyloliquefaciens* presentó propiedades bioactivas con potencial aplicación en el desarrollo de nuevos agentes antitumorales.

Palabras Clave: antioxidante; citotoxicidad; genotoxicidad; microorganismos marinos.

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INTRODUCTION

The marine environment is a rich source of unique bioactive molecules with broad chemical and functional diversity. Marine organisms live under stressful ecological conditions, and they are able to produce secondary metabolites for adapting, reproducing, communicating, and defending themselves (Ambrosino et al., 2019).

Microbial communities living in these habitats have been recognized to display diverse bioactive compounds (Cheng et al., 2020; Glöckner et al., 2012). In particular, heterotrophic bacteria attract interest upon their decomposing activity and their participation in several processes within the marine ecosystem (Madigan et al., 2017). As part of this group, the genus *Bacillus* stands out for producing molecules with recognized bioactive potentialities. Extracts and molecules obtained from the genus have been reported with antimicrobial, antifungal, antioxidant, and anticancer activities (Blunt et al., 2018; León et al., 2010; Prazdnova et al., 2015; Velasquez et al., 2018). Also, metabolites identified in *Bacillus* have the ability to modulate chronic inflammation (Gautam and Jachak, 2009) and the expression of different genes involved in the immune response (Kondratyuk et al., 2012). Particularly, this genus has a tremendous ability to sense and respond to its surroundings to survive in a stressful environment by producing different molecules (Hassan et al., 2020). As oxidative stress is directly or indirectly involved in various pathological conditions in humans (Forman and Zhang, 2021). Bioproducts derivate from *Bacillus* could be a promise as effective agents for chronic disease prevention and treatment by reducing oxidative stress, including cancer. Thus, the search for new marine-derived antioxidant molecules from *Bacillus* can have a great prospect for obtaining novel drugs.

The evaluation of the metabolic capacities of microorganisms isolated from Cuban marine ecosystems shows its potential applications in the biotechnology and pharmaceutical industries (Bernal et al., 2015; Barrios-San Martín et al., 2012). The screening of some bioactivities of 38 microorganisms from the Marine Bacteria Collection (CBM) at the Institute of Marine Sciences (ICIMAR) showed that a culture of *B. amyloliquefaciens*, CBM-116 strain, has the capacity to produce the enzyme L-asparaginase (Hernández-Balmaseda et al., 2019). Tumor cell growth, unlike normal cells, depends on exogenous L-asparagine. Thus, the L-asparaginase activity, which inhibits amino acid formation, is considered a target in cancer therapy (Rudrapati and Audipudi, 2015). The present work aimed to characterize the formation of the metabolites produced by the fermented culture of CBM-116 strain and to evaluate the *in vitro* antioxidant and potential toxicity of the extract (M116) obtained from this marine microorganism.

MATERIAL AND METHODS

Microorganism

The study was performed by using *Bacillus amyloliquefaciens*, CBM-116 strain, deposited in the Marine Bacteria Collection (batch 2018-116), ICIMAR, Havana, Cuba.

B. amyloliquefaciens CBM-116 growth kinetic characterization

Kinetic characterization of submerged cultures in batch at sieve scale started from an inoculum obtained in nutrient broth at 125 r/min and 30 ± 2°C within an orbital sieve (Infors HT Ecotron). After 12 h culture of the inoculum, the fermentation volumes were inoculated at 0.2 optical density (OD) units measured at 650 nm in a spectrophotometer (Shimadzu UV 1201). Then, microbial growth was expressed as OD units and recorded at different time intervals during the 168 h of fermentation.

The content of total soluble carbohydrates in the cell-free fermented broth was determined as described (Dubois et al., 1956), the soluble proteins according to Bradford (1976), and the phenolic compounds as described by the British Pharmacopoeia (Pharmacopoeia B and Commission BP, 2010). In addition, the kinetic parameters: specific growth rate (μ), substrate consumption (Qs), and product formation (Qp) were determined. The product/biomass yield (YP/X) and the maximum product productivity (PP) were calculated (López Santín and Gódia Casablancas, 1998). The data of two experiments with three replications each were analyzed.

