

## Full Length Research Paper

## ***In vitro* antitumoral activity of soluble protein extracts of *Bacillus thuringiensis***

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There are many studies about the antitumour effects of *Bacillus* toxins from different strains or subspecies in different parts of the world. Proteins that selectively kill tumor cells *in vitro* have potential as anticancer agents. The aim of this study was to evaluate the cytotoxic activity of soluble proteins extracts (SPE) from Mexican strains of *B. thuringiensis* on murine lymphoma L5178YR cell line. *In vitro*, L5178YR cells were treated with different concentrations of specific primers (SPE) from *B. thuringiensis* (0 to 39.85 µg/mL) and cellular viability was evaluated by MTT method and orange acridine/ethidium bromide staining. The mechanism of cell death was evaluated through caspase-3 activation by flow cytometry and TUNEL assays. The study results shows that SPE from *B. thuringiensis* (GM1-3 h, GM1-24 h, GM1-48 h, GM18-3 h, GM18-24 h, GM18-48 h, HD512-3 h, HD512-24 h, and HD512-48 h) affected the cell viability of L5178YR in a dose-dependent manner which presented higher cytotoxic effect of SPE collected at 3 h independent of the strain used; and the 7% SDS-PAGE presented an electrophoretic profile of proteins in a range of 10 to 100 kDa of the SPE *B. thuringiensis*. The cytotoxicity is through a mechanism of apoptosis because the caspase-3 activation and TUNEL assays corroborated this result. In conclusion, SPE derived from early culture (3 h) of *B. thuringiensis* (*Bt*) GM1, GM18 and HD-512 have *in vitro* cytotoxic potential on murine lymphoma L5178YR cell line through a mechanism of cell death by apoptosis.

**Key words:** *Bacillus thuringiensis*, soluble protein extracts, cancer.

### INTRODUCTION

The entomopathogenic *Bacillus thuringiensis*, a gram-positive bacterium, is naturally found in the soil. It is characterized by crystal production during sporulation, containing Cry proteins, encoded by the *Cry* genes, with

a wide division into classes and subclasses according to their insecticide activity, and presently classified according to the percent amino acid identity between Cry protein sequences (Mezzomo et al., 2015; Mohamed et

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al., 2010; Palma et al., 2014a). Besides the cry proteins, known as  $\delta$ -endotoxins, *B. thuringiensis* isolates can synthesize other toxins, such as  $\beta$ -exotoxin, phospholipases, proteases, chitinases and enterotoxins (Berlitz et al., 2012).

*B. thuringiensis* also produces proteins during its vegetative state. Some of these are insecticidal, and are called vegetative insecticidal proteins (VIP). VIP were named as such because, unlike the Cry and Cyt proteins, they are mainly produced during the vegetative growth phase of  $\delta$ -endotoxins, *B. thuringiensis* cultures, although their secretion can also be extended into the sporulation stage. vip genes have shown no DNA sequences homology to cry and cyt genes, suggesting they bind to different receptors (Estruch et al., 1996; Lee et al., 2003). Four basic types of VIP toxins (Vip I, II, III, and IV) have been described, although they can be present in a variety of forms within each class (Yu et al., 1997).

The Cry and Cyt proteins toxins of *B. thuringiensis* have different activities including; antimicrobial (Cahan et al., 2008), insecticidal (Höfte and Whiteley, 1989), toxicity against nematode (Wei et al., 2003), antitumoral, (Chan et al., 2012; Jung et al., 2007) and adjuvant of the immune system (Román Calderón et al., 2007). *B. thuringiensis* spore crystals have shown toxicity for lymphocytes and promoting cytotoxic and genotoxic effects for the erythroid lineage of bone marrow at high concentrations which is not commonly found in the environment, indicated that these *B. thuringiensis* spore crystals (Mezzomo et al., 2015) were not harmless to mammals (Mezzomo et al., 2015; Okumura et al., 2014).

Since 1970s, Prasad and Shethna had carried out research on the antitumour effects of *B. thuringiensis* toxins from different strains or subspecies (Wong, 2010). Recently, the named cell-free supernatant of *B. thuringiensis* demonstrated as a suitable biostimulation agent for enhancing chlorpyrifos biodegradation in chlorpyrifos-contaminated soils (Aceves-Diez et al., 2015). But scarce studies about the anticancer activity of soluble protein extracts (SPE) from *B. thuringiensis* have been carried out; the aim of this study was to determine the cytotoxic effect of SPE from Mexican strains of *B. thuringiensis* on lymphoma murine L5178YR.

