








Article

In Vitro and In Vivo Antitumor Activity of a Chloroform Partition from *Ibervillea sonorae* (S. Watson) Greene Endophytic *Bacillus subtilis* Extracts

Ricardo Romero-Arguelles ¹ , César Iván Romo-Sáenz ^{1,2,*} , Patricia Tamez-Guerra ¹ , Diego Fonseca-Rivera ¹, Joel H. Elizondo-Luevano ¹ , Nancy Edith Rodríguez-Garza ^{1,3} , Orquidea Pérez-Gutiérrez ¹, Celia María Quiñones-Flores ², Carlos Arzate-Quintana ² , Lydia Paulina Loya Hernández ² and Ricardo Gómez-Flores ^{1,*} 

¹ Department of Microbiology and Immunology, School of Biological Sciences, Autonomous University of Nuevo Leon, San Nicolas de los Garza 66455, Nuevo Leon, Mexico; ricardoromeroarguelles@gmail.com (R.R.-A.); patricia.tamezgr@uanl.edu.mx (P.T.-G.); diego.fonsecarvr@uanl.com.mx (D.F.-R.); joel.elizondoly@uanl.edu.mx (J.H.E.-L.); nancy.rodriguez@usal.es (N.E.R.-G.); operezg@uanl.edu.mx (O.P.-G.)

² School of Medicine and Biomedical Sciences, Autonomous University of Chihuahua, Chihuahua 31109, Mexico; cquinonezf@uach.mx (C.M.Q.-F.); carzate@uach.mx (C.A.-Q.); lploya@uach.mx (L.P.L.H.)

³ Infectious and Tropical Diseases Group (e-INTRO), Salamanca Biomedical Research Institute-Tropical Diseases Research Centre of the University of Salamanca (IBSAL-CIETUS), School of Pharmacy, University of Salamanca, 37007 Salamanca, Spain

* Correspondence: cromo@uach.mx (C.I.R.-S.); rgomez60@hotmail.com (R.G.-F.)



Academic Editors:
Carlos Gamarra-Luques and
Claudio Ferrante

Received: 31 March 2025

Revised: 8 May 2025

Accepted: 13 May 2025

Published: 14 May 2025

Citation: Romero-Arguelles, R.; Romo-Sáenz, C.I.; Tamez-Guerra, P.; Fonseca-Rivera, D.; Elizondo-Luevano, J.H.; Rodríguez-Garza, N.E.; Pérez-Gutiérrez, O.; Quiñones-Flores, C.M.; Arzate-Quintana, C.; Hernández, L.P.L.; et al. In Vitro and In Vivo Antitumor Activity of a Chloroform Partition from *Ibervillea sonorae* (S. Watson) Greene Endophytic *Bacillus subtilis* Extracts. *Plants* **2025**, *14*, 1474. <https://doi.org/10.3390/plants14101474>

Copyright: © 2025 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

Abstract: Cancer is a complex disease due to its high variability and resistance to conventional treatments. The search for new therapies has prompted the study of less invasive natural sources, such as endophytic bacteria from medicinal plants. *Bacillus subtilis* is known to produce bioactive metabolites with promising pharmacological properties. This study evaluated the antitumor activity of the endophyte *B. subtilis* from *Ibervillea sonorae* against murine L5178Y-R lymphoma cells within in vitro and in vivo models. *B. subtilis* methanol extract was fractionated in hexane, chloroform, and methanol, with the chloroform partition showing the highest tumor cell growth inhibition ($IC_{50} = 34.62 \pm 0.180 \mu\text{g/mL}$) and the highest selectivity index ($SI = 15.53$) when compared with the hexane and methanol partitions. The in vivo study showed that mice treated with 10 mg/kg of the chloroform partition significantly ($p < 0.01$) reduced the tumor volume and weight without affecting tumor-free body weight. The maximum tolerated dose test indicated that 10 mg/kg was safe and well tolerated. These results indicate that *B. subtilis* may be a promising source of selective antitumor compounds.

Keywords: *Bacillus subtilis*; endophyte; medicinal plants; lymphoma

1. Introduction

Cancer continues to represent a major public health problem worldwide, with an estimated morbidity rate of 18.1 million and a mortality rate of 9.6 million per year. Some of the main characteristics of cancer include uncontrolled cell proliferation, the potential to evade control mechanisms, and cellular dimorphism [1,2]. In particular, lymphoma is a group of malignant neoplasms of lymphocytes with more than 90 subtypes, currently classified into Hodgkin's and non-Hodgkin's lymphoma [3]. Surgery, chemotherapy, and radiotherapy are the conventional treatments for this type of disease. However, they remain expensive

and negatively impact the patient's health due to their side effects, myelosuppression, and neurological, cardiac, pulmonary, and renal toxicity, reducing patients' quality of life and discouraging them from undergoing current treatments, favoring the progression of cancer [4,5]. Therefore, there is a need to design new strategies that promote the control and elimination of this disease, which are more effective, selective, and less toxic than conventional therapies. The search for new drugs to treat this type of pathology has led to the discovery of new sources of bioactive molecules with selectivity and specificity characteristics [6,7].

In the last two decades, various endophytic bacteria associated with different plants have produced a wide range of bioactive compounds with therapeutic effects, especially antitumor activity [6,8]. Endophytic bacteria in cucurbits, such as *Ibervillea sonora*, produce bioactive compounds with antitumor effects, specifically against in vitro lymphoma models [9]. This represents a source of new drugs with biological activity, especially antitumor activity. Therefore, in the present work, the antitumor potential of the chloroform partition of *B. subtilis* (ISE-B27), an endophyte of *I. sonora*, was evaluated against murine L5178Y-R lymphoma cells (ATCC CRL-1722) in an in vitro and in vivo model using BALB/c mice.

