Chapter 6

Evaluation of the therapeutic potential of purified L-asparaginase from *Bacillus indicus*

6.1 Introduction

The cancer cells in contrast to normal cells have the potential to remodel their metabolism (metabolic reprogramming) to suit their escalated proliferation rates (Garcia-Bermudez *et al.*, 2020). The increased proliferation imposes substantial nutrient requirements from external environments. The deprivation of nutrients from these extracellular surroundings by utilizing nutrient hydrolyzing enzymes is an effective strategy to specifically target cancer cells (Vander Heiden 2011; Fung and Chan 2017). L-asparagine is one such nutrient that is essentially required by all types of leukemia cells from the extracellular environment to fulfill their growth (Ueno *et al.*, 1997). The inability to synthesize their own L-asparagine owing to the absence of the enzyme asparagine synthetase and the concomitant L-asparaginase mediated breakdown of L-asparagine in the serum upon application makes them extremely vulnerable to nutrient starvation (Sindhu and Manonmani, 2018; Jiang *et al.*, 2021). The L-asparagine depletion induces halt of overall protein synthesis and ultimately leads to cancer cell death (Zhang *et al.*, 2014; Métayer *et al.*, 2019).

Due to the accompanying side effects of the commercial L-asparaginase preparations, there is a need for alternative sources of micro-organisms that can produce the L-asparaginase enzyme with anti-cancer properties. These anti-cancer effects must be effective against the acute lymphoblastic leukemia, against which the L-asparaginase is known as the therapeutic drug. Leukemia cancer starts in the normal cells that would differentiate into diverse types of blood cells. Most commonly, leukemia starts in early forms of white blood cells. Leukemia

are further differentiated on the basis of their origin; whether they starts in myeloid cells (myeloblastic leukemia) or lymphoid (lymphoblastic leukemia) cells. Also leukemia is classified as acute (fast growing) or chronic (slow growing). Acute lymphoblastic leukemia (ALL) is also called acute lymphocytic leukemia**.** "Acute" means that the leukemia can progress quickly, and if not treated, would probably be fatal within a few months. "Lymphocytic" means it develops from early (immature) forms of lymphocytes**,** a type of white blood cell. The anticancer effect of a potential compound can be successfully tested on human derived cancer cell lines. Human cancer cell lines are the essential models widely utilized in laboratories to study the biology of particular cancer, and to also test the therapeutic efficacy of new anticancer therapeutics (Gillet *et al.*, 2013). Although, several type of leukemia cell lines are present to test the effectiveness of a potential drug, viz. Jurkat, MOLT-4, HL-60, K-562, CCRF-CEM, THP-1, RS-4, Kasumi-1, the present studies were specifically performed using the MOLT-4 cell line due to its acute lymphoblastic leukemia (T-lymphoblast) origin.

6.2 Materials and Methods

6.2.1 L-asparaginase enzyme preparation

The purified and characterized L-asparaginase enzyme from *Bacillus indicus* MTCC 4374 as described in chapter 4 was utilized for the determination of its therapeutic potential against the acute lymphoblastic leukemia MOLT-4 cell line.

6.2.2 Chemicals and equipments utilized

Cell culture media – Roswell Park Memorial Institute-1640 medium (RPMI-1640), Foetal Bovine Serum (MP Biomedicals, Germany), Trypsin-EDTA solution (HiMedia, India), 1x phosphate-buffered saline (HiMedia, India), Propidium Iodide staining solution – 2mg/mL,

(Thermo scientific), Acridine orange – 2mg/mL, (Thermo scientific), Dimethyl Sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Cell culture treated T-25 flasks from Biolite (Thermo Fisher Scientific Inc., USA), 6 well cell culture plates (Thermo scientific), Annexin V- AbFlour^{TM} 488 Apoptosis Detection Kit (Abbkine, Inc.) containing AbFlour 488 Annexin V and Propidium Iodide, 10mL serological pipettes and 96-well plates from Nunc (Thermo Fisher Scientific Inc., USA), Fluorescence microscope - XDFL series (Sunny Instruments, China), Analysis Software used in fluorescence microscopy – ImageJ (Fiji) software, Version 1.53c, Flow cytometer used - Cytomics FC500 Flow cytometer (Beckman Coulter, USA), Analysis Software used in flow cytometry - FlowJo X 10.0.7.

6.2.3 Antitumor potential of the purified L-asparaginase - MTT cytotoxicity assays

To determine the therapeutic potential of the purified L-asparaginase from *Bacillus indicus* MTCC 4374, the anti-leukemic effect was evaluated against the human acute lymphoblastic leukemia (MOLT-4) cancer cell line. The leukemia MOLT-4 cells was acquired from NCCS Pune, India and grown as a suspension in RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS) and incubated in humidified air containing 5% CO_2. The antitumor properties of the asparaginase preparation were assessed on the procured cell line using the MTT-based cytotoxicity assay. The assay is based on the metabolic activity of living cells which causes the conversion of yellow tetrazolium salt to a purple-colored insoluble formazan product that can be quantified spectrophotometrically. 80 µL of cell suspension was incubated with 20 µL of different concentrations (0.1 - 100 µM) of the purified L-asparaginase for a treatment period of 24 hours at 37 ºC. Then, 10 µl MTT reagent was added and further incubated for 3 hours. After MTT treatment, insoluble formazan precipitates were solubilized using DMSO and the resulting absorbance was computed at 570 nm. The known anti-cancer drug doxorubicin was taken as the positive control.