## MATERIALS AND METHODS

### *B. thuringiensis* strains

The *B. thuringiensis* strain GM1 (serovar 7 *aizawai*, isolated of garden soil from México), GM18 (serovar 24A24B *neolenensis*, isolated of agriculture soil from México) and HD-512 (serovar 15 *Dakota*) were from the culture collection of Howard Dulmage. All strains were kept under dry in the Bank of Laboratory of Immunology and Virology from Faculty of Biological Science of University Autonomous of Nuevo León, México. They were grown at 30°C on nutrient broth (pH 7.6) consisting of meat extract (10 g), polypeptone (10 g), NaCl (2 g), and distilled water (1000 mL). The cultures were kept in a rotatory shaker at 14 x g by 3, 24, and 48 h at 30°C. Then the supernatants of strains were collected by

centrifugation at 7519 x g by 10 min. The SPE were precipitated by acetone in a proportion of 1:1 of supernatant and incubated 30 min at 70°C. Thereafter, centrifuged at 30,074 x g/30 min, the supernatant was eliminated and the pellet was resuspended in sterile distilled water and washed three times with sterile distilled water and stored at 20°C until use. The treatments were named according to the strain and time recollection; GM1-3h, GM1-24h, GM1-48h, GM18-3h, GM18-24h, GM18-48h, HD512-3h, HD512-24h and HD512-48h.

### MTT method

L5178YR cells obtained from ATCC (American Type Culture Collection) were cultured in RPMI medium with 10% FBS, and  $5 \times 10^3$  cells/well were plated on 96 flat-bottom well plates, and the SPE of *B. thuringiensis* were diluted in the same medium and added at concentrations ranging from 0 to 39.85  $\mu\text{g/mL}$ . The plates were then incubated for 24 h at 37°C, and 5% CO<sub>2</sub> atmosphere. Thereafter, the supernatants were removed, and cells were washed twice with RPMI-1640 medium. Cell viability was determined by the MTT method. Quantification was obtained by the absorbance reading at a wavelength of 570 nm and cellular viability was expressed as percentage.

### Acridine orange/ethidium bromide

L5178YR cancer cells were briefly seeded at  $1 \times 10^6$  cells/well into 6-well plates in RPMI-1640 with 10% FBS and treated with SPE of *B. thuringiensis* at doses mentioned previously. After one washing with phosphate-buffered saline (PBS), the cells were stained with 2  $\mu\text{L}$  of a mixture (1:1) of acridine orange-ethidium bromide (100  $\mu\text{g/mL}$ ) in PBS. The cells were incubated for five minutes in the dark at room temperature and washed with phosphate-buffered saline; then were viewed under a Nikon inverted fluorescent microscope (TE-Eclipse 300) with an attached camera and photographs were taken under fluorescent conditions. Detection of apoptosis was based on morphological and fluorescent characteristics of the stained cells. Viable cells were indicated by bright green color, apoptotic cells by orange/brown color, and necrotic cells by red color.

### Protein determination

Protein concentration was measured by the method of Bradford using bovine serum albumin as standard (Bradford, 1976).

### SDS-PAGE

The protein profile of the soluble supernatant from each strain evaluated was performance according to the Laemmli method gel electrophoresis (Lane, 1978).

### Flow cytometry analysis

L5178YR cells ( $1 \times 10^6$ ) were treated with SPE of *B. thuringiensis* at doses mentioned previously, and after 24 h of incubation, the active caspase-3 was detected using the PE-conjugated active caspase-3 apoptosis kit (BD Pharmingen™), by flow cytometry analysis (Accuri Flow Cytometer, Beckton Dickinson).

### TUNEL assay

Nuclear DNA fragmentation of apoptotic cells was measured by the

**Table 1.** Relative cell viability of L5178YR treated with SPE of *B. thuringiensis* by MTT. L5178YR ( $5 \times 10^3$  cells) were treated with different concentrations of SPE from *B. thuringiensis*, incubated for 24 h at 37°C, and 5% CO<sub>2</sub> atmosphere. Cell viability was determined by the MTT method. Quantification was obtained by the absorbance reading at a wavelength of 570 nm and cellular viability was expressed as percentage. Results were given as the mean  $\pm$  SD of three independent experiments (\* $p < 0.05$ ).

