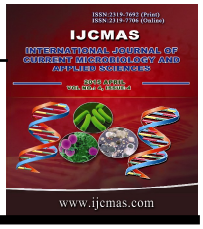
Int.J.Curr.Microbiol.App.Sci (2015) 4(4): 701-712

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 4 (2015) pp. 701-712**
http://www.ijcmas.com



Original Research Article

Cytotoxic Effect of Purified L-asparaginase from *Salinicoccus sp.* M KJ997975

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A B S T R A C T

Keywords

L-asparaginase, Salinicoccus sp., antileuckemic agent, cytotoxicity, MTT assay

L-asparaginase belongs to the group of hydrolytic enzymes that is responsible for hydrolysis of L-asparagine into L-aspartic acid and ammonia. It had been used as an effective therapeutic agent against acute lymphocytic leukemia. In the current study purified L-asparaginase from *Salinicoccus* sp. M KJ997975 was tested for *in vitro* cytotoxic activity using 3- (4, 5 - dimethyl - 2 - thiazolyl) - 2, 5- diphenylteterazolium bromide (MTT) assay against HeLa and Jurkat cell line. L-asparaginase inhibited the growth of both celllines with an IC_{50} of 0.171 IU/ml and 0.096 IU/ml respectively. Purified LA from *Salinicoccus* sp. M KJ997975 did not show any effect on normal human lymphocytes indicating that the purified enzyme may have high potential for cytotoxicity on cancer cells.

Introduction

L-asparagine (LA) is an essential amino acid for growth of tumor cells. The development of LA as a therapeutic agent began in 1953 and today it is one of the most biotechnologically and biomedically important therapeutic enzymes accounting for about 40% of the total worldwide enzyme sales (Capizzi *et al.*, 1970). LA is broadly distributed among the plants, animals and microorganisms. Out of these microbes are considered as better source of this enzyme, because of easy cultivation, extraction and purification on large scale is also viable (Verma *et al.*, 2007). Acute lymphoblastic leukemia (ALL) is a type of leukemia, in which there is over production of immature white blood cells. ALL is most common in childhood. Currently LA is used in the treatment of ALL. LA treatment for acute lymphoblastic leukemia is a major breakthrough in modern oncology as it induces complete remissions in over 90% children within four weeks. Cancer cells differentiate themselves from normal cells in diminished expression of asparaginase synthase. Hence, they are not capable of producing L-asparagine, and mainly depend on the L-asparagine from the circulating plasma pools. The effective depletion of L-asparagine results in death of leukemic cells. ELSPAR, ONCASPAR, ERWINASE and KIDROLASE are some of the brands of LA,

approved by FDA for the treatment of acute lymphoblastic leukemia and lymphosarcoma (Naggar *et al.,* 2014). Though this enzyme is useful in ALL treatment, it is associated with several toxicities like its need for frequent intramuscular injection and its high rate of allergic reactions. Efficacy of cancer chemotherapy is often affected by emergence of resistant cancer cells. PEG-LA derived from *E. coli* had shown decreased immunogenicity and increased circulating half-life (Natra *et al.,* 2007). Antiproliferative effects of native LA from *Aspergillus terreus* was observed on leukemia cell lines RS411 and HL60 (Loureiro, 2012). LA from *S. halstedii* showed anti-tumor activity and cytotoxic effect against cancer cell line *in vitro* and *in vivo*. The reduction of tumor size in albino mice may be attributed to the elevation of CAT and SOD activities as well as the diminishing of MDA (El-Sabbagh, 2013). Potential cytotoxicity was reported from the glutaminase free purified L-asparaginase of *Thermus thermophilus* on HeLa and SK-N-MC cell lines (Prista *et al*., 2001). Purified glutaminase free LA from *Penicillium brevicompactum* NRC 829 inhibited the growth of human hepatocellular carcinoma cell line (Hep-G2), with IC_{50} value of 43.3µg/ml (Elshafei *et al.,* 2010). Antitumor effects of L-asparaginase in acute lymphoblastic leukemia was reported due to curcumin by constitutive inhibition of AKT and AKT-regulated gene products (Wang et al., 2012). Antiproliferative effect of recombinant L-asparaginase from thermotolerant E.coli strain was reported on leukemic cell lines like RS 4, 11 and HL 60 (Muharram *et al.,* 2014). Effects of cold-stored LA on cell proliferation and cytotoxicity were measured in feline (MYA-1,F1B) and canine (17–71,OSW) lymphoma cells. Cold-stored E.coli-derived LA depletes asparagine and retains enzymatic activity (Wypij and Pondenis, 2013). LA from *Bacillus circullans* was checked for its antineoplastic effect on cancer cells. Flow cytometry experiments indicated an increase in sub G-1 cell population when treated with LA (Prakasham *et al.,* 2009).