M116 extract preparation and chemical characterization

At 72 h of culture, the fermented broth of CBM-116 strain was filtered through 0.22 μm membranes and it was concentrated to dryness by rotary evaporation under reduced pressure at 1 atm and 60°C. The chemical characterization of the resulting dry extract (named M116) included the qualitative determination of phenols, flavonoids, sugars and amine groups (Miranda and Cuellar, 2000). Three replications of each determination were analyzed.

Evaluation of the antioxidant activity of the M116 extract

Free radical scavenging activity

The radical scavenging capacity of 1,1-diphenyl-2-picrylhydracil (DPPH•) was determined as described (Brand-Williams et al., 1995) with slight modifications. M116 extract was evaluated between 125-2000 µg/mL. The reaction was left in the dark for 30 min and the UV absorbance was measured at 517 nm. Determinations were performed in triplicate. Distilled water was used as negative control. The percent of inhibition was calculated as: % DPPH inhibition = [(Abs control - Abs sample)/Abs control] x 100, where: Abs control: Abs of blank +DPPH and Abs sample: Abs of extract +DPPH.

IC_{50} values were calculated as the extract concentration required to scavenge 50% of DPPH• by plotting the percentage of inhibition *versus* log concentration.

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was done according to Benzie and Strain (1996) with modifications. FRAP reagent was prepared by mixing 0.1 mol/L of sodium acetate buffer (pH 3.6), 10 mmol/L of TPTZ (2, 4, 6-tris(2-pyridyl)-s-triazine) and 20 mmol/L of ferric chloride (10: 1: 1, v: v: v). The extract (2 mg/mL) and five successive dilutions were prepared. Test samples (30 µL) and water (90 µL) were allowed to react with 900 µL of the FRAP solution for 4 min. Readings of the colored product were then done at 540 nm. The blank consisted of 120 µL of water and 900 µL of reagent. According to the standard curve of ascorbic acid, the results were expressed as µmol equivalent of ascorbic acid (100-1000 µmol/L). In each experiment, three replications were analyzed.

Evaluation of the cytotoxic and genotoxic potential of M116 in *C. crescentus*

The NA1000 p3213 *lacZ* strain *C. crescentus* was donated by the Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo, Brazil. Cells were grown in Peptone Yeast Extract (PYE) medium supplemented with $CaCl_2$ (0.5 mmol/L), tetracycline (2 µg/mL) and incubated for 16 h at 30 ± 2°C and 120 r/min in an orbital sieve (Ely, 1991).

Cell culture (6×10^7 cells/mL, OD = 0.4 at 600 nm) was centrifuged at 12 × 4 g for 5 min in a MiniSpin centrifuge. The cell pellet was re-suspended in PYE medium to obtain an M116 extract concentration ranging from 1 to 2000 µg/mL. Cells in the PYE medium were used as a negative control, and cells exposed to 45 J/m² UV-C light doses as a positive control. Treated and controls cells were incubated for 30 min at 4°C and afterward for 2 h at 30°C at 120 rpm with constant shaking. Subsequently, samples were centrifuged at 12 × 4 g for 5 min in a MiniSpin centrifuge, and the pellet was re-suspended in PYE medium for further evaluation. For the mutagenicity test, one more step was required that guarantee the fixation of mutations. All cell suspensions already washed were kept overnight in the same incubation conditions.

Cytotoxicity was expressed as a percentage of viable colonies in regard to untreated control. DNA primary damage was evaluated by using SOS Chromotest (Galhardo et al., 2005), and the genotoxicity criterion was estimated by the β-galactosidase activity. The mutagenic potential of the extract was evaluated by the resistance test to rifampicin of *C. crescentus* (Rif^R) (Galhardo et al., 2005). The mutated colonies frequency (MCF) was determined from the viability of the culture during 48 h after exposure to extract concentrations.