Treatments	6.65 $\mu\text{g/mL}$ RCV (%)	26.55 $\mu\text{g/mL}$ RCV (%)	39.85 $\mu\text{g/mL}$ RCV (%)
GM1-3 h	36.76*	2.75*	8.69*
GM18-3 h	78.15	13.04*	8.80*
HD512-3 h	60.65*	14.10*	16.12*
GM1-24 h	61.19*	64.80*	34.89*
GM18-24 h	71.58	55.67*	57.05*
HD512-24 h	94.80	105.30	38.81*
GM18-48 h	89.18	57.68*	55.04*
HD512-48	67.89*	63.73*	44.64*
Control	100	100	100

TUNEL assay (DeadEnd Fluorometric TUNEL System; Promega, Madison, WI). Briefly, culture medium was discarded, cells were fixed in 4% formaldehyde, permeabilized in 0.2% Triton X-100 and incubated with TdT incubation buffer for 60 min in a 37°C humidified incubator for 3'-OH labeling. Immediately, analyze samples under a fluorescence microscope using a standard fluorescein filter was set to view the green fluorescence of fluorescein at  $520 \pm 20$  nm; view red fluorescence of propidium iodide at  $>620$  nm.

#### Statistical analysis

Data represent the mean  $\pm$ SD of triplicates from three independent experiments. Statistical differences were obtained using the analysis of variance, and the Dunnett's tests (SPSS v. 17.0 program). The results were considered statistically significant if the \* $p$  value was  $< 0.05$ .

## RESULTS

### Cell viability assays

The SPE of *B. thuringiensis* treatments (GM1-3h, GM1-24h, GM1-48h, GM18-3h, GM18-24h, GM18-48h, HD512-3h, HD512-24h, and HD512-48h) affected the cell viability of L5178YR in a dose-dependent manner, except *Bt* HD512-24h at doses of 6.65 and 26.55  $\mu\text{g/mL}$  (Table 1). We selected the SPE of *B. thuringiensis* treatments from GM1 3h, GM-18 3h, and HD-512 3h, because were the best treatments that affected the cell viability to carry out the experiments with acridine orange/ethidium bromide staining, shown that these treatments induced cell death by apoptosis characteristic by orange color in cells (Figure 1).

### SDS-PAGE

The SDS-PAGE of SPE of *B. thuringiensis* from GM1 3h,

GM-18 3h and HD-512 3h shows an electrophoretic profile of proteins in a range of 10 to 100 kDa. Being of special interest, the presence of protein major the amount of molecular weight of 16.69 kDa (Figure 2).

### Apoptosis assays

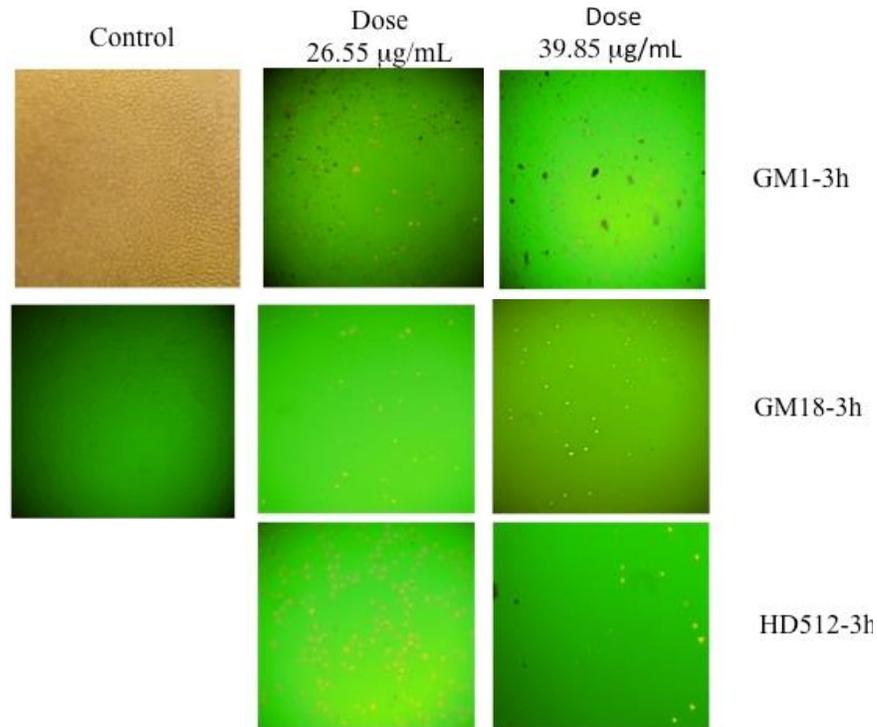
In order to confirm the type of cellular death induced by SPE of *B. thuringiensis*, shown by TUNEL assay the presence of apoptosis (Figure 3). This data was corroborated by caspase-3 activation in treatments with GM1-3h at 26.55  $\mu\text{g/mL}$  (20.4%) GM1-3h at 39.85  $\mu\text{g/mL}$  (28.9%), GM18-3h at 26.55  $\mu\text{g/mL}$  (23.4%), GM18-3h at 39.85  $\mu\text{g/mL}$  (19 %), HD512-3h at 26.55  $\mu\text{g/mL}$  (27.3%) and HD512-3h at 39.85  $\mu\text{g/mL}$  (36.9%) characteristic of apoptosis (Figure 4).