Material and Methods

Chemicals

All the chemicals used were of high analytical grade procured from Hi-media, Sigma Aldrich and Merck. Solvents were procured from SRL, S.D. Fine. The standard L-asparaginase - Lucanase is a commercial product from Kyowa Hakko Kirin Co.Ltd, Tokyo, Japan , recommended for treatment of acute lymphoctytic leukemia in humans.

Microorganisms:

The organism used in the study was isolated from forest soil of Tungareshwar, Vasai, Maharashtra. The organism was identified as *Salinicoccus* sp. M KJ997975 by 16S rRNA sequencing method (Bhat *et al.,* 2014).

Study of cytotoxic effect of purified L-asparaginase (Camping *et al*., 1991)

Cell culture of Cancer cell lines:

Jurkat cells (human T lymphocyte cells) were grown in RPMI supplemented with 10% FBS, 200mM- L-glutamine, 2% penicillin-streptomycin and 2.5 µg/ml amphotericin-B solution. HeLa cells were grown in 90 % DMEM supplemented with 10% FBS, 2% penicillin-streptomycin and 2.5 µg/mL amphotericin-B. Both cell lines were incubated at 37°C in a humidified atmosphere of 95% air, 5% CO_2. Following 24-48 hrs of incubation period the spent medium was removed and the cell count was carried out using the Luna automated cell counter (Logos Biosystems, India) based on

trypan blue dye exclusion method. Cytotoxicity of the prepared samples on the cancer cells was determined using MTT (-3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay.

In vitro cell viability assay by MTT on Jurkat and HeLa cell lines:

90µL cell suspension seeded in 96-well microplates (Corning,USA) at a density of 25,000 cells/well for Jurkat cell lines and 0.2 x 10^6 cells/ plate of HeLa cells were incubated for 24h, after which the cells were exposed to an increasing concentration of test samples for 24 h. Cells were seeded in triplicates and incubated in a CO_2 incubator. Cells were then incubated with 10% MTT (5mg/ml) for 3 h and 100µL dimethyl sulfoxide (DMSO) was added. The untreated cells, standard LA, Lucase from *E.coli* and standard anticancer drug 6- Mercaptopurine (6 µM) (for Jurkat cells) and curcumin (for HeLa cells) treated cells were used for comparison. Cell viability was determined by measuring the absorbance on a microplate reader (SPECTRO star Nano, BMG LABTECH, Germany) at 570nm. Cell viability was calculated as a percentage of viable cells at different test concentrations relative to the control (untreated). The test sample concentration that resulted in 50% inhibition of cell growth was calculated as the half maximal inhibitory concentration (IC_{50}) by constructing a dose-response curve.

Isolation of normal human peripheral blood lymphocytes and estimation of cell viability by dye exclusion method (Mosamann, 1983)

18 µL of suspension of separated lymphocytes was mixed with 2 µL of (15.17 IU/ml) purified LA and incubated at 37^0C for different time intervals (10 min, 30 min, 24 h, 48 h). After incubation 18 µL of cell suspension was mixed with 2 µL of trypan blue. For control 18 µL of cell suspension was mixed with 2 µL of DMSO. The haemocytometer was loaded with 20 µL of sample and cell counting was done. Percentge cell viability was calculated.

Statistical analysis:

The results were expressed as mean $\pm$ standard deviation (S.D.). Chi square test was applied to the primary and secondary screening data. Statistical significance of MTT assay was analyzed by one way ANOVA.

Result and Discussion

MTT assay is a non radioactive colorimetric assay which is used to measure the cell viability in response to a variety of cytotoxic stimuli. The assay is based on the reduction of the yellow, water soluble tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl tetrazolium bromide (MTT) within metabolically active cells. The reduction of the tetrazolium salt occurs by the action of mitochondrial dehydrogenases present only in viable cells, yields a purple formazan product which can be quantified spectrophotometrically. The percentage viability of the treated cells was calculated by comparing it with the control cells which were not treated .

The *in vitro* cytotoxic effect of LA isolated from *Salinicoccus* sp. M KJ997975 was verified on two human tumor cell lines namely Jurkat and HeLa by MTT assay. It showed cytotoxic effect on both the cell lines. Commercially available brand of LA Lucase from *E.coli* showed IC_{50} value of 0.022 IU/ml and 0.053 IU/ml againt HeLa and Jurkat cell lines respectively (Figure 2 and 4).