6.2.4 Assessment of live/dead cells by acridine orange/propidium iodide (AO/PI) dual staining

The accurate assessment of cell viability can be performed using AO/PI dual staining. Acridine orange (AO) can permeate the intact membranes of all the nucleated cells and renders green fluorescence. The propidium iodide (PI) will only bind the dead cells with poor membrane integrity and renders red fluorescence. The net outcome is the accurate live/dead cell discrimination qualitatively. Cultured MOLT-4 cells (~ 3×10^5 cells/mL) in a 6 well plate were incubated at 37 ºC for 24 hours. The spent medium was then aspirated and washed. The cells were then treated with the known concentration of purified L-asparaginase and incubated for 24 hours. 500 µL of AO/PI solution (200 µg/mL) was added to every well and incubated for 5 min. The resulting cells were then visualized using fluorescence microscopy.

6.2.5 Assessment of apoptotis using Annexin V/Propidium iodide assay

The induction of apoptosis by a test compound can be readily identified using the Annexin V/PI assay (Vermes et al. 1995; Van Engeland et al. 1998). Annexin V apoptosis is a form of programmed cell death to remove unwanted, damaged, or senescent cells from tissues. In normal cells, the negative phospholipids reside on the inner side of the cellular membrane while the outer surface of the membrane is occupied by uncharged phospholipids. After a cell has entered apoptosis, the negatively charged phosphatidylserine (PS) are transported from the inner to the outer leaflet of the plasma membrane, thus exposing PS to the external cellular environment. The human anticoagulant, Annexin V, is a 35-36 kDa Ca^{2+}-dependent phospholipid-binding protein that has a high affinity for PS. Annexin V labeled with a fluorophore or biotin can identify apoptotic cells by binding to PS exposed on the outer leaflet. Propidium iodide (PI) is a fluorescent nucleus dye, impermeable to live cells but stains dead cells with red fluorescence by binding tightly to the nucleic acids in the cell. After

staining a cell population with AbFlour^{TM} 488 annexin V and PI in the provided binding buffer, early apoptotic cells show green fluorescence of the cellular membrane, dead cells show red fluorescence of the nucleus and green fluorescence of the cellular membrane, and live cells show little or no fluorescence. Detection can be analyzed by flow cytometry or by fluorescence microscopy.

The MOLT-4 cells were initially seeded (~3 x 10^5 cells/mL) and incubated at 37 ºC for 24 hours. The cells were then treated with the test L-asparaginase for 24 hours incubation period. The untreated cells were taken as negative control while doxorubicin-treated cells served as the positive control. After washing twice with PBS, 100 µl of cell suspension was incubated with 5 µl of AbFlour 488 conjugated Annexin V dye and 2 µl of PI. The flow cytometry of the samples were then performed on the Cytomics FC500 flow cytometer (Beckman Coulter, U.S.A).

6.3 Results and Discussion

6.3.1 Cytotoxic evaluation of the anti-leukemic potential purified L-asparaginase

The MOLT-4 cell line belonging to the human acute lymphoblastic leukemia cells were cultured on RPMI-1640 culture medium to study the anti-cancer effects of the purified L-asparaginase. The quantitative determination of cytotoxicity by the purified L-asparaginase was carried out using the metabolic activity based MTT (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. **Figure 6.1** shows the cultured MOLT-4 cells in suspension before and after the L-asparaginase treatment. The cytotoxic activity against leukemia cells by the purified asparaginase was analyzed in terms of IC_{50}, the concentration of potential drug to kill 50% of the cells after 24 h of L-asparaginase treatment. The varying concentrations of the purified L-asparaginase (10^{-7} – 10^{-4} M) from *Bacillus indicus* MTCC 4374 were evaluated to study the cytotoxic potential against leukemia *in vitro* (**Figure 6.2**). After 24 h of the incubation period, the purified L-asparaginase was found to be highly efficient in the killing of leukemia MOLT-4 cells with an IC_{50} of 1.21 µM. This IC_{50} corresponds to 0.67 IU/mL of purified L-asparaginase activity. The IC_{50} shown by the purified L-asparaginase was found to be better than the commercial L-asparaginase preparation from *Erwinia chrysanthemi* and comparable to the L-asparaginase of *Escherichia coli*. Earlier studies performed on the L-asparaginases of *E. chrysanthemi* and *E. coli* depicted an IC_{50} of 2 IU/mL and >1 IU/mL, respectively against MOLT-4 (acute lymphoblastic leukemia) cell line (Abakumova *et al.*, 2012). Moreover, the cytotoxicity shown by the purified L-asparaginase against the MOLT-4 cells was higher (depicted by the lower IC_{50}) compared to several other reported cytotoxic L-asparaginases. The L-asparaginase of *Enterobacter cloacae* showed an IC_{50} of 7.1 IU/mL against MOLT-4 leukemia cells (Husain *et al.*, 2016). Similarly, the utilization of L-asparaginase from *Streptomyces fradiae* against Caco2 cancer cells reported an IC_{50} of 4 IU/mL (El-Naggar *et al.*, 2016). The purified enzyme

also revealed better cytotoxic properties than the standard anti-leukemic drug doxorubicin, which showed an IC_{50} of 6.4 µM. The toxicity was found to be dose-dependent as the increase in concentration was accompanied by an increase in growth inhibition. The calculations of IC_{50} were performed considering the non-linear regression nature of the drug dose-response studies.