2. Results

2.1. In Vitro Antitumor Activity of *B. subtilis* Extracts Against L5178Y-R Cells

We obtained hexane, chloroform, and methanol partitions from *B. subtilis* (ISE-B27) crude methanol extract, resulting in the highest yield (1187 mg) for the chloroform partition, followed by the methanol partition with 1011 mg and the hexane partition with 124 mg. The partitions were evaluated against murine L5178Y-R lymphoma and human peripheral blood mononuclear cells (PBMCs), obtaining the highest IC₅₀ and SI for the chloroform partition of 34.62 ± 0.180 µg/mL and 15.53, respectively (Table 1), whereas the IC₅₀ of the hexane and methanol partitions were 148.1 ± 0.330 µg/mL and 117.7 ± 0.310 µg/mL; however, their respective SIs were 3.37 and 4.24 (Figure 1).

Table 1. In vitro performance and antitumor activity of *B. subtilis* methanol extract partitions.

Partition	Performance	L5178Y-R ¹	PBMC ¹	SI
Hexane	124 mg/g	148.1 ± 0.330	$>500 \pm 0.037$	3.37
Chloroform	1187 mg/g	34.62 ± 0.180	537.7 ± 2.731	15.53
Methanol	1011 mg/g	117.7 ± 0.310	$>500 \pm 0.003$	4.24

¹ IC₅₀ (µg/mL). Data represent the mean \pm SD. Vincristine was used as a positive control, causing 80% L5178Y-R growth inhibition.

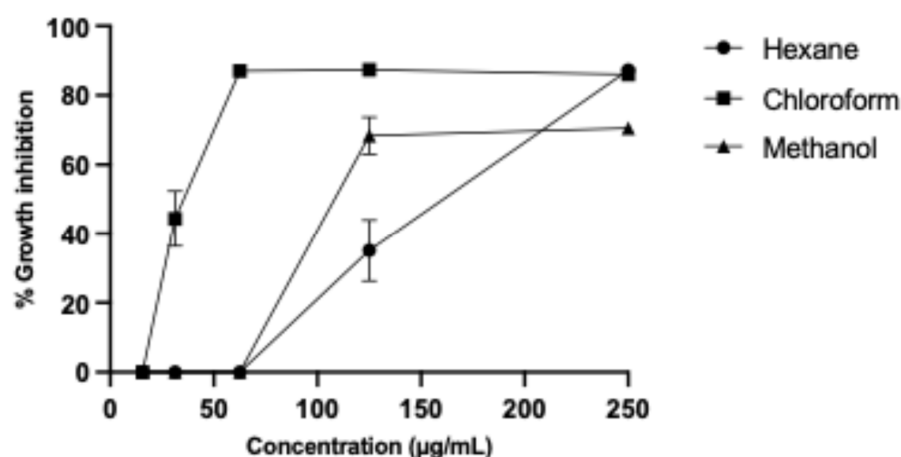


Figure 1. L5178Y-R lymphoma cell growth inhibition by *B. subtilis* partitions.

2.2. Biological Activity of *B. subtilis* Extracts

We evaluated the hemolytic and anti-hemolytic effect on erythrocytes, using the most effective and selective extract partitions against L5178Y-R cells (Table 1). The extract partition that showed the lowest hemolytic activity was chloroform with an IC_{50} of $1443 \pm 0.490 \mu\text{g/mL}$, followed by methanol-based partitioning with an IC_{50} of $223.2 \pm 0.370 \mu\text{g/mL}$, and the hexane-based partition with an IC_{50} of $74.01 \pm 0.270 \mu\text{g/mL}$. In addition, evaluation of the anti-hemolytic activity demonstrated that the methanol-based partition possessed the highest protective potential with an IC_{50} of $89.35 \pm 0.290 \mu\text{g/mL}$, whereas the hexane and chloroform partitions presented $IC_{50} > 500 \mu\text{g/mL}$ (606.5 ± 0.440 and $11,847 \pm 0.610 \mu\text{g/mL}$, respectively (Table 2)). However, the partitions did not show antioxidant activity (Table 2).

Table 2. Hemolytic, anti-hemolytic, and antioxidant activity of ISE-B27 extract partitions.

Partition	Hemolysis	Anti-Hemolysis (AAPH)	Antioxidant Activity (DPPH)
Hexane	74.01 ± 0.270	606.5 ± 0.440	0
Chloroform	1443 ± 0.490	$11,847 \pm 0.610$	0
Methanol	223.2 ± 0.370	89.35 ± 0.290	0

2.3. Phytochemical Analysis of *B. subtilis* Extract

For the characterization of the main chemical groups present in the chloroform partition, a series of chemical tests were performed, revealing the presence and absence of various families of compounds. Table 3 shows the results of the chemical tests of the ISE-B27 (*B. subtilis*) extract chloroform partition. The test was positive for triterpenes, coumarins, tannins, and alkaloids, and negative for sesquiterpene lactones, quinones, saponins, flavonoids, and carbohydrates (Table 3).

Table 3. Phytochemical composition of chloroform-based partition.

Classes	Chloroform Partition
Triterpenes	++ ¹
Coumarins	+++
Sesquiterpene lactones	-
Quinones	-
Saponins	-
Flavonoids	-
Tannins	++
Carbohydrates	-
Alkaloids	+++

¹ ++, low intensity (50%); +++, medium intensity (75%); -, negative reaction.

2.4. Maximum Tolerated Dose of *B. subtilis* Extract in Tumor-Free Mice

To determine the dose to be used in the in vivo model the maximum tolerated dose test was performed, in which the extract was administered at different concentrations. This resulted in a 100% survival of the treated group at 10 mg/kg chloroform-based partition of the ISE-B27 bacterial extract and vehicle. Furthermore, the group treated at 50 mg/kg had a survival rate of 33.3%, whereas the highest concentrations of 100 mg/kg and 1000 mg/kg were significantly toxic, resulting in mice death. Mice treated with the *B. subtilis* extract at 10 mg/kg and vehicles did not show significant differences in weight loss (Figure 2). To determine whether *B. subtilis* extract treatment does not cause liver damage, liver function was evaluated by clinical chemical analysis of the group treated with 10 mg/kg, showing no liver damage (Table 4).