Table.1 Percentage viability of lymphocytes treated with purified LA from *Salinicoccus* sp. M KJ997975 assessed by trypan blue Dye Exclusion Method at different time intervals

Time/Concentration	10 min	30 min	24 h	48 h
DMSO control	100%	100%	100%	100%
0.157 IU/ml	92%	92%	90%	90%
0.078 IU/ml	95%	95%	92%	92%
0.039 IU/ml	98%	98%	98%	98%
0.019 IU/ml	98%	98%	98%	98%

Figure.1 Effect of standard LA (Lucase from E.coli) on HeLa cell lines in the form of % survival. Values are expressed as Mean + S.D. In figure symbols represent statistical significance ** p<0.001

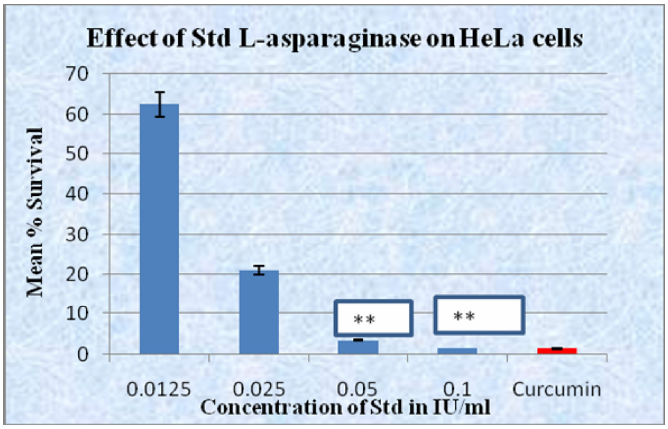


Figure.2 Effect of purified LA from *Salinicoccus* sp. M KJ997975 on HeLa cell lines in the form of % survival. Values are expressed as Mean + S.D. In figure symbols represent statistical significance ** p<0.001

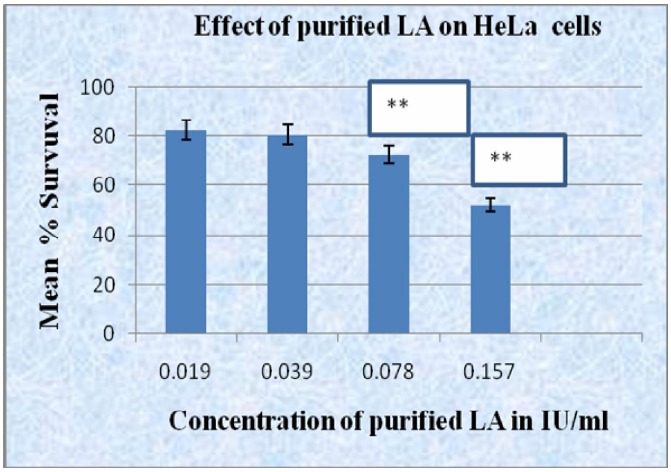


Figure.3 Effect of standard LA (Lucase from E.coli) on Jurkat cell lines as % survival. Values are expressed as Mean + S.D. In figure symbols represent statistical significance ** p<0.001

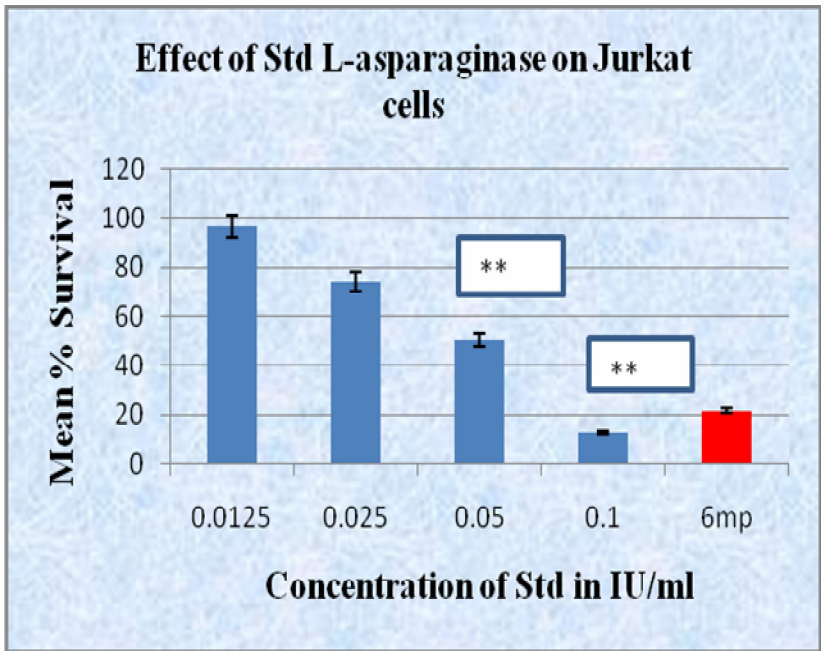


Figure.4 Effect of purified LA from Salinicoccus sp. M KJ997975 on Jurkat cell lines in the form of % survival Values are expressed as Mean + S.D. In figure symbols represent statistical significance ** p<0.001