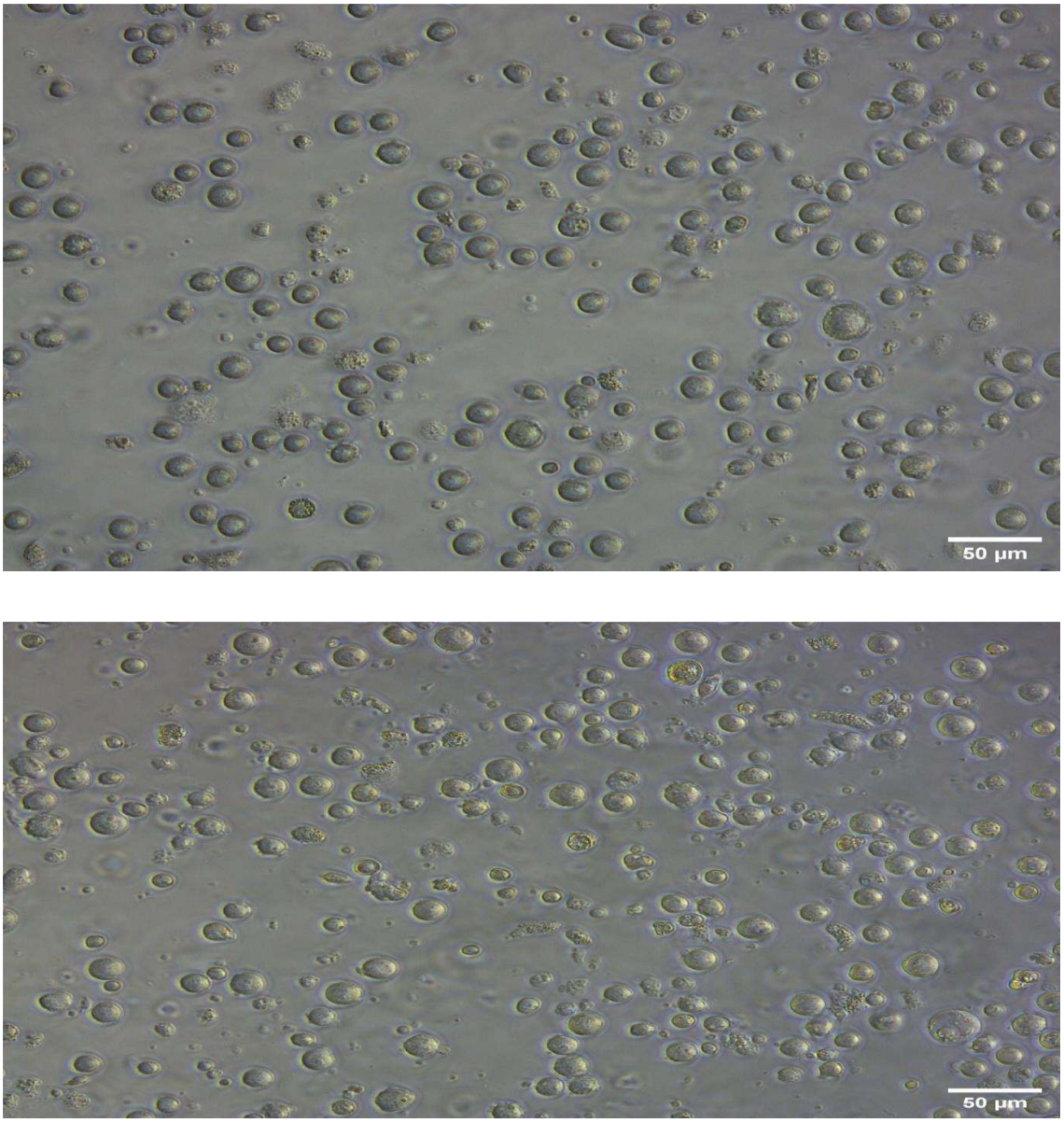

Figure 6.1: Inverted microscopy images of MOLT-4 cells (50 µm scale). (a) Untreated cells (b) cells after purified L-asparaginase treatment.