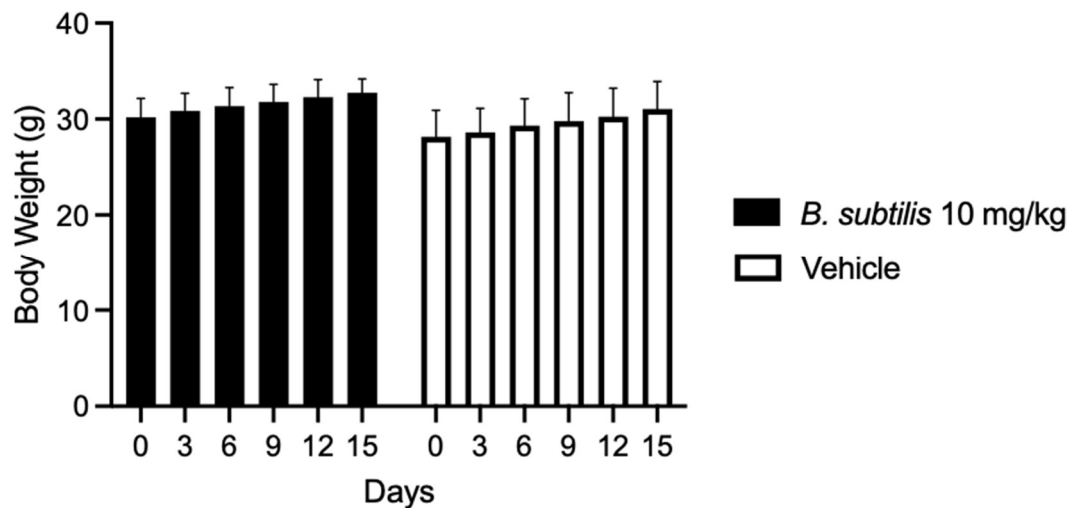


Figure 2. Body weight variation in mice treated with *B. subtilis* extract and vehicle for 15 days.

Table 4. Liver function at 15 days following administration of treatments at the maximum tolerated dose test in tumor-free mice.

Test	Treatment (10 mg/kg)	Reference Values
Albumin	3.06 ± 0.05	2.0–4.6 g/dL [10]
Total proteins	4.06 ± 0.15	4.3–6.4 g/dL [10]
Alkaline phosphatase	174.00 ± 17.34	44–118 U/L [10]
Aspartate aminotransferase	159.5 ± 21.92	69–191 U/L [10]
Alanine transaminase	46.46 ± 4.05	26–120 U/L [10]
Total bilirubin	0.08 ± 0.02	0.3–0.8 mg/dL [10]

2.5. In Vivo Effect of *B. subtilis* Extract Against L5178Y-R Cells

Mice were implanted with L5178Y-R cells and treated with the chloroform-based partition (10 mg/kg) of the bacteria ISE-B27 for 14 d after tumor implantation. We observed a significant reduction in tumor volume ($p < 0.01$) and tumor weight ($p < 0.001$) in mice treated with the chloroform partition compared to untreated tumor-bearing mice (Figure 3), without affecting tumor-free weight (Figure 4). In addition, as shown in Table 5, liver function was not affected by this treatment in tumor-bearing mice in the maximum tolerated dose test.

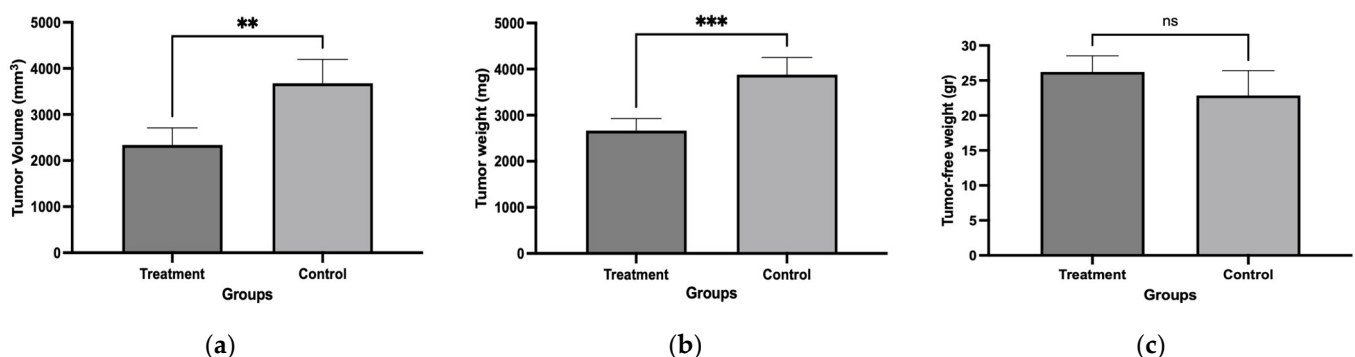


Figure 3. Tumor volume, tumor weight, and tumor-free weight of female BALB/c mice treated with chloroform-based partition (10 mg/kg) from ISE-B27 (*B. subtilis*) bacterial extract. (a) Tumor volume, (b) tumor weight, and (c) tumor-free weight. ** $p < 0.01$; *** $p < 0.001$; ns: not significant.

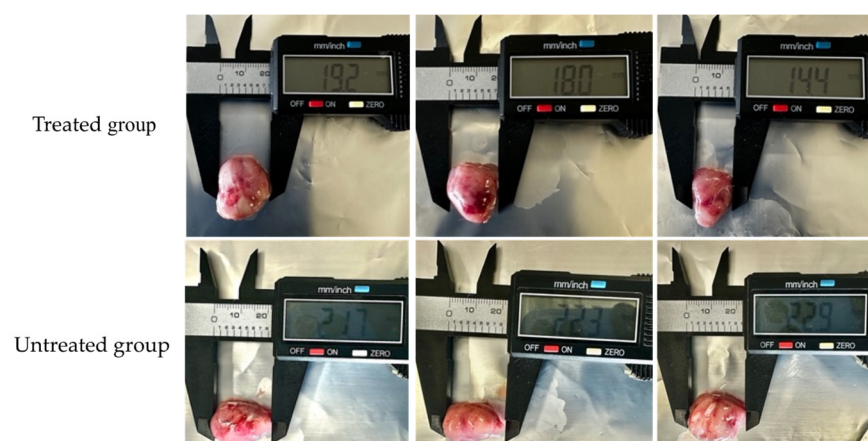


Figure 4. Effect of chloroform-based partition (10 mg/kg) from ISE-B27 (*B. subtilis*) bacterial extract on tumor size. Tumors of the untreated group were significantly larger compared to those of the treated group.