## DISCUSSION

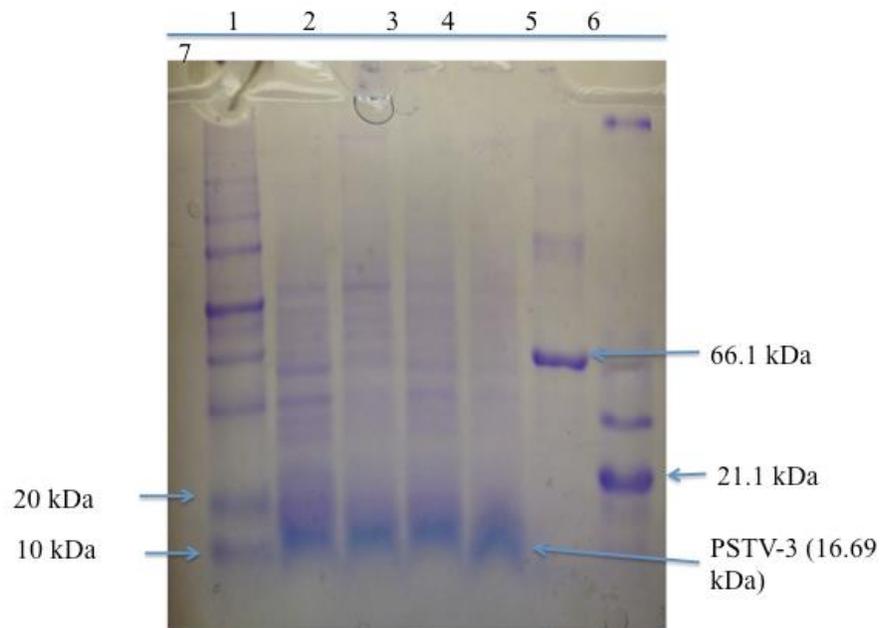
Parasporins are parasporal proteins produced by *B. thuringiensis* that are capable of killing cancer cells (Okassov et al., 2015; Okumura et al., 2014), similar to this study results obtained with SPE from *B. thuringiensis* that shows cytotoxic activity on L5178YR murine lymphoma.

In the literature reviewed, no data was referred to the *in vitro* action of SPE of *B. thuringiensis* strains GM1, GM18 and HD-512. Studying vegetative proteins from Malaysian, strains of *B. thuringiensis israelensis* (*Bt* 11, *Bt* 12, *Bt* 15, *Bt* 16, *Bt* 17, *Bt* 21 and *Bt* 22) and *Bacillus sphaericus* H-25 strains (Bs 1 and Bs 2) were found, and screened for cytotoxic activity being indiscriminately cytotoxic to both CEM-SS (human T lymphoblastoid) and HeLa (human uterus cervical cancer) cell lines (Ramasamy et al., 2008).