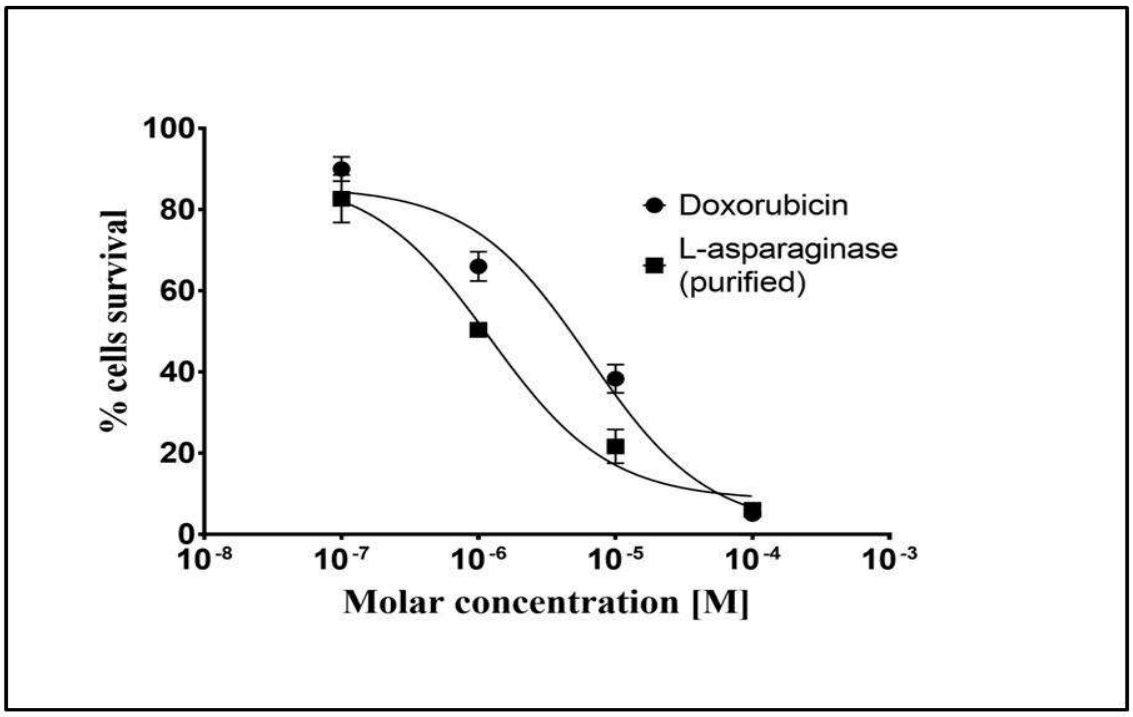

Figure 6.2: Anti-leukemic effect exerted by the purified L-asparaginase on human leukemia MOLT-4 cells (Sharma and Mishra, 2023).

6.3.2 Live / dead cells assay using the AO / PI dual staining

The AO/PI method using fluorescence microscopy is a reliable live/dead cell assay that has superiority over the trypan blue dye exclusion method (Bank 1988; Mascotti et al. 2000). The ability of propidium iodide to bind the non-viable cells or cells with compromised cell membranes ensures the accurate detection of dead cells among the viable cells that fluoresce green. The detailed images of untreated and treated MOLT-4 cells by the purified L-asparaginase were captured using fluorescence microscopy. **Figure 6.3** denotes the cell density of MOLT-4 cells before the L-asparaginase treatment. The untreated MOLT-4 leukemic cells show a higher cell density of living cells with very few dead cells. After the 24 h incubation of leukemic cells, a decrease in the cell density and appearance of orange-red spots were visible uniformly. These red orange spots depicted the dead cells with no

membrane integrity and the propidium iodide dye binding imparted the red florescence (**Figure 6.4)**.