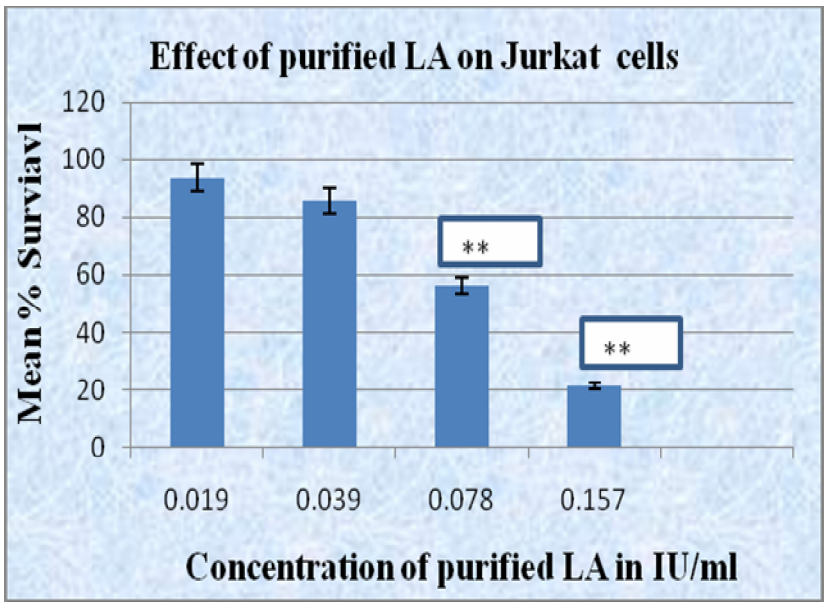
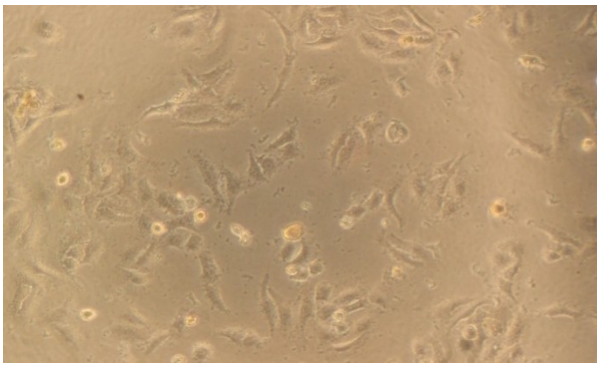


Figure.5 Effect of various concentrations of standard LA from E. coli on HeLa cell line
a. Effect of Std. LA with concentration at 0.1IU/ml after 24 h on HeLa cell lines. b. Less formazon crystal formation with Std. LA at 0.1IU/ml concentration after 24 h HeLa cell lines.c. Effect of Std. LA with concentration at 0.05 IU/ml after 24 h HeLa cell lines.d. Less formazon crystal formation with Std. LA at 0.05 IU/ml after 24 h HeLa cell lines. e. Effect of Std. LA with concentration at 0.025 IU/ml -24 h on HeLa cell lines. f. More formazon crystal formation with Std. LA at 0.025 IU/ml -24 h on HeLa cell lines



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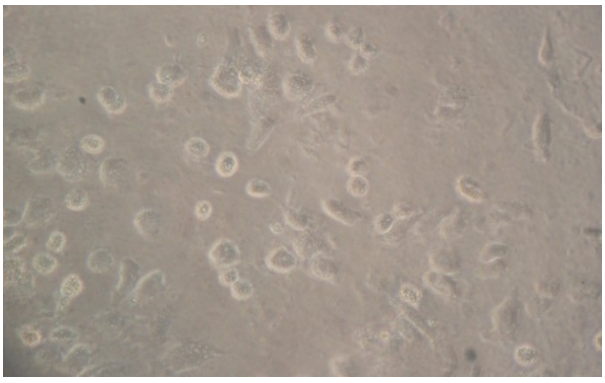

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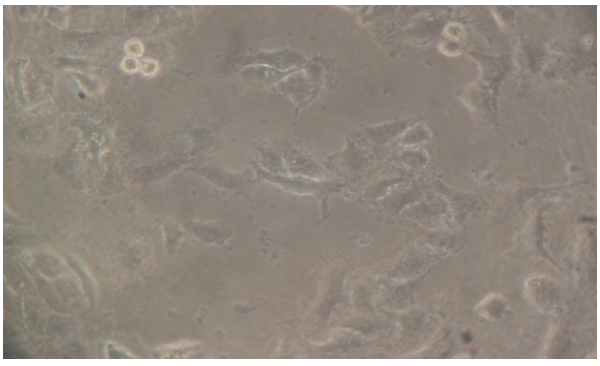

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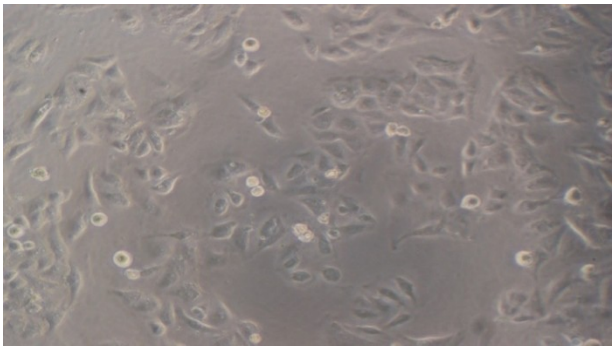

