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EVALUATION OF CYTOTOXIC ACTIVITY OFL-ASPARAGINASE FROM MARINE BKJM$_2$ AGAINST JURKAT J6 AND PA$_1$ CELLLINES

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ABSTRACT

Cancer is most prevalent disease occurring in human beings now a days. Cancer remains the second leading cause of death in the world. In its projection, the Indian Council of Medical Research (ICMR) said in 2016 the total number of new cancer cases is expected to be around 14.5 lakh and the figure is likely to reach nearly 17.3 lakh new cases in 2020. L-Asparaginase is an anti-neoplastic agent used in the chemotherapy of acute lymphoblastic leukemia. L-Asparaginase producing marine microorganisms are isolated from coastal area of Kerala. Among 10 isolates were isolate, out of 10 isolates only one showed a potent positive result for L-Asparaginase production on screening and named as BKJM$_2$. Marine isolate (BKJM$_2$) was tested for invitro cytotoxic activity using MTS assay against Jurkat J6(Leukemia cell line) and 3-(4, 5-dimethyl-2-thiazolyl)-2, 5- diphenyl tetrazolium bromide (MTT) on Pa$_1$ (Ovarian cancer cell line).The IC$_{50}$ (Inhibitory concentration 50) was calculated as 8.27% of the purified enzyme. L-Asparaginase inhibited the growth of both cell lines. L-Asparaginase from BKJM$_2$ did not show any effect on normal human cells indicating that the purified enzyme have high potential for selective cytotoxicity on cancer cells.

KEYWORDS: *L-Asparaginase, Cyto toxic effect, Jurkat j6 cell line, Pa$_1$ cells and MTT Assay.*

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INTRODUCTION

Marine microbes represent a potential source for commercially important bioactive compounds[1]. L-Asparaginase is the first enzyme with anti-tumor activity intensively studied in human beings. It is used for the treatment of malignances of the multiorgans[2]. It is widely used as a therapeutic agent for treating acute lymphoblastic leukemia in children [3].The amino acid L-Asparagine, is essentially required for the survival of both normal and Cancer cells. The normal cells can synthesize L-Asparagine her own but cancer cells don't synthesize L-Asparagine. The commonest therapeutic practice to treat this condition is to intravenously administer L-Asparaginase in order to deplete the blood asparagines level and exhaust its supply to cancer cells. It constitutes one of the most bio technologically and biomedically important groups of therapeutic enzyme accounting 40% of the total worldwide enzyme sales [4].This enzyme also used as a therapeutic agent in the treatment of Hodgkin disease, acute myelocytic leukemia, acute mylomonocytic leukemia, chronic lymphocytic leukemia, lympho sarcoma treatment and malanosarcoma[5]. L-Asparaginase (L-Asparaginase aminohydrolase, E.C.3.5.1.1) is broadly distributed among plants, animals, microorganisms and also found in rodent's serum. Among this microorganisms can produce L-Asparaginase frequently, because they can be easily cultured and the procedures of purification of LA from them are suitable for the production of enzyme at the industrial scale [6]. In spite of several studies done on L-Asparaginase, very few reports were available on fungal based production of enzyme, especially with $BKJM_2$ isolate; no literature is available nationally and internationally. Hence in the present study, it has been aimed to produce L-asparaginase from marine fungi of Kerala costal region Veli.

MATERIALS AND METHODS

Sample collection

Two marine water samples were collected from coastal area of Kerala (Shangumugham Beach, Veli Back Water) at the depth of 10 cm. The samples were collected in sterile air tight bottles under sterile conditions, labeled and transported to the laboratory for further study [7].

Isolation of Marine isolates

For isolation of L-Asparaginase producing marine fungal species, the samples were serially diluted and plated on Glucose- aspargine agar & glycerol asparagine agar medium and then incubated at 37^0 C for 24-72 hrs[8].The $BKJM_2$ colony characteristics and morphology was represented in(Table-1) and (Figure-1).

Microscopic identification of $BKJM_2$

The isolate was identified on the basis of their morphology using standard taxonomic keys and monographs. The pure culture isolate were characterized morphologically by Lacto phenol cotton blue staining [9]. Represented in (Figure 2 & 3).

Screening for L-asparaginase positive cultures

All the isolates were screened for L-Asparaginase production. The isolated strains were inoculated in glycerol asparagine agar supplemented with 0.3ml of 2.5% phenol red dye at P^H-6.5. The formation of pink coloured zone around the colony shows the positive results for L-Asparaginase production [10-11]. (Figure 4 & 5) showing the zone of inhibition around the colony.

Study of Cytotoxic effect of purified L-Asparaginase
Growth of cancer cell line

Jurkat J6 and PA_1 cell lines were procured from NCCS (Pune). Jurkat J6 cells (human T lymphocyte cells) were grown in RPMI supplemented with 10% FBS, 200Mm L-glutamine, 2% streptomycin and 2.5µg/ml amphotericin-B solution. Pa_1 cells were routinely maintained in Modified Eagle's Medium (MEM) 90%+10% Fetal Bovine Serum (FBS) with 1% pen- strep solution at 5% CO_2 and 37^0C in a humidifying incubator [12].

Cytotoxic effect of L-Asparaginase on Jurkat J6 and Pa_1 cell lines by MTS and MTT Assay

MTS and MTT assay are the colorimetric method for measuring the activity of enzymes in living cells that reduce MTT