Evaluation of the cytotoxic potential of M116 extract in cell lines

MDCK (canine kidney), J774 (murine macrophages), MCF-7 (human breast adenocarcinoma), A549 (human lung carcinoma), 4T1 (murine mammary carcinoma), and CT26 (murine colorectal carcinoma) cell lines were obtained from American Type Culture Collection (ATCC). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS). All cell culture reagents were purchased from Life Technologies (Praisley, UK). Cells were cultured at 37°C in 5% CO_2 humidified.

Cell viability was assessed by using the 3-(4, 5-dimethylthiozol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma Aldrich, St. Louis, MO, USA) (Mosmann, 1983) and neutral red (Sigma Aldrich, St. Louis, MO, USA) assays (INVITOX Protocol, 1990). In both assays, the cells were exposed to different M116 concentrations (1-1000 µg/mL) for 48 h. The percentage of cell viability was calculated in relation to non-treated cells (assumed as 100% value). Then, IC_{50} values were calculated as the concentration of extract that inhibited 50% of cell viability after exposure time. The selectivity index (SI) was defined as the ratio of the IC_{50} value observed in tumor cell lines to the IC_{50} value observed in the non-malignant cells.

Statistical analysis

Data were expressed as mean ± standard deviation of at least two experiments and analyzed using the GraphPad Prism 5 (2007) program (GraphPad Software, Inc.). Normality was checked using the Kolmo-

gorov-Smirnov test, followed by the Bartlett test or the maximum F to asses' homoscedasticity. Duncan's multiple range test was used to analyze data from the culture kinetic characterization. Comparison of the means among treatments was carried out by using simple classification analysis of variance (ANOVA) plus Dunnett parametric *posthoc* test when significant differences were found. Values of *p<0.05, **p<0.01, ***p<0.001 were considered statistically significant.

RESULTS

Growth kinetic characterization of CBM-116 culture

The cell growth of the CBM-116 culture showed variations in biomass concentrations over time, as has been described by the different phases of microbial growth (Fig. 1). The latency phase was not detected, an exponential growth was observed right from the start, and extended until 8 h, time in which the maximum specific growth rate (μ = 0.2243 ± 0.0126 h^{-1}) was reached. Subsequently, the specific growth rate decreased until it became zero (Fig. 1), which corresponded to the negative acceleration phase and the beginning of the stationary phase, which in turn lasted until the end of the experiment.

The exo-cellular concentration of phenolic compounds during the culture growth did not show significant differences over time, while the concentrations of total soluble carbohydrates significantly decreased from the first hour's culture (p<0.05, Duncan's test), showing values of Qs = 0.1460 ± 0.0027 h^{-1} (Fig. 1). Soluble protein content increased over time in two phases (Fig. 1). The first one lasted 10 h and achieved an increase Qp rate around 0.0114 ± 0.0012 h^{-1}, which was related to the biomass growth. Later, the protein level remained stable until approximately 24 h when Qp was zero. After that, a second increase in protein concentration was observed but with a significantly lower Qp (0.0020 ± 0.0002 h^{-1}) than the one in the first 10 h stage (p<0.05, Duncan's test). The maximum content of soluble proteins occurred between 48 and 72 h. Afterward, protein levels remained constant until the end of the fermentation process, when a maximum productivity of 0.0092 ± 0.0005 g/Lh was achieved. The protein/biomass yield was 0.2629 ± 0.0133. At the end, from the fermented culture, a time of 72 h was selected to obtain M116 extract.

Chemical nature of the M116 extract obtained from CBM-116

The qualitative analysis of metabolites evidenced that phenolic compounds were the major component of M116 extract, in correspondence with the polyphenols, total soluble carbohydrates, and proteins were found in the extract (Table 1).

Table 1. Concentration of metabolites in M116 extract.

Metabolites	Concentration (mg/g)
Total phenols	78.89 ± 0.58
Soluble proteins	4.29 ± 0.72
Total soluble carbohydrates	3.30 ± 0.62

Data are expressed as the mean ± SD (n = 3).