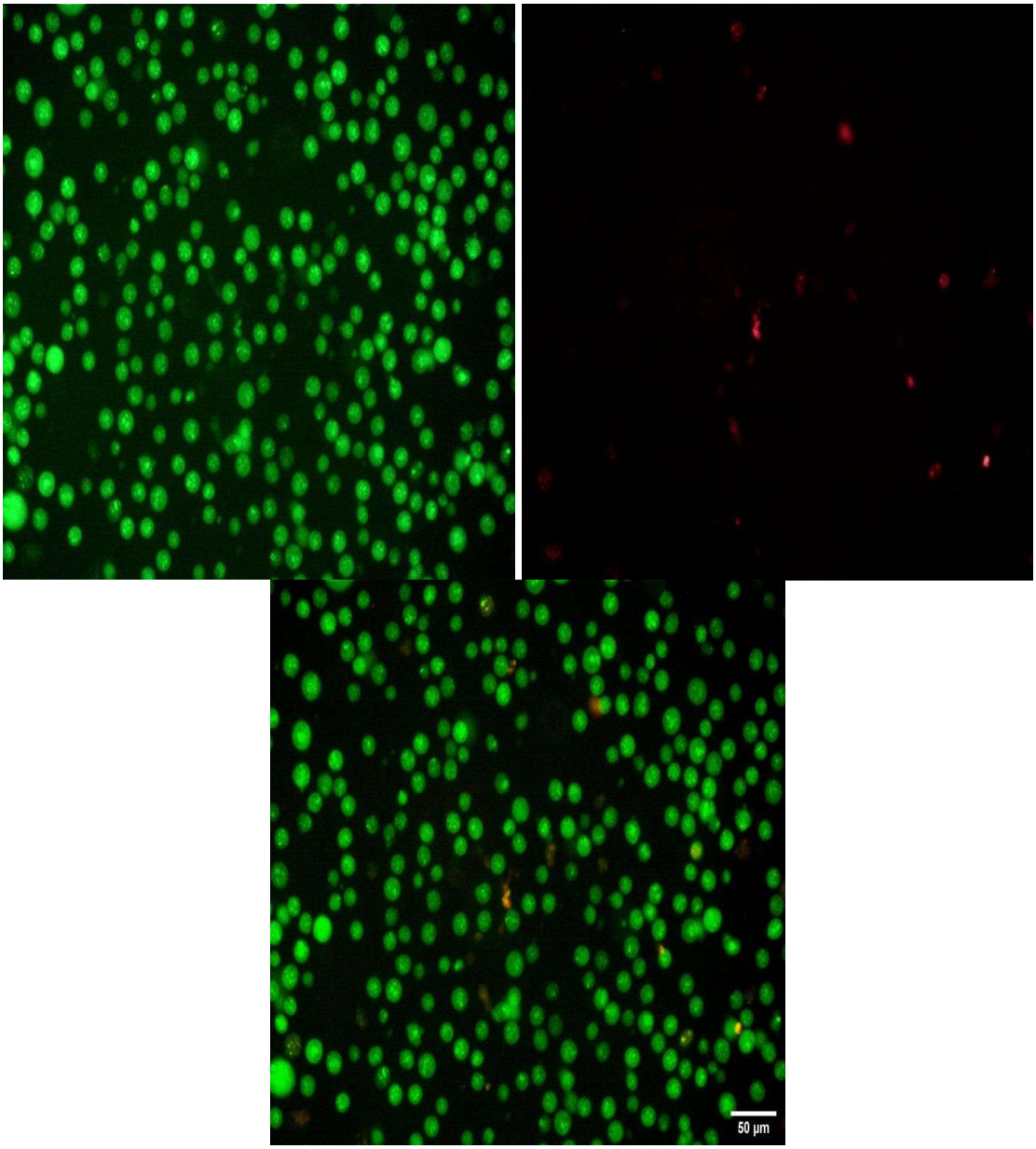

Figure 6.3: Live/dead cell assay using acridine orange/propidium iodide staining of MOLT-4 cells. Untreated cells stained with (a) acridine orange; (b) propidium iodide; (c) combined (merged) image stained with both acridine orange and propidium iodide (Sharma and Mishra, 2023).