Table 5. In vivo liver function.

Test	Treated Group (10 mg/kg)	Untreated Group	Vehicle	Negative Control	Reference
Albumin	3.36 ± 0.11	3.53 ± 0.25	3.65 ± 0.05	3.10 ± 0.36	2.0–4.6 g/dL [10]
Total proteins	4.64 ± 0.15	5.26 ± 0.41	4.85 ± 0.12	4.51 ± 0.66	4.3–6.4 g/dL [10]
Alkaline phosphatase	57.40 ± 18.44	68.66 ± 30.85	127.75 ± 24.12	190.22 ± 37.12	44–118 U/L [10]
Aspartate aminotransferase	233 ± 24.33	307.66 ± 54.04	93.33 ± 12.50	140.04 ± 56.56	69–191 U/L [10]
Alanine transaminase	81.10 ± 17.34	69.29 ± 5.79	31.73 ± 2.40	67.86 ± 37.83	26–120 U/L [10]
Total bilirubin	0.08 ± 0.008	0.08 ± 0.02	0.07 ± 0.005	0.05 ± 0.01	0.3–0.8 mg/dL [10]

3. Discussion

Cancer represents one of the main causes of death worldwide [11]. Despite advances in cancer therapies they still have disadvantages, such as drug resistance and side effects [12], which has prompted researchers to discover new selective antitumor agents of high therapeutic efficacy and with marginal side effects [13]. Gram-positive bacteria, such as actinomycetes and endophytic *Bacillus* from medicinal plants, synthesize compounds for pharmaceutical application against cancer, such as aromatic compounds, polysaccharides, and lipopeptides [13–16].

Results of the present study demonstrated the in vitro and in vivo antitumor activity of *B. subtilis*, which is an endophyte of *I. sonorae*, a cucurbit native to northern Mexico, whose methanol and ethyl acetate extracts contain cucurbitacins, with cytotoxic properties against the tumor cell lines HepG2, HeLa, L5178Y-R, A549, M12A, LS180, and MDA-MB-231 [17–20].

Currently, the cancer incidence and mortality rate are increasing worldwide and lymphoma is one of the eleven most prevalent types of cancer [11]. Our study showed significant tumor cell growth inhibition by chloroform-based partitions of the endophytic bacterium *B. subtilis* (ISE-B27) methanol extract, exerting an IC_{50} of 34.62 ± 0.180 μ g/mL against L5178Y-R cells. This result is comparable with other studies, such as one isolating a dipeptide from *Bacillus pumilus* AMK1 using chloroform which had significant cytotoxic and apoptotic activity against HepG2 liver cancer cells [21]. Another study found soluble proteins from *Bacillus thuringiensis* strains GM1, GM18, and HD-512 with cytotoxic activity against L5178Y-R cells [22].

In addition, the evaluation of hemolytic activity in the present study showed that the partition of chloroform from the bacterial extract exerted an IC_{50} of 443 ± 0.490 μ g/mL, which is consistent with results obtained by others where ethyl acetate fractions did not

possess hemolytic activity in human erythrocytes [23]. We also found that the chloroform partition had in vivo antitumor potential, which agrees with recent studies using a mouse xenograft model with sarcoma 180 [24]. Another study reported a reduction in tumor size and weight using a mouse xenograft of human colon cancer treated with compounds synthesized from *B. polyfermenticus* [25]. Moreover, iturin A, extracted from *B. subtilis* methanol extract, was shown to reduce 58% of the tumor weight at a dose of 3 mg/kg/day in a xenograft model of HepG2 cells [26].

Marginal information has been reported on the interaction that bacteria play in the production of bioactive compounds in medicinal plants. This study contributes to the knowledge of the relationship that these bacteria may have with the plant's biological activity. It is then essential to investigate the compounds that exert this activity, as well as their tumor selectivity and cell death mechanisms.

4. Materials and Methods

4.1. *Bacillus* Strain

We used the bacterial strain *B. subtilis* ISE-B27, an endophyte of *I. sonorae* [9]. It was activated on malt extract agar (ISP2) (Sigma-Aldrich, St. Louis, MO, USA) for three days at 28 °C.

4.2. Preparation of Extracts

B. subtilis ISE-B27 was fermented in 120 mL of ISP2 broth (Sigma-Aldrich, St. Louis, MO, USA) for three days under shaking (120 rpm) at 28 ± 2 °C. Cultures were then centrifuged at 4500 rpm for 10 min, separating the biomass from the supernatant. Methanol extracts were prepared by macerating for 48 h and then suspending 50 g of the biomass in 100 mL of methanol [27]. Next, the solvent was filtered and evaporated in a rotary evaporator (Buchi R-3000; Brinkman Instruments, Inc., Westbury, Long Island, NY, USA). Fractionation was performed from the crude methanol extract in a Soxhlet apparatus with 500 mL of hexane, chloroform, and methanol for 24 h with each solvent. Once the fractionated extracts were obtained, they were evaporated and reconstituted with dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) and stored at −20 °C until use. Extract yields were analyzed using the following formula: % yield = [(gram of dry extract/gram of biomass) (100)].

4.3. Cell Lines

We used the murine L5178Y-R lymphoma cell line (ATCC CRL-1722) (ATCC, Manassas, VA, USA) and human peripheral blood mononuclear cells (PBMCs) as controls, which were obtained from 20 mL of blood from a healthy volunteer donor. Blood was diluted at a ratio of 1:9 with phosphate-buffered saline (PBS) and placed in a tube containing 15 mL of Ficoll-Paque PLUS (GE Healthcare Life Sciences, Pittsburgh, PA, USA), taking care that the phases did not mix. It was then centrifuged at $400 \times g$ for 30 min at 20 °C, after which the lymphocyte layer was carefully collected and washed with PBS at $100 \times g$ for 10 min at 20 °C, the supernatant was discarded, and PBMCs were suspended in RPMI 1640 culture medium (Life Technologies, Inc., Grand Island, NY, USA) supplemented with 10% inactivated fetal bovine serum (Life Technologies, Inc., Grand Island, NY, USA) and 1% antibiotic/antimycotic solution (Life Technologies, Inc., Grand Island, NY, USA). Cells were then incubated at 37 °C in an atmosphere of 5% CO₂ in 95% air.