Antioxidant activity

Screening the antioxidant activity of the substances may require a combination of different methods to describe the background of their antioxidant properties (Gulcin, 2020). The antioxidant potential of M116 was determined by using DPPH free radical scavenging and reductive capacity (FRAP) assays. The extract did not show free radical-scavenging activity at any of the tested concentrations (125-2000 µg/mL). As it was expected, the positive control of the experiment, ascorbic acid, showed an antioxidant effect with IC_{50} values of 48.6 ± 3.2 µg/mL (data not shown). But, when FRAP assay was used, the data revealed a reductive capacity for the extract in a concentration-dependent manner (Table 2), with a linear dependence (Linear regression R^2 = 0.9569).

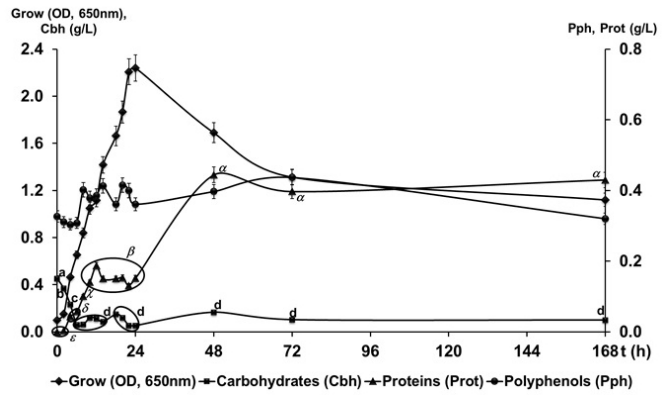

Figure 1. Type fermentation profile of the CBM-116 strain on a sieve scale in nutrient broth medium, 125 r/min, 30 ± 2°C, pH = 7.

Different letters indicate statistically significant differences, p<0.05 (Duncan's test).