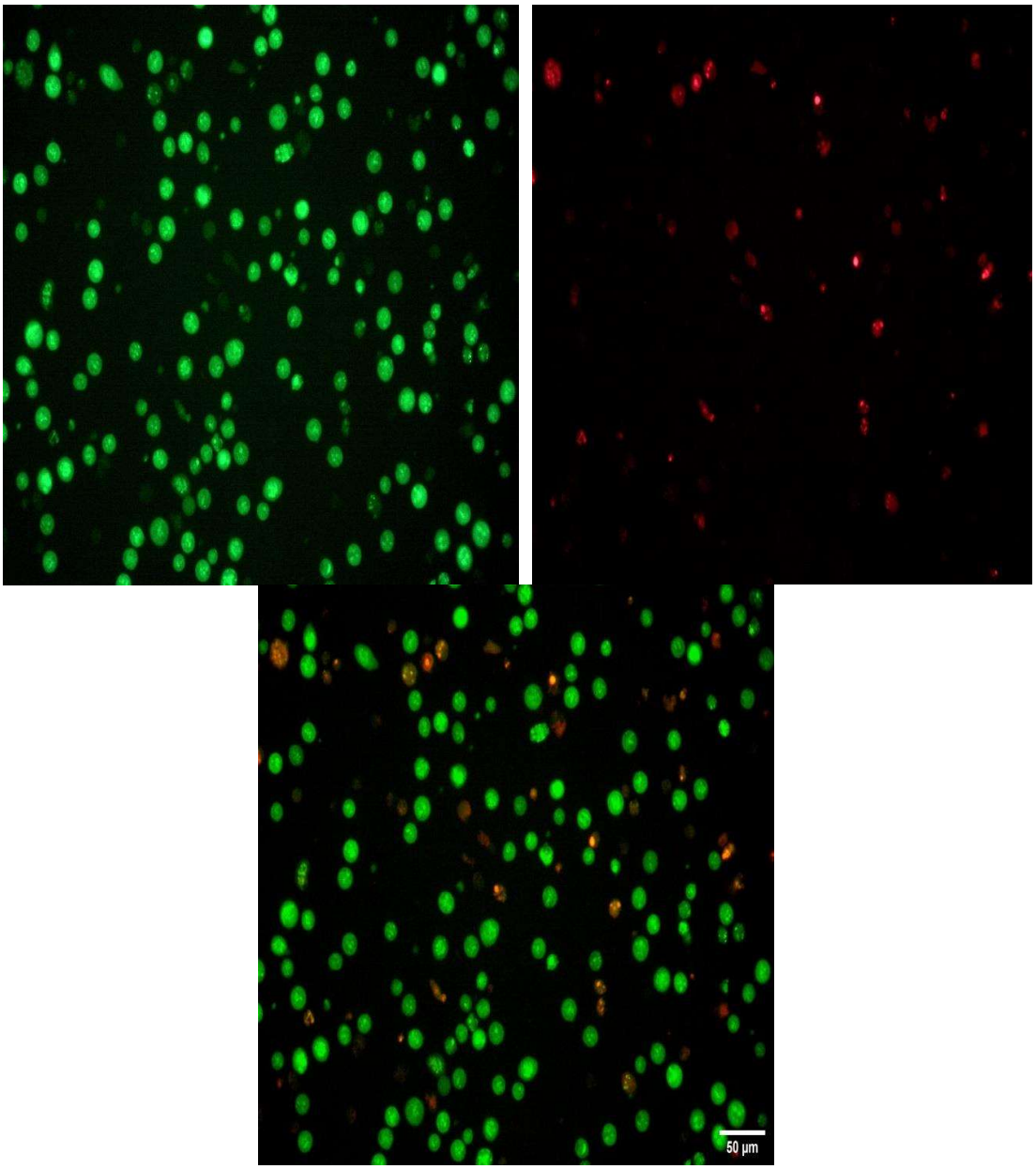

Figure 6.4: Live/dead cell assay using acridine orange/propidium iodide staining of MOLT-4 cells after purified L-asparaginase treatment. Treated cells stained with (a) acridine orange; (b) propidium iodide; (c) combined (merged) image stained with both acridine orange and propidium iodide (Sharma and Mishra, 2023).

6.3.3 Quantitative confirmation of apoptotic process using flow cytometry

The use of Annexin V conjugated with AbFlour 488 and propidium iodide (PI) is a reliable technique for detecting apoptosis using flow cytometry. The fluorescence of the Annexin V conjugated with AbFlour 488 was detected using a 525 nm band filter and collected in the FL1 detector, while the PI fluorescence was detected using a 620 nm band filter and collected in FL3 detector. The dual staining assay can accurately detect and quantify the percentage of live cells, early apoptotic cells and late apoptotic cells. The outcomes demonstrated the therapeutic potential of purified asparaginase preparation from *Bacillus indicus* MTCC 4374 to induce apoptosis in the MOLT-4 leukemic cells **(Figure 6.6).** The Annexin V/PI staining exemplified the apoptotic cells percentage of 21.4% in the MOLT-4 leukemic cells after 24 hours of L-asparaginase treatment in the present study. The usage of the standard drug doxorubicin as the positive control led to the apoptotic cell percentage of 14.84% (**Figure 6.7)**. In this context, the purified L-asparaginase proved to be a potent therapeutic agent against leukemic cells. The possible underlying mechanism of the apoptotic induction by the purified L-asparaginase is the deprivation of the essential nutrient L-asparagine by catalyzing its hydrolysis. Moreover, the inability of the tumor cells to synthesize their L-asparagine resulted in the halt of protein synthesis that further led to the induction of apoptosis.