4.4. Effect of Extracts on L5178Y-R Cell Growth

In 96-well flat-bottom plates (Corning Inc., Corning, NY, USA) in complete RPMI 1640 culture medium, L5178Y-R cell suspensions (100 µL) were cultured at a density of 1×10^4 cells/well and PBMCs were cultured at a density of 1×10^5 cells/well. After 24 h,

cells were incubated in triplicate for 48 h at 37 °C in 5% CO₂ with 1:2 serial dilutions of 1 mg/mL stock extracts (final concentrations ranging from 15.6 µg/mL to 500 µg/mL), in a final volume of 200 µL. Tumor cell growth was then assessed by the colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide reduction assay (MTT; Affymetrix, Cleveland, OH, USA), adding 15 µL/well MTT (final concentration 0.5 mg/mL). Formazan crystals were dissolved in DMSO, and optical densities (ODs) were measured at 570 nm on a MULTISKAN GO microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) [28]. The percentage of cell growth inhibition was calculated as follows: % growth inhibition = $100 - ((\text{OD}_{570} \text{ in extract-treated cells} / \text{OD}_{570} \text{ in untreated cells}) (100))$. The positive control was 0.05 µg/mL vincristine sulfate (VC; Hospira, Warwickshire, UK). Concentrations were plotted on a log scale against the percentage of growth inhibition to determine IC₅₀ values, which were used to determine the selectivity index (SI). This index was calculated by dividing the IC₅₀ of normal cells by that of tumor cells [29].

4.5. Antioxidant Activity

The antioxidant activity of each extract partition was evaluated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method [30]. In a 96-well plate, we incubated 100 µL of extract at different concentrations and 100 µL of DPPH (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at room temperature in the dark, using DMSO as a negative control and ascorbic acid as a standard at concentrations ranging from 10 µg/mL to 100 µg/mL. ODs were then read at 517 nm in a MULTISKAN GO microplate reader (Thermo Fisher Scientific, Waltham, MA, USA), and the percentage of DPPH radical inhibition was calculated.

4.6. Hemolytic and Anti-Hemolytic Activity

The hemolytic and anti-hemolytic activity of the extracts was determined as previously described [31]. We obtained 20 mL of blood from a healthy volunteer (approved by the Ethics Committee of the Facultad de Ciencias Biológicas, UANL protocol CI-09-2022; informed consent obtained in accordance with NOM-253-SSA1-2012) in tubes with EDTA anticoagulant, after which the erythrocytes were washed three times with PBS at pH 7.2 and 5% of erythrocytes were suspended in sterile PBS. Next, we incubated the extracts (15.625 µg/mL to 250 µg/mL) plus the erythrocyte suspension in 2 mL tubes in triplicate, using distilled water as a positive control and PBS as a negative control, at 37 °C for 30 min, after which we centrifuged at 4 °C for 5 min at 13,000 rpm. To assess the anti-hemolytic activity, we incubated a suspension of red blood cells with 150 mM of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) (Sigma-Aldrich, St. Louis, MO, USA) plus extracts, using PBS as a negative control and erythrocytes with AAPH as a positive control, at 37 °C for 5 h at 200 rpm and centrifuged under the conditions described above. In both cases, once the sample was centrifuged, 200 µL of the supernatant was obtained and placed in a 96-well microplate to measure the OD at 540 nm. The percentage of hemolysis and anti-hemolysis was calculated as follows: % hemolysis or % AAPH inhibition (anti-hemolysis) = $[(\text{OD}_{540} \text{ treatment} - \text{OD}_{540} \text{ negative control}) / (\text{OD}_{540} \text{ positive control} - \text{OD}_{540} \text{ negative control})] \times 100$.

4.7. Phytochemical Analysis of the Extract

The phytochemical profile of the extract was analyzed by qualitative tests to detect the presence of different compounds, using each extract dissolved in methanol. For the detection of xanthophylls, 0.2 mL of HCl was added to 0.5 mL of extract: a green or purple coloration indicated a positive result. To identify unsaturated carbon-carbon bonds we used the Baeyer test, in which 5 drops of 1% KMnO₄ were added to 10 drops of each extract. The test is positive if a color changes from purple to reddish brown occurs together with a precipitate. Carbohydrates were detected by the Molisch test. For this, the Molisch

reagent was added to 10 drops of extract and concentrated H_2SO_4 ; the presence of a purple ring at the interface was interpreted as positive. The presence of flavonoids was detected by the Shinoda test, in which 10 drops of HCl and a small piece of Mg were added to 20 drops of extracts; the presence of an orange, red, or purple color indicated a positive result. Alkaloids were detected by the Dragendorff test, in which five drops of Dragendorff reagent were added to five drops of extract: the presence of an orange-brown precipitate indicated a positive test. The phenolic hydroxyl group was determined by the FeCl_3 test, in which two drops of reagent were added to three drops of extract; the test is positive if a red, blue, or purple color or precipitate appears. For sterols and triterpenes, the Liebermann–Burchard test was used. A total of 10 drops of the Liebermann–Burchard reagent were added to 20 drops of extract; the presence of green or blue colors was positive for sterols, whereas a purple or pink color was positive for triterpenes. Coumarins were detected by adding 10 drops of 10% NaOH to the extract, which produced a color change, followed by slowly adding 1 drop of concentrated HCl until a discoloration was observed, indicating a positive test. To determine the presence of lactones, the Baljet test was used, in which five drops of Baljet reagent were added to five drops of extract; the presence of a red, orange, or violet precipitate was a positive result [32].