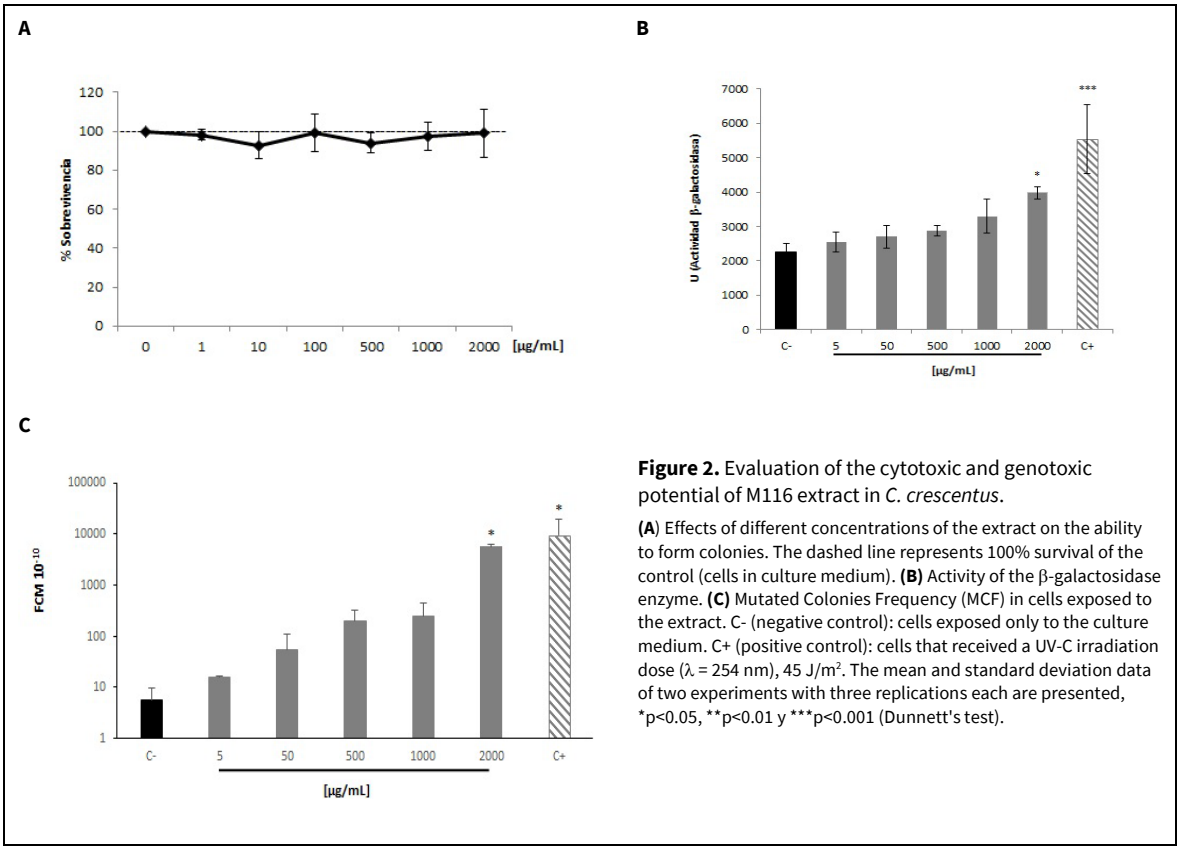

Figure 2. Evaluation of the cytotoxic and genotoxic potential of M116 extract in *C. crescentus*.

(A) Effects of different concentrations of the extract on the ability to form colonies. The dashed line represents 100% survival of the control (cells in culture medium). **(B)** Activity of the β-galactosidase enzyme. **(C)** Mutated Colonies Frequency (MCF) in cells exposed to the extract. C- (negative control): cells exposed only to the culture medium. C+ (positive control): cells that received a UV-C irradiation dose (λ = 254 nm), 45 J/m^2. The mean and standard deviation data of two experiments with three replications each are presented, *p<0.05, **p<0.01 y ***p<0.001 (Dunnett's test).

Table 2. Antioxidant activity of M116 extract as a measure of the iron-reducing capacity.

M116 [µg/mL]	FRAP [µM ascorbic acid equivalents]*
125	NE
250	104.56 ± 10.18
500	256.78 ± 13.47
1000	330.11 ± 16.44
2000	453.44 ± 15.03

Data are expressed as the mean ± SD, NE: no effect.

Evaluation of the cytotoxic and genotoxic potential of M116 extract in *C. crescentus*

The exposure of *C. crescentus* up to 2000 µg/mL of M116 did not lead to significant changes in growth regarding non-treated cells (assumed as 100% growth) (Fig. 2A). These results suggested that the extract was not toxic under the experimental conditions.

On the other hand, M116 extract at 2000 µg/mL concentration shows evidence of genotoxicity. This dose increased the β-galactosidase activity regarding the negative control and induced primary damage to the DNA, according to the induced SOS response, a damage tolerance mechanism at the cellular level (Fig. 2B). MCF results in the Rif^R assay showed that the M116 extract induced mutagenicity in a concentration-dependent manner (Fig. 2C).

Cytotoxic potential of M116 extract in cell lines

Viability of tumor CT26, 4T1, MCF-7, and A549 cells and non-tumor MDCK and J774 cells was determined after 48 h exposure to the M116 extract. M116 extract did not cause observable toxicity on non-tumor cells (Fig. 3). Cell viability decreased in a concentration-dependent manner for tumor cells ($p<0.01$, Dunnett's test) (Fig. 3). As can be observed, statistical differences were found up to 250 µg/mL for 4T1 and CT26 cells, 500 µg/mL for A549 and 1000 µg/mL for MCF-7 cells respect to controls (assumed as 100% viability). After exposure to the maximum tested extract concentration (1000 µg/mL) a 10% of reduction of cell viability was observed in MCF-7 and a 30% in A549 cells. Thus, the IC_{50} value to the extract in these cells was not possible to be calculated once the 50% of inhibition of cell viability was not reached. Meanwhile, higher inhibition percentages were found for 4T1 and CT26 cells, in the order of 65 and 70%, respectively. According to these observations, the IC_{50} was calculated. In the MTT assay, values in the order of 384 µg/mL for CT26 and 488 µg/mL for 4T1 cells were found. Similar results were observed when Neutral red assay data was analyzed (Table 3). M116 ex-

tract showed cytotoxicity against these tumor lines, but it was not considered a potential anticancer candidate due to a lack of selectivity against them. As the non-malignant cells were almost unaffected by the extract, to calculate the selectivity index (SI), we assumed that for these cells, the IC_{50} was reached at the maximum concentration evaluated (1000 µg/mL). From this assumption, the selectivity index for 4T1 and CT26 tumor cell lines must be values above 2.0 and 2.5, respectively.