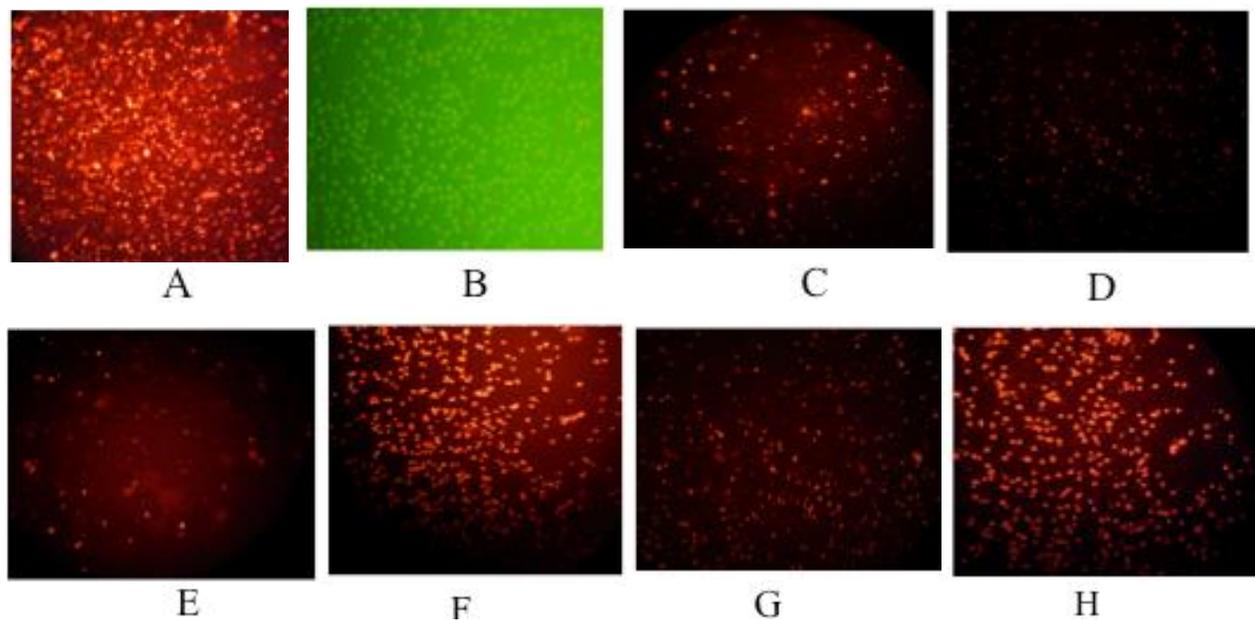
The SPE collected at 3 h shows several proteins



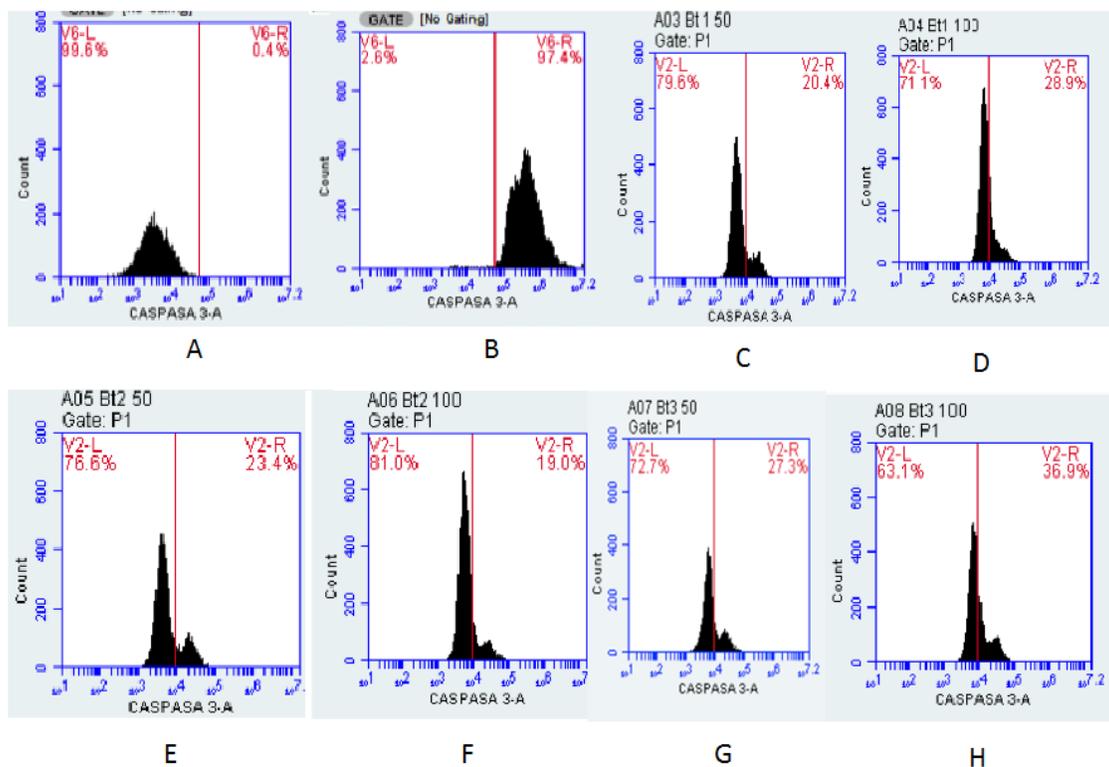
**Figure 1.** Relative cell viability of L5178YR treated with SPE of *B. thuringiensis* by acridine orange/ethidium bromide staining. L5178YR ( $1 \times 10^6$  cells) were treated with different concentrations of SPE from *B. thuringiensis*, incubated for 24 h at 37°C, and 5% CO<sub>2</sub> atmosphere. Cell viability was determined by acridine orange/ethidium bromide method. The cells were viewed under fluorescent microscope. Viable cells were indicated by bright green color, apoptotic cells by orange/brown color, and necrotic cells by red color.