4.8. Animals

Female BALB/c mice aged 6 to 7 wk were provided by the Bioterium of the Immunology and Virology Laboratory of the School of Biological Sciences at the Autonomous University of Nuevo Leon, Mexico. The animal study protocol was approved by the Research Ethics and Animal Welfare Committee of the Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León (protocol code CEIBA-2021-011). They were housed in microventilated cages enriched with cardboard tubes for recreational purposes, access to water and food ad libitum, in a stress- and pathogen-free environment, and at a temperature of 22 °C, with light and dark cycles of 12 h and a relative humidity of 45%. Established guidelines for the care and welfare of animals in cancer research were followed [33]. To determine the evaluation criteria of the animals, a clinical score was used that evaluated body weight, hair condition, posture, and activity [34].

4.9. Maximum Tolerated Dose Test

We performed the maximum tolerated dose experiment following the protocol described above [34]. Extracts were suspended in a vehicle composed of 90% PEG-300 (Sigma-Aldrich), 5% DMSO, and 5% of 96% ethanol [35]. Three doses of 10 mg/kg of the chloroform-based extract were intraperitoneally administered to each mouse, in addition to maintaining a vehicle-treated group and the control group. The observation period was 15 d, during which the weight and clinical score of each mouse were recorded. For blood studies, we intraperitoneally administered 25 mg/kg of sodium pentobarbital (Aranda Salud Animal, Querétaro, Mexico) as an anesthetic. A cardiac puncture was then performed to obtain a blood sample, followed by euthanasia of the animal. The blood samples were stored in tubes without heparin and centrifuged at 3000 rpm for five minutes to separate the serum and perform liver function tests. Animals were euthanized by cervical dislocation if a weight loss $\geq 20\%$ or a clinical score ≥ 3 occurred.

4.10. In Vivo Antitumor Activity

L5178Y-R lymphoma was maintained by intraperitoneally inoculating 0.2 mL of L5178Y-R cells (5×10^6 cells/mouse) into 4- to 6-week-old female BALB/c mice as previously described [36]. Ascites cell suspensions were washed twice in PBS by centrifugation at 2000 rpm for 10 min and adjusted to 2×10^5 cells/mL in PBS, after which 6- to 7-week-old female BALB/c mice received a subcutaneous administration (upper right thigh) of 0.2 mL

of this lymphoma suspension. After 7 days of tumor inoculation, mice bearing the L5178Y-R tumor were selected and treated with *B. subtilis* extract. Controls were tumor-bearing mice not treated with extracts (untreated), tumor-bearing mice treated with vehicle (a solution containing 90% PEG-300, 5% DMSO, and 5% of 96% ethanol) (vehicle), and untreated normal mice (untreated control without tumor). Overall survival and tumor volume (length) (width)²/2 were then determined. Tumor measurements (length and width) were obtained with a vernier caliper.

4.11. Statistical Analysis

Results were expressed as mean \pm SD of three replicate determinations per treatment (in vitro study) and five mice (in vivo study) per experimental group. Statistical analyses were performed using GraphPad Prism 9 software (GraphPad Software Inc., San Diego, CA, USA). Statistical analysis determined the distribution of the data obtained for each variable (in vivo and in vitro data) studied using the Kolmogorov–Smirnov test, and subsequently a comparison was made by analysis of variance (ANOVA). For data with a predominant normal distribution, the one-way test was used. In cases where a difference was found between the groups, the Student *t* test was used to evaluate the level of significance of the difference in the mean \pm SD of the control group against each of the treatment concentrations ($p < 0.05$).

5. Conclusions

Results of the present study demonstrated the pharmacological potential of chloroform partitions of *Bacillus subtilis* ISE-B27 extracts, an endophyte of *Ibervillea sonora*, and the its potential to become a promising source of compounds with therapeutic potential against cancer by it significantly reducing tumor growth without affecting the general health of mice.

Author Contributions: Conceptualization, R.R.-A. and C.I.R.-S.; methodology, R.R.-A., D.F.-R. and R.G.-F.; software, D.F.-R. and C.M.Q.-F.; validation, C.I.R.-S., P.T.-G. and O.P.-G.; formal analysis, C.A.-Q., J.H.E.-L. and N.E.R.-G.; investigation, R.R.-A., L.P.L.H. and R.G.-F.; resources, C.I.R.-S. and R.G.-F.; data curation, R.R.-A.; writing—original draft preparation, R.R.-A. and C.I.R.-S.; writing—review and editing, R.G.-F.; visualization, R.R.-A., L.P.L.H. and R.G.-F.; supervision, C.I.R.-S.; project administration, C.I.R.-S. and R.G.-F.; funding acquisition, C.I.R.-S. and R.G.-F. All authors have read and agreed to the published version of the manuscript.

Funding: We received financial support through SNII-445572 to C.I.R.-S. and SNII-9942 to R.G.-F.

Data Availability Statement: Data are contained within the article.

Acknowledgments: The authors thank CONAHCYT-MEXICO for scholarships CVU: 3367959 to R.R.-A., CVU: 708487 to D.F.-R., CVU: 418935 to J.H.E.-L., CVU: 1119878 to L.P.L.-H., and CVU: 1179478 to N.E.R.-G.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Stewart, B.W.; Kleihues, P. (Eds.) *World Cancer Report*; IARC Publications: Lyon, France, 2003.
2. Bray, F.; Ferlay, J.; Soerjomataram, I.; Siegel, R.L.; Torre, L.A.; Jemal, A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA A Cancer J. Clin.* **2018**, *68*, 394–424. [\[CrossRef\]](#)
3. Molyneux, E.M.; Rochford, R.; Griffin, B.; Newton, R.; Jackson, G.; Menon, G.; Harrison, C.J.; Israels, T.; Bailey, S. Burkitt's lymphoma. *Lancet* **2012**, *379*, 1234–1244. [\[CrossRef\]](#)
4. Jemal, A.; Bray, F.; Center, M.M.; Ferlay, J.; Ward, E.; Forman, D. Global cancer statistics. *CA A Cancer J. Clin.* **2011**, *61*, 69–90. [\[CrossRef\]](#)