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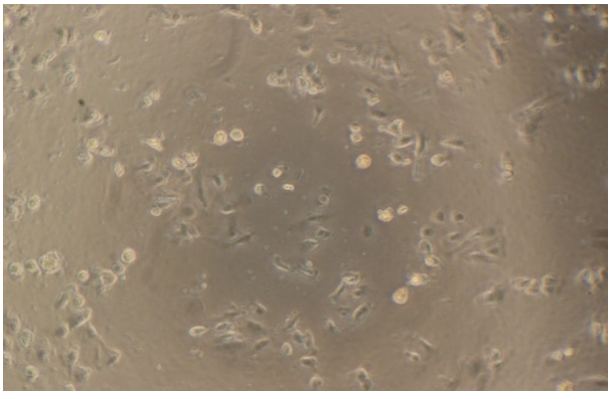

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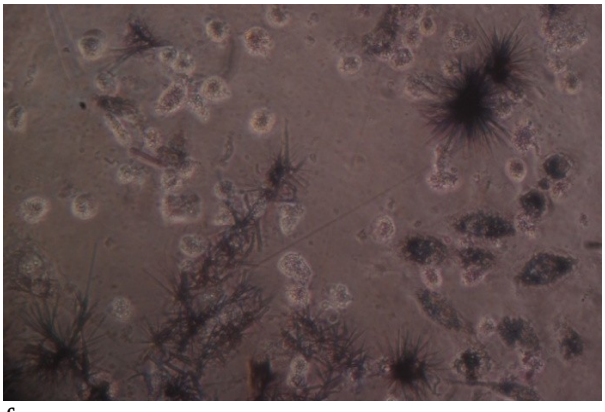

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Figure.6 Effect of various concentrations of purified LA from Salinicoccus sp. M KJ997975 on HeLa cell line. a. Effect of Purified LA with concentration at 0.15 IU/ml after 24 h on HeLa cell lines. b. Formazon crystal formation with purified LA at 0.15 IU/ml after 24 h on HeLa cell line. c. Effect of Purified LA with concentration at 0.078 IU/ml after 24 h on HeLa cell lines. d. More formazon crystal formation with purified LA at 0.078 IU/ml after 24 h on HeLa cell lines. e. Effect of Purified LA with concentration at 0.039 IU/ml -24 h on HeLa cell lines. f. More formazon crystal formation with purified LA at 0.039 IU/ml -24 h on HeLa cell lines.