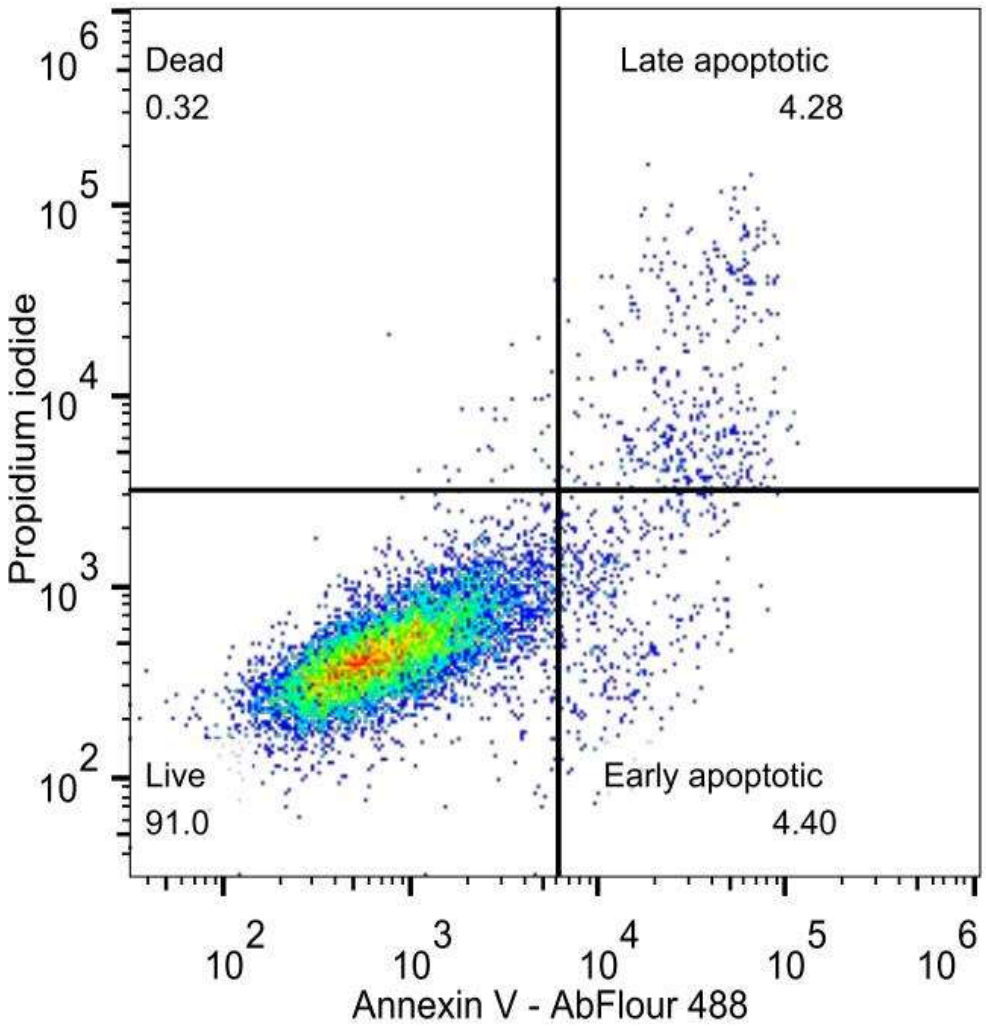

Figure 6.5: Flow cytometry analysis depicting the apoptosis after 24 h treatment on untreated MOLT-4 leukemia cells (Sharma and Mishra, 2023).

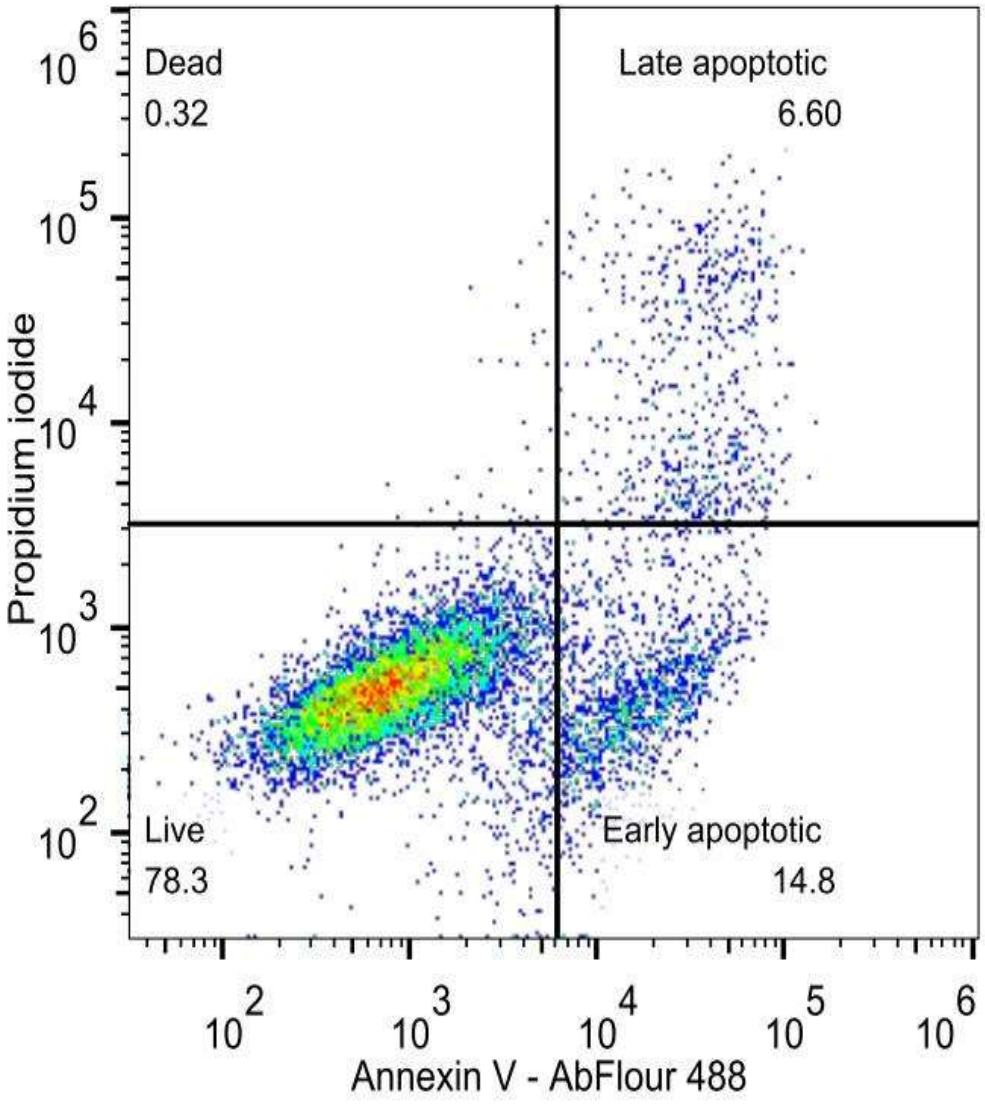

Figure 6.6: Flow cytometry analysis depicting the apoptosis induced by the purified L-asparaginase after 24 h treatment on MOLT-4 leukemia cells (Sharma and Mishra, 2023).