5. Cavalcanti, I.D.L.; Soares, J.C.S.; Cavalcanti, I.D.L.; Soares, J.C.S. Conventional cancer treatment. In *Advances in Cancer Treatment: From Systemic Chemotherapy to Targeted Therapy*; Springer Nature: Berlin/Heidelberg, Germany, 2021; pp. 29–56.
6. Singh, A.; Singh, D.K.; Kharwar, R.N.; White, J.F.; Gond, S.K. Fungal endophytes as efficient sources of plant-derived bioactive compounds and their prospective applications in natural product drug discovery: Insights, avenues, and challenges. *Microorganisms* **2021**, *9*, 197. [\[CrossRef\]](#)
7. Raimi, A.; Adeleke, R. Bioprospecting of endophytic microorganisms for bioactive compounds of therapeutic importance. *Arch. Microbiol.* **2021**, *203*, 1917–1942. [\[CrossRef\]](#)
8. Numan, M.; Shah, M.; Asaf, S.; Ur Rehman, N.; Al-Harrasi, A. Bioactive compounds from endophytic bacteria *Bacillus subtilis* strain EP1 with their antibacterial activities. *Metabolites* **2022**, *12*, 1228. [\[CrossRef\]](#)
9. Romero-Arguelles, R.; Romo-Sáenz, C.I.; Morán-Santibáñez, K.; Tamez-Guerra, P.; Quintanilla-Licea, R.; Orozco-Flores, A.A.; Gomez-Flores, R. In vitro antitumor activity of endophytic and rhizosphere gram-positive bacteria from *Ibervillea sonidoe* (S. Watson) Greene against L5178Y-R lymphoma cells. *Int. J. Environ. Res. Public Health* **2022**, *19*, 894. [\[CrossRef\]](#)
10. Hedrich, H. (Ed.) *The Laboratory Mouse*; Academic Press: Cambridge, MA, USA, 2004.
11. Sung, H.; Ferlay, J.; Siegel, R.L.; Laversanne, M.; Soerjomataram, I.; Jemal, A.; Bray, F. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA A Cancer J. Clin.* **2021**, *71*, 209–249. [\[CrossRef\]](#)
12. Raguz, S.; Yagüe, E. Resistance to chemotherapy: New treatments and novel insights into an old problem. *Br. J. Cancer* **2008**, *99*, 387–391. [\[CrossRef\]](#)
13. Zheng, L.P.; Zou, T.; Ma, Y.J.; Wang, J.W.; Zhang, Y.Q. Antioxidant and DNA damage protecting activity of exopolysaccharides from the endophytic bacterium *Bacillus Cereus* SZ1. *Molecules* **2016**, *21*, 174. [\[CrossRef\]](#)
14. Ghosh, A.; Sutradhar, S.; Baishya, D. Delineating thermophilic xylanase from *Bacillus licheniformis* DM5 towards its potential application in xylooligosaccharides production. *World J. Microbiol. Biotechnol.* **2019**, *35*, 34. [\[CrossRef\]](#)
15. Nafie, M.S.; Awad, N.M.; Tag, H.M.; Abd El-Salam, I.M.; Diab, M.K.; El-Shatoury, S.A. Micromonospora species from rarely-exploited Egyptian habitats: Chemical profile, antimicrobial, and antitumor activities through antioxidant properties. *Appl. Microbiol. Biotechnol.* **2021**, *105*, 2427–2439. [\[CrossRef\]](#)
16. Ek-Ramos, M.J.; Gomez-Flores, R.; Orozco-Flores, A.A.; Rodríguez-Padilla, C.; González-Ochoa, G.; Tamez-Guerra, P. Bioactive products from plant-endophytic Gram-positive bacteria. *Front. Microbiol.* **2019**, *10*, 463. [\[CrossRef\]](#)
17. Alarcon-Aguilar, F.J.; Calzada-Bermejo, F.; Hernandez-Galicia, E.; Ruiz-Angeles, C.; Roman-Ramos, R. Acute and chronic hypoglycemic effect of *Ibervillea sonidoe* root extracts-II. *J. Ethnopharmacol.* **2005**, *97*, 447–452. [\[CrossRef\]](#)
18. Torres-Moreno, H.; Marcotullio, M.C.; Velázquez, C.; Ianni, F.; Garibay-Escobar, A.; Robles-Zepeda, R.E. Cucurbitacin IIb, a steroidal triterpene from *Ibervillea sonidoe* induces antiproliferative and apoptotic effects on cervical and lung cancer cells. *Steroids* **2020**, *157*, 108597. [\[CrossRef\]](#)
19. Vidal-Gutiérrez, M.; Torres-Moreno, H.; Hernández-Gutiérrez, S.; Velazquez, C.; Robles-Zepeda, R.E.; Vilegas, W. Antiproliferative activity of standardized phytopreparations from *Ibervillea sonidoe* (S. Watson) Greene. *Steroids* **2021**, *169*, 108824. [\[CrossRef\]](#)
20. Torres-Moreno, H.; Velázquez, C.A.; Garibay-Escobar, A.; Curini, M.; Marcotullio, M.C.; Robles-Zepeda, R.E. Antiproliferative induction and apoptosis of cucurbitacin-type triterpenes from *Ibervillea sonora*. *Crops Ind. Prod.* **2015**, *77*, 895–900. [\[CrossRef\]](#)
21. Karanam, G.; Arumugam, M.K.; Sirpu Natesh, N. Anticancer effect of marine sponge-associated *Bacillus pumilus* AMK1 derived dipeptide Cyclo (-Pro-Tyr) in human liver cancer cell line through apoptosis and G2/M phase arrest. *Int. J. Pept. Res. Ther.* **2020**, *26*, 445–457. [\[CrossRef\]](#)
22. Molina, M.A.F.; Gamboa, E.M.; Calderon, M.E.R.; Benavides, P.Z.; Morales, L.G.R.; Monsivais, L.Z.; Cerda, E.E.C.N.; Rivera, C.A.S.; Guerra, R.T.; Padilla, C.R.I.G. In vitro antitumor activity of soluble protein extracts from *Bacillus thuringiensis*. *Afr. J. Microbiol. Res.* **2016**, *10*, 324–329. [\[CrossRef\]](#)
23. Nighat, F.; Mushtaq, Z.; Maqsood, M.; Shahid, M.; Hanif, M.A.; Jamil, A. Cytotoxic, α -amylase inhibitory and thrombolytic activities of organic and aqueous extracts of *Bacillus clausii* KP10. *Pak. J. Pharm. Sci.* **2020**, *33*, 135.
24. Dey, G.; Bharti, R.; Banerjee, I.; Das, A.K.; Das, C.K.; Das, S.; Jena, B.C.; Misra, M.; Sen, R.; Mandal, M. Pre-clinical risk assessment and therapeutic potential of antitumor lipopeptide 'Iturin A' in an in vivo and in vitro model. *RSC Adv.* **2016**, *6*, 71612–71623. [\[CrossRef\]](#)
25. Ma, E.L.; Choi, Y.J.; Choi, J.; Pothoulakis, C.; Rhee, S.H.; Im, E. The anticancer effect of probiotic *Bacillus polyfermenticus* on human colon cancer cells is mediated through ErbB2 and ErbB3 inhibition. *Int. J. Cancer* **2010**, *127*, 780–790. [\[CrossRef\]](#) [\[PubMed\]](#)
26. Zhao, H.; Yan, L.; Guo, L.; Sun, H.; Huang, Q.; Shao, D.; Jiang, C.; Shi, J. Effects of *Bacillus subtilis* iturin A on HepG2 cells in vitro and vivo. *AMB Express* **2021**, *11*, 67. [\[CrossRef\]](#) [\[PubMed\]](#)
27. Prihanto, A.A.; Firdaus, M.; Nurdiani, R. Endophytic Fungi Isolated from Mangrove (*Rhizophora mucronata*) and Its Antibacterial Activity on *Staphylococcus aureus* and *Escherichia coli*. *J. Food Sci. Eng.* **2011**, *1*, 386–389.
28. Mosmann, T. Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. *J. Immunol. Methods* **1983**, *65*, 55–63. [\[CrossRef\]](#)