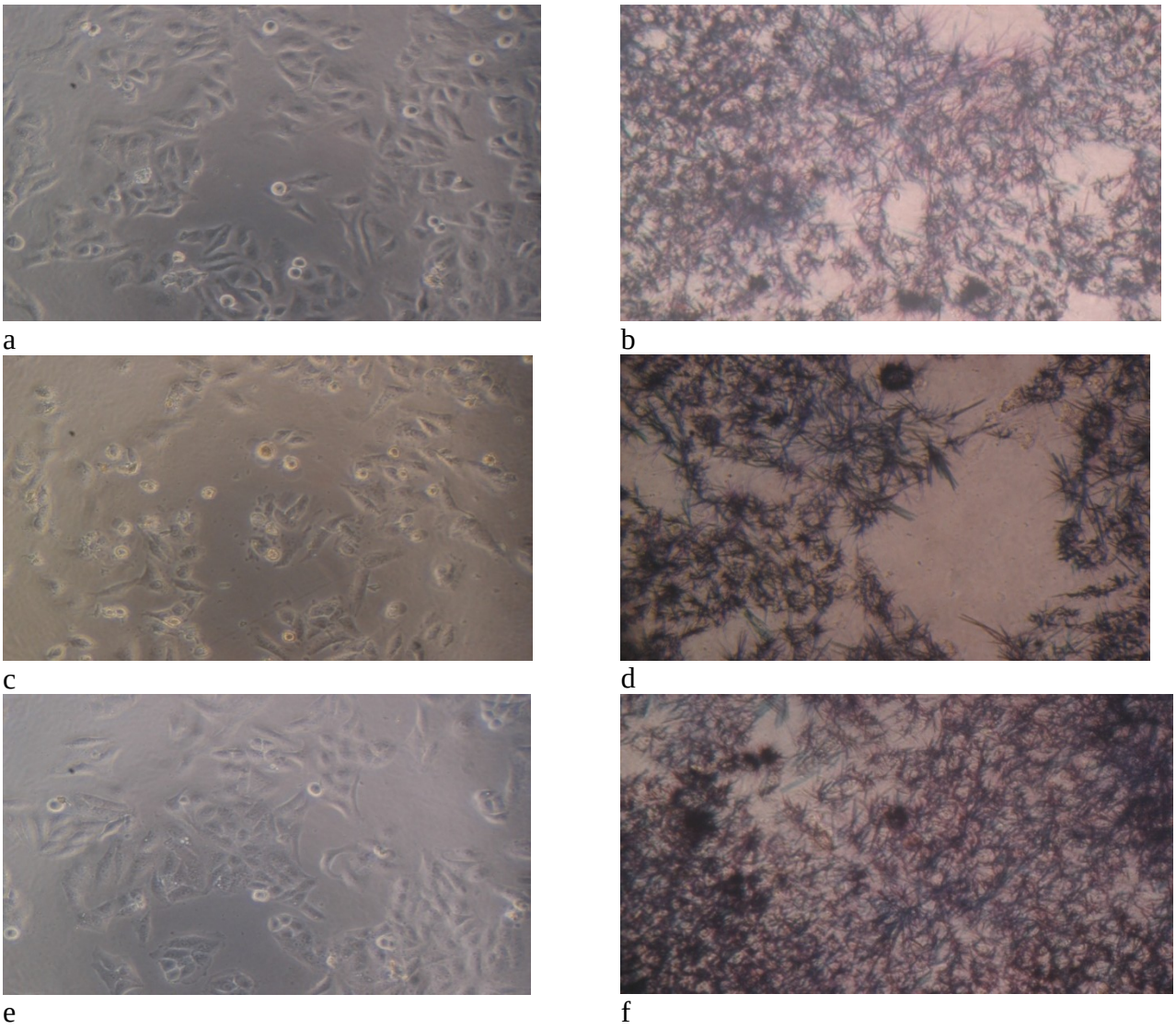


Figure.7 Effect of various concentrations of purified LA from Salinicoccus sp. M KJ997975 and effect of control on HeLa cell linea. Effect of Purified L-asparaginase with concentration at 0.019 IU/ml after 24 h on HeLa cell lines.b.More formazon crystal formation with purified L-asparaginase at 0.019 IU/ml after 24 h on HeLa cell lines.c. Medium control- Effect of medium control on HeLa cell lines.d. More formazon crystal formation with medium control for HeLa cell lines.e. Vehicle control –Effect of Vehicle (Distilled water) on HeLa cell line.f. More formazon crystal formation with vehicle control for HeLa cells.