DISCUSSION

One of the main challenges in obtaining new natural medicines lies in designing flows that are the most efficient, viable, and environmentally friendly compounds. The diversity of marine microorganisms' origin is wide; they can be isolated from sediment, sludge or invertebrate symbionts. This made them a worldwide source to find novel natural products with sustainable supply. For instance, compounds derived from the culture of marine microorganisms can be obtained relatively easy by fermentation process (Engel et al., 2002). Only by adjusting some fermentation parameters, changing components in the culture medium, and/or modifying the microorganism genome may it be possible to optimize the processes for obtaining metabolites to increase productivity (Glöckner et al., 2012). For the next ten years, these compounds are estimated to dominate the field of natural marine products (Blunt et al., 2018). Thus, these structures have been proposed to have different pharmacological properties and possible therapeutic uses (Mayer et al., 2021; Newman and Cragg, 2017).

Kinetic parameters addressed here to establish proper batch culture conditions for the CBM-116 strain at the sieve scale constituted basic information for subsequent studies, aimed to enhance the efficiency of the fermentation process and increase yield and productivity at other scales. The strain growth reached µ value after 8 h, which was fast enough, and it was probably related to the genotypic and phenotypic adaptation to the culture conditions at the inoculum stage, thus shortening the latency stage. This increase in biomass corresponded to the assimilation of glycosidic compounds as carbon sources and preferential energy for the metabolism of low or non-demanding microorganisms.

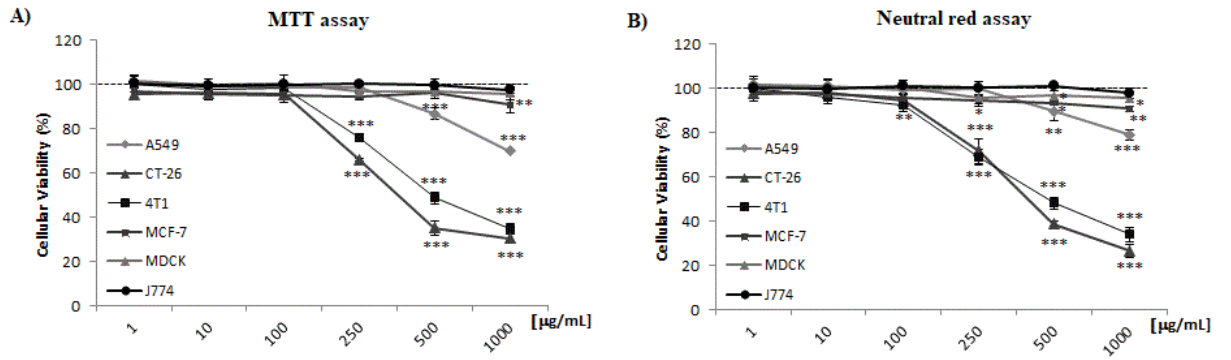

Figure 3. Effects of the M116 extract on cell viability by the MTT **(A)** and Neutral red **(B)** assays.
The percentage of cell viability compared to the control is shown, cells not exposed to the extract (100% cell viability), *p<0.05, **p<0.01, ***p<0.001 (Dunnett's test).

Table 3. Cytotoxicity evaluation of M116 extract in different cells.

Cell line	Origin	MTT assay IC_{50} [µg/mL] (SI)	Neutral red assay IC_{50} [µg/mL] (SI)
MDCK	Canine kidney	>1000	>1000
J774	Murine macrophages	>1000	>1000
MCF-7	Human breast (adenocarcinoma)	>1000	>1000
A549	Human lung carcinoma	>1000	>1000
4T1	Murine mammary carcinoma	488 (>2.0)	478.6 (>2.1)
CT26	Murine colorectal carcinoma	384 (>2.6)	398 (>2.5)

Selectivity index (SI) was calculated by dividing the IC_{50} value into normal cells by the IC_{50} value on cancer cells. The SI value indicates the sample selectivity of the cell lines tested.