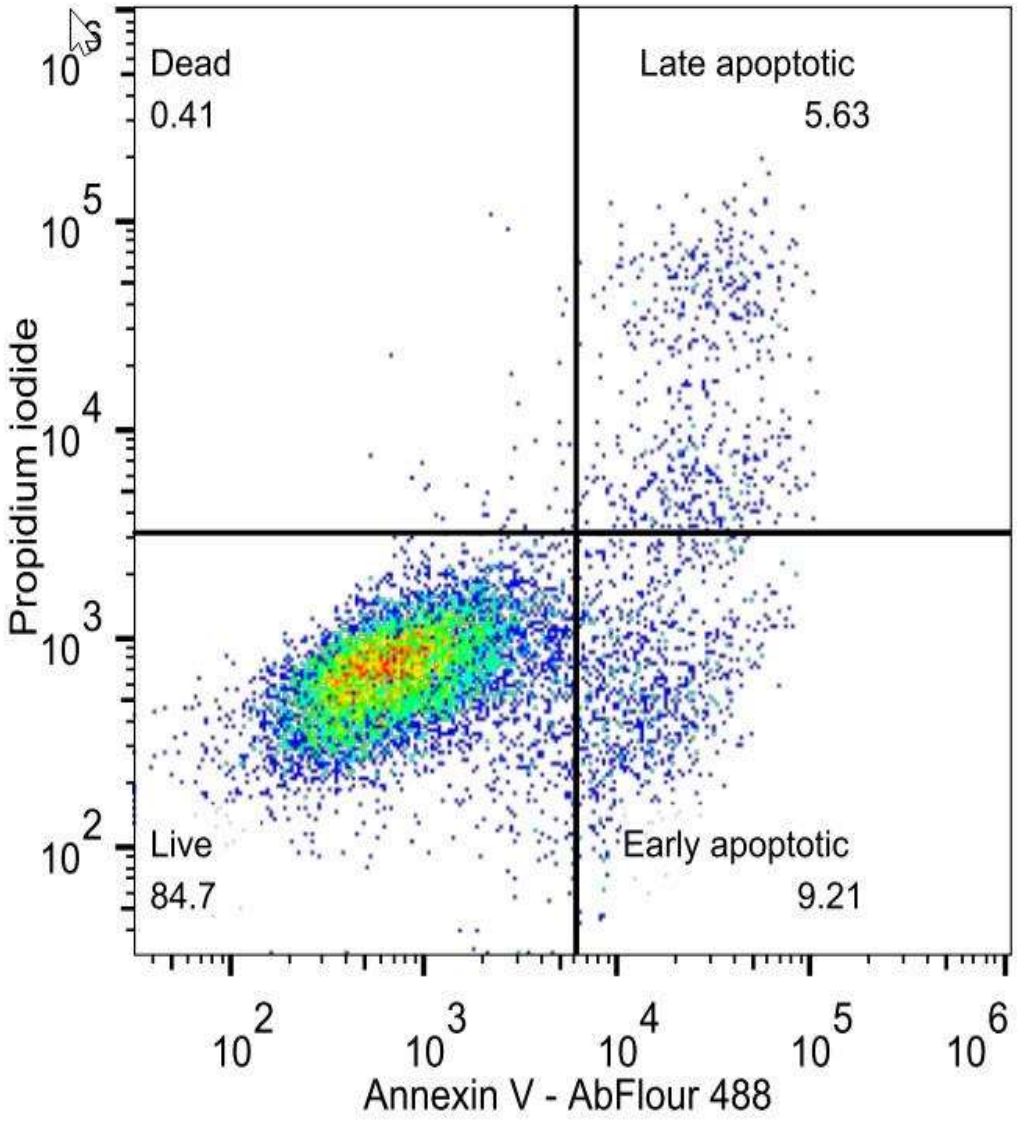

Figure 6.7: Flow cytometry analysis depicting the apoptosis induced by the doxorubicin (positive control) after 24 h treatment on MOLT-4 leukemia cells (Sharma and Mishra, 2023).

6.4 Conclusion

The one of the ideal characteristics for the new L-asparaginase preparation is to show the high potency against the leukemic cancer cells and also to carry out their cell death through

apoptosis mechanism. The purified L-asparaginase demonstrated excellent cytotoxic activity against MOLT-4 leukemia cells with an IC_{50} of 1.21 µM *in vitro* in comparison to the standard anti-leukemic drug doxorubicin, which showed an IC_{50} of 6.4 µM. The cytotoxic activity was found to be dose-dependent as the increase in concentration was accompanied by an increase in growth inhibition. The obtained outcomes were further validated through fluorescence microscopy of live/dead cells using the Acridine orange/Propidium iodide dual staining. The Annexin V/Propidium iodide assay using flow cytometry analysis evidently revealed the apoptosis inducing potential of the purified L-asparaginase preparation in the acute lymphoblastic leukemia (MOLT-4) cells. The plausible mechanism of the apoptotic induction by the purified L-asparaginase is the deprivation of the essential nutrient L-asparagine to the MOLT-4 leukemia cells. The current study clearly displayed the tremendous anti-leukemic potential of L-asparaginase from *Bacillus indicus* for its utilization as a potent anti-leukemic agent.