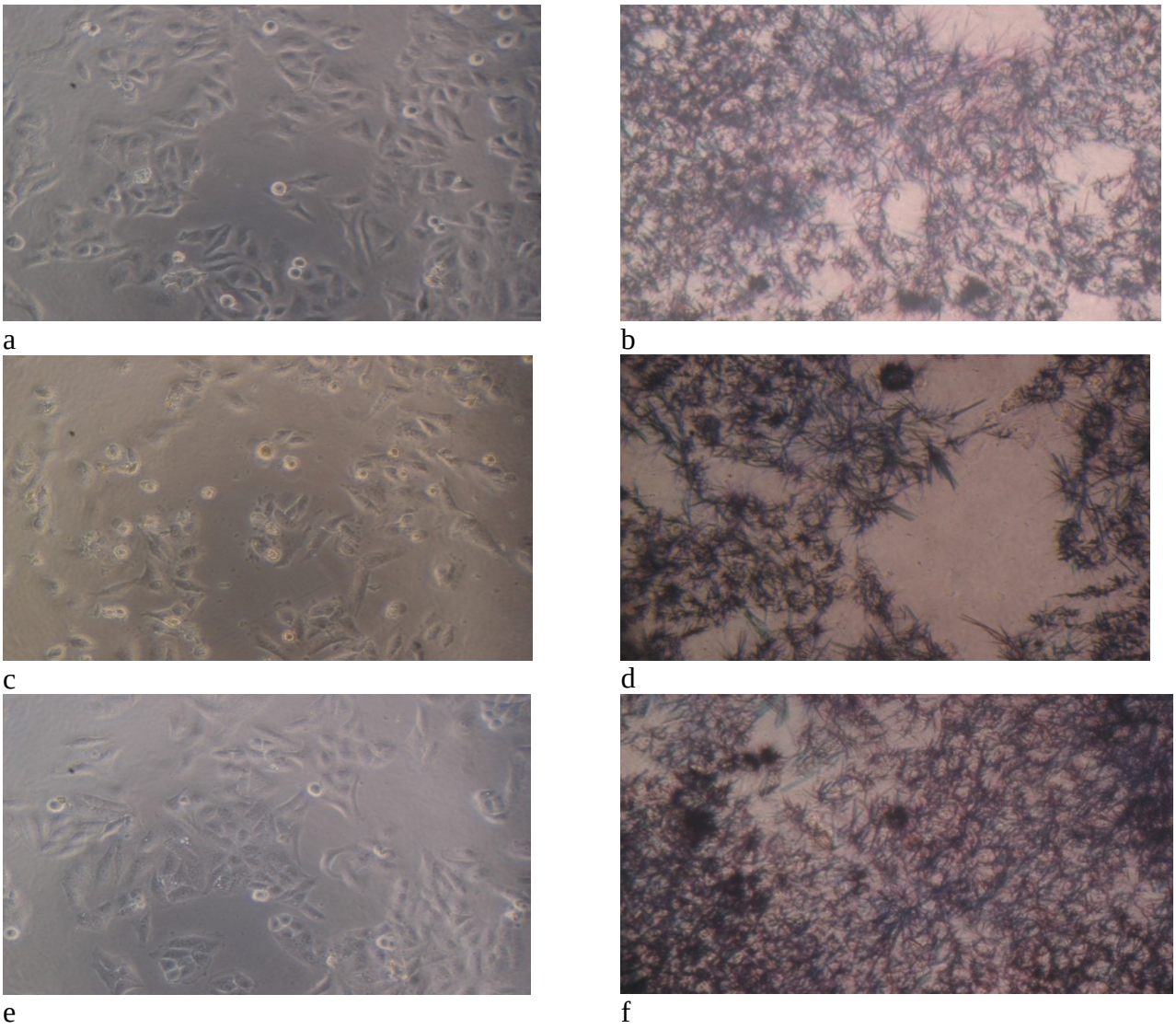
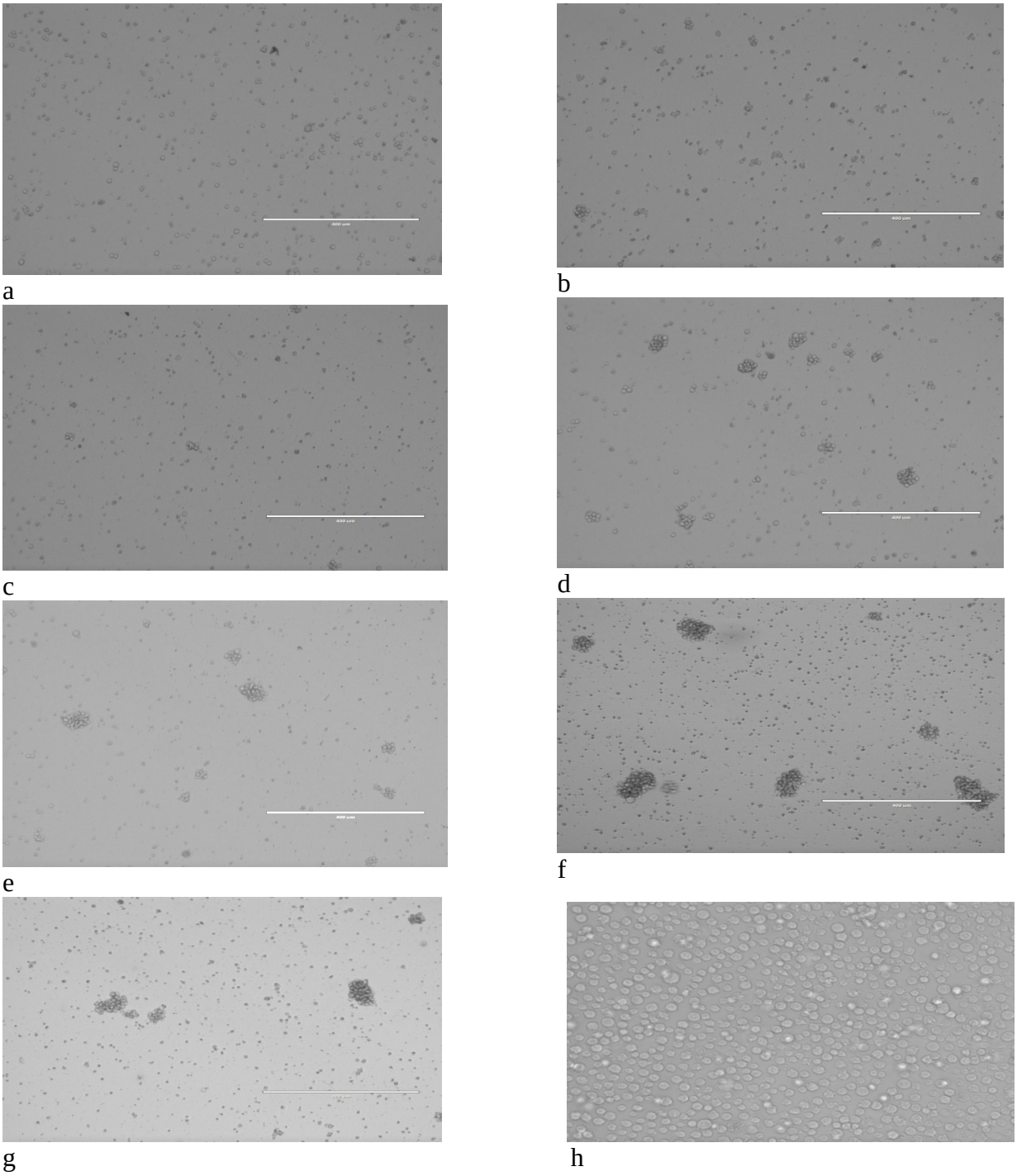