Kinetics of soluble protein formation in the fermented broth of CBM-116 strain showed a consecutive reaction according to Deindorfer classification (Doraiswamy and Kulkarni, 1987), as it was expected for the production of secondary metabolites with microbial origin (López Santín and Gódia Casablancas, 1998). The chemical structures of these compounds derived from secondary metabolism correspond to one expected for microbial metabolism, which includes aliphatic chains, isolated or condensed aromatic rings, heterocyclic rings, peptides, and oligosaccharides, among others (Pan et al., 2019; Subramani and Sipkema, 2019).

The most abundant compounds identified within the M116 extract were the phenolic structures, characterized by at least one aromatic ring and one or more hydroxyl groups. Likewise, flavonoids and phenolic acids with interesting pharmacological properties have been identified in extracts from other marine microorganisms (Pan et al., 2019). The phytochemical analysis showed that metabolites of a protein nature were identified in the CBM-116 culture. The dryness process for obtaining the extract from the culture included rotary evaporation using reduced pressure, a heat transfer process capable of denaturing the protein structures. Thus, it may explain the differences observed between the culture and the extract's chemical composition.

The data also showed that higher concentrations tested M116 extract, which was not cytotoxic. It induced mutagenicity and DNA primary damage in *C. crescentus*. The antitumor capacity of different therapeutic agents used commonly in oncology contemplates damage to the DNA, as is the case of platinum derivatives, anthracyclines, and nitrogenous base analogs, recognized mutagenic and genotoxic agents in humans (Khabour et al., 2014).

For two cancers cell lines (CT-26 and 4T1), toxicity effects of the bioactive compounds present in the M116 extract were observed than the bacterial cells of *Caulobacter crescentus*, which could be due to the differences in the permeability of their cell membranes. Although the genotoxic and mutagenic potentials are only significant at 2000 µg/mL, there is a clear tendency to increase genetic material alterations in the range of concentrations in which the viability of tumor cells was determined. So, DNA damage should not be discarded as a possible mechanism by which this natural product induces cell death.

Phenolic compounds identified in some extracts obtained from plants and symbiotic microorganisms show antitumor properties (Ramadhan et al., 2018). As it was mentioned, tumor cell growth depends on exogenous L-asparagine. Thus, the microbial synthesis of this enzyme has been proposed to become an indicator of antineoplastic activity, particularly in acute lymphoblastic leukemia (Jain et al., 2012; Qeshmi et al., 2018). The capacity of the CBM-116 culture to produce L-asparaginase suggests a possible antitumor activity for its metabolites (Hernández-Balmaseda et al., 2019).

There is increasing evidence that oxidative processes promote carcinogenesis, although these mechanisms are not well understood. The antioxidants may be able to cause the regression of premalignant lesions and inhibit their development into cancer (Fishbein et al., 2020). The evaluation of the bioactive potentialities of the CBM-116 extract obtained by fermentation of a *B. amyloliquefaciens* culture showed an iron-reducing capacity.

The model of scavenging the stable DPPH radical has been used to evaluate the free radical scavenging ability of one substance (Brand-Williams et al., 1995; Sirivibulkovit et al., 2018). The antioxidant effect of one analyzed sample on DPPH radical scavenging may be due to their hydrogen donating ability, and it reduces the stable violet DPPH radical to the yellow DPPH-H. Substances that are able to perform this reaction can be considered antioxidants and, therefore, radical scavengers (Dehpour et al., 2009). On the other hand, FRAP assay is based on the ability of antioxidants to reduce Fe^{3+} to Fe^{2+} in the presence of tripyridyltriazine (TPTZ), forming the intense blue Fe^{2+}–TPTZ complex with an absorption maximum at 593 nm; the absorbance increase is proportional to the antioxidant content (Gupta, 2015). As our data showed, the M116 extract had no scavenging activity, but it showed ferric-reducing antioxidant properties (Table 2), which suggested potential antioxidant activity for this extract. Recently, Rajan et al. (2021) reported that among bacteria isolated from mangrove sediments, the extracellular extracts of *B. amyloliquefaciens* exhibited potential antioxidant activity against free radical species. Antioxidant exopolysaccharidess have also been isolated from *Bacillus licheniformis* UD061 (Fang et al., 2013). These findings are in line with our observations, corroborating the antioxidant potential of this genus.