**Figure 2.** 7% SDS-PAGE of SPE of *B. thuringiensis*. Lane 1: Molecular weight marker Promega V849A, Lane 2 and 3: GM1, Lane 4: GM-18, Lane 5: HD-512, Lane 6: BSA; Lane 7: Trypsin inhibitor.



**Figure 3.** L5178YR stained by the TUNEL labeling. A) L5178YR TUNEL positive control cells showing abundant red color characteristic of cellular death, B) untreated control L5178YR cells showing abundant green color characteristic of viability, C) GM1-3h 26.55  $\mu\text{g/mL}$ , D) GM1-3h 39.85  $\mu\text{g/mL}$ , E) GM18-3h 26.55  $\mu\text{g/mL}$ , F) GM18-3h 39.85  $\mu\text{g/mL}$ , G) HD512-3h 26.55  $\mu\text{g/mL}$ , H) HD512-3h 39.85  $\mu\text{g/mL}$ .



**Figure 4.** Representative flow cytometry histograms of caspase-3 activation in apoptotic L5178YR cells. L5178YR cells treated with different doses of SPE from *B. thuringiensis* to determine the activation of caspase-3 by flow cytometry analysis, Accuri Flow Cytometer (Beckton Dickinson). The histograms shown the distribution of: A) caspase-3 negative control cells, B) doxorubicin positive control, C) GM1-3h 26.55  $\mu\text{g/mL}$ , D) GM1-3h 39.85  $\mu\text{g/mL}$ , E) GM18-3h 26.55  $\mu\text{g/mL}$ , F) GM18-3h 39.85  $\mu\text{g/mL}$ , G) HD512-3h 26.55  $\mu\text{g/mL}$  and H) HD512-3h 39.85  $\mu\text{g/mL}$ .

detected by SDS-PAGE in a range of 10 to 100 kDa with cytotoxic activities open the gates to study the sequencing and synthesis of amino acids from each protein being of special interest the proteins with a molecular weight of 16.69 kDa by its abundant expression. Similar to this as reported by Palma et al. (2014b), that describes the insecticidal activity of a novel *B. thuringiensis* Cry-related protein with a deduced 799 amino acid sequence (~89 kDa) and ~19% pairwise identity 95-kDa-aphidicidal protein (sequence number 204) and ~40% pairwise identity to the cancer cell killing Cry proteins (parasporins Cry41Ab1 and Cry41Aa1), respectively. This determined that SPE of *B. thuringiensis* induced cellular death through apoptosis. It has been reported that PS1Aa1 induces apoptosis through caspase-3 in HeLa cells (Okassov et al., 2015).

## Conclusion

The SPE derived from early culture (3 h) of *B. thuringiensis* strains GM1, GM18, and HD-512 have *in vitro* cytotoxic potential on L5178YR lymphoma murine cell line through mechanism of apoptosis. This could offer another modality of cancer therapy, as a novel tumoricidal agent derived from prokaryotic cells but definitive assessment must wait until these observations are clarified through experiments in several tumor cancer cell lines *in vitro* and *in vivo*.

## Conflict of Interests

The authors have not declared any conflict of interests.

## ACKNOWLEDGMENTS

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