Figure.8 Effect of standard LA from E. coli and purified LA from Salinicoccus sp. M KJ997975 along with control on Jurkat cells.a. Effect of 6MP (Standard anticancer drug) on Jurkat cell lines. b. Effect of standard LA (Lucase) from E.coli with concentration 0.1 IU/ml on Jurkat cell lines. c. Effect of standard LA (Lucase) from E.coli with concentration 0.05 IU/ml on Jurkat cell lines. d. Effect of standard LA (Lucase) from E.coli with concentration 0.025 IU/ml on Jurkat cell lines, e. Effect of purified LA from Salinicoccus sp.M KJ997975 with concentration 0.151 IU/ml on Jurkat cell lines.f. Effect of purified LA from Salinicoccus sp.M KJ997975 with concentration 0.078 IU/ml on Jurkat cell lines.g. Effect of purified LA from Salinicoccus sp.M KJ997975 with concentration 0.039 IU/ml on Jurkat cell lines h. Untreated Jurkat cells.



Purified enzyme from *Salinicoccus* sp. M KJ997975 showed IC_{50} value of 0.171 IU/ml and 0.096 IU/ml againt HeLa and Jurkat cell lines respectively (Figure 1 and 3) shows the effect of. The results were found to be very significant ($p < 0.001$) when compared to commercially available of LA on both cells and anticancer agent 6 Mercaptopurine on Jurkat cells and curcumin on HeLa cells . Purified LA did not elicit any immunostimulatory response in normal human lymphocytes *in vitro* (Table 1).

The viability of normal human lymphocytes isolated from peripheral blood under the influence of purified LA in varying concentrations was assessed by trypan blue dye exclusion method and was then compared with the control cells. The viability of cells treated with purified LA was found to decrease, as shown by the results obtained from trypan blue dye exclusion method. Purified LA were not found to affect the viability of the cells significantly. At concentrations of under 0.157 IU/ml cell viability of over 90% was observed in the first 12 hours of incubation. Other concentrations (0.078, 0.039, 0.019 IU/ml) did not have much effect on lymphocytes as % of viability was found between 92 % to 95% (Table 1). Thus purified LA from *Salinicoccus* sp. M KJ997975 did not show any cytotoxic effect on normal human lymphocytes.

Antitumor activity of LA from *Erwinia caratovora* has been studied on Jurkat, Molt 4, human chronic myloid leukemia K562 cells, HL 60 and other human soild tumors. LA significantly increased the number of apoptotic cells to 40% (Jurkat cells) and 99% (HL60 cells) suggesting that the enzyme cytoxicity is associated with only L-asparagine deficiency (Abakumova *et al*., 2012). The purified L-asparaginase from *Bacillus licheniformis* showed cytotoxic effect against Jurkat clone E6-1, MCF-7 and K-562 (Mahajan *et al.,* 2014). *In vitro* cytotoxicity of LA from pathogenic strain *Helicobacter pylori* against different cell lines reported that AGS and MKN 28 gastric epithelial cells being the most affected (Cappelletti *et al.,* 2008). L-asparaginase has been reported to show sensitivity against fibrosarcoma and liposarcoma (Tardito *et al.*, 2007). There are reports on LA causing selective death of asparagine dependent tumor cells and also induces apoptosis in tumor cells (Kelo, 2009). The enzyme isolated from *Salinicoccus* KJ997975 also exhibited cytotoxicity against both the cell lines tested but it was found more effective against Jurkat cell line.

Several groups of microorganisms have potential of LA production and enzymes derived from microorganisms are the major sources for practical and clinical use. The *in vitro* cytotoxic effect of LA isolated from *Salinicoccus* sp. KJ997975 was verified on two human cancer cell lines namely Jurkat and HeLa. In this study it showed cytotoxic effect on both the cell lines though it was more potent inhibitor of Jurkat cell line proliferation.

Commercially available brand of LA, Lucase from *E.coli* used as standard showed IC_{50} value of 0.022 IU/ml and 0.053 IU/ml againt HeLa and Jurkat cell lines respectively. Purified enzyme from *Salinicoccus* sp. M KJ997975 showed IC_{50} value of 0.171 IU/ml and 0.096 IU/ml againt HeLa and Jurkat cell lines respectively. Purified enzyme did not show any effect on normal human lymphocytes implying that it may prove to be a novel source for LA isolated for chemotheraputic purpose. Flowcytometric analysis and *in vivo* cytotoxicity assays are warrented to prove the efficacy of this new source of LA.

Acknowledgments

We would like to thank The Director, School of Biotechnology & Bioinformatics, D.Y.Patil University Navi Mumbai, for support and providing necessary facilities to pursue the research work.

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