29. Ramírez-Villalobos, J.M.; Romo-Sáenz, C.I.; Morán-Santibañez, K.S.; Tamez-Guerra, P.; Quintanilla-Licea, R.; Orozco-Flores, A.A.; Romero-Arguelles, R.; Tamez-Guerra, R.; Rodríguez-Padilla, C.; Gomez-Flores, R. In vitro tumor cell growth inhibition induced by *Lophocereus marginatus* (Dc.) S. Arias and Terrazas endophytic fungi extracts. *Int. J. Environ. Public Health Res.* **2021**, *18*, 9917. [\[CrossRef\]](#)
30. Kačániová, M.; Vukovic, N.L.; Čmiková, N.; Bianchi, A.; Garzoli, S.; Ben Saad, R.; Ben Hsouna, A.; Elizondo-Luévano, J.H.; Ahl, H.A.H.S.-A.; Hikal, W.M.; et al. Biological activity and phytochemical characteristics of star anise (*Illicium verum*) essential oil and its anti-Salmonella activity on sous vide pumpkin model. *Foods* **2024**, *13*, 1505. [\[CrossRef\]](#)
31. Elizondo-Luévano, J.H.; Castro-Ríos, R.; Vicente, B.; Fernández-Soto, P.; López-Aban, J.; Antonio, M.U.R.O.; Chávez-Montes, A. In vitro antischistosomal activity of the Argemone Mexicana methanolic extract and its main component berberine. *Iran. J. Parasitol.* **2021**, *16*, 91. [\[CrossRef\]](#)
32. Rodríguez-Garza, N.E.; Quintanilla-Licea, R.; Romo-Sáenz, C.I.; Elizondo-Luevano, J.H.; Tamez-Guerra, P.; Rodríguez-Padilla, C.; Gomez-Flores, R. In Vitro Biological Activity and Lymphoma Cell Growth Inhibition by Selected Mexican Medicinal Plants. *Life* **2023**, *13*, 958. [\[CrossRef\]](#)
33. Workman, P.; Aboagye, E.O.; Balkwill, F.; Balmain, A.; Bruder, G.; Chaplin, D.J.; Double, J.A.; Everitt, J.; Farningham, D.A.H.; Glennie, M.J.; et al. Guidelines for the welfare and use of animals in cancer research. *Br. J. Cancer* **2010**, *102*, 1555–1577. [\[CrossRef\]](#)
34. Clark-Pérez, D.L.; Romo-Sáenz, C.I.; Ramírez-Villalobos, J.M.; Tamez-Guerra, P.; Caballero-Hernández, D.; Delgado-Miranda, A.L.; García, A.; Gomez-Flores, R. In Vitro and In Vivo Antitumor Activity of *Lophocereus marginatus* (DC.) S. Arias & Terrazas Endophytic *Aspergillus versicolor* and *Metarhizium anisopliae* Extracts Against the Murine Lymphoma L5178Y-R. *Microorganisms* **2024**, *12*, 2310. [\[CrossRef\]](#) [\[PubMed\]](#)
35. Matias, M.; Silvestre, S.; Falcao, A.; Alves, G. Considerations and Pitfalls in Selecting the Drug Vehicles for Evaluation of New Drug Candidates: Focus on in Vivo Pharmac-Toxicological Assays Based on the Rotarod Performance Test. *J. Pharm. Pharm. Sci.* **2018**, *21*, 110–118. [\[CrossRef\]](#) [\[PubMed\]](#)
36. Gomez-Flores, R.; Quintanilla-Licea, R.; Hernandez-Martinez, C.H.; Samaniego-Escamilla, M.; Tamez-Guerra, P.; Monreal-Cuevas, E.; Tamez-Guerra, R.; Rodriguez-Padilla, C. Survival of Lymphoma-Bearing Mice by *Pachycereus Marginatus* Cactus Extracts and Elucidation of Bioactive Compounds. *Nat. Prod. Commun.* **2019**, *14*, 1934578X19845814. [\[CrossRef\]](#)

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.