However, all these findings, after 48 h exposure of different cell lines to M116 extract, caused just 30 to 70% growth inhibition, where the maximum inhibition was found for the CT26 colon cancer cells (IC_{50} values were 384 and 398 µg/mL by MTT and neutral red assays, respectively). Under our experimental conditions, the extract cannot be considered a classic cytotoxic agent. As per Boik criteria (Boik, 2001), a compound is cytotoxic when it is able to exhibit IC_{50}

values under 20 µg/mL and moderately cytotoxic between 20 to 100 µg/mL. Thus, under our experimental conditions, M116 is not a cytotoxic agent. Meanwhile, the possibility that M116 induces toxicity in other tumor cell lineages or under other conditions should not be discarded. Studies on silver nanoparticles synthesized using *Bacillus amyloliquefaciens* show strong cytotoxic effects on A549 lung carcinoma cells (Samuel et al., 2020). Not only the cytotoxicity must be the mechanism responsible for justify the anticancer properties of one compound (Batra and Sharma, 2013), more for the case of ones with antioxidant and L-asparaginase activities. Reinforcing this hypothesis, for some natural agents not highly cytotoxic the modulation of other targets related to the development of the tumorigenesis and metastatic process has been recognized as antitumor mechanism (Park et al., 2021; Qeshmi et al., 2018; Xin et al., 2017).

As one of the major therapeutic options for cancer treatment, chemotherapy has limited selectivity against cancer cells. The selective index denotes the capacity of an experimental chemical compound or compounds (e.g., extracts) to efficiently kill a specific cancer cell with minimal toxicity on the non-cancerous cells. SI value >3 indicates high selectivity of a compound (Maungchanburi et al., 2022; Prayong et al., 2008). SI values greater than 1 are already recognized as indicators of greater toxicity to neoplasms than non-malignant cells, and high SI values are preferred for any potential cancer drug candidate (Robles-Escajeda et al., 2016; Tronina et al., 2023). SI values above 2 were found after M116 exposure for both colon tumor cells (CT26) and breast tumor cells (4T1), which could suggest a possible antitumor activity for the extract, in agreement with the antitumor potential described for the genus (Chen et al., 2013).

The results here presented suggest that the extract obtained by fermentation of *B. amyloliquefaciens* could be an interesting candidate for developing anti-tumor agents with lower toxicity. Further studies will be performed to isolate the active metabolites and elucidate the mechanisms responsible for the effects found in M116.

CONCLUSION

The *B. amyloliquefaciens* CBM-116 strain from the Cuban marine platform was capable of producing bioactive metabolites. In particular, the M116 dry extract from this marine organism could be a promissory candidate for obtaining new antitumor molecules.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	IC	FFL	IR	YLL	MVI	JAH	CC	VC	LP	EC	AS	MDF	RRN	IHB	EO
Concepts or ideas		x	x							x	x		x	x	x
Design	x	x	x	x		x	x			x			x	x	x
Definition of intellectual content	x	x	x	x	x	x				x	x	x	x	x	x
Literature search	x	x	x	x	x	x				x	x	x	x	x	x
Experimental studies	x	x	x	x	x		x	x	x	x				x	
Data acquisition	x			x	x	x	x	x	x					x	
Data analysis	x	x	x			x	x		x		x	x	x	x	x
Statistical analysis	x	x		x	x	x						x	x	x	x
Manuscript preparation	x	x	x			x				x			x	x	x
Manuscript editing	x		x			x								x	
Manuscript review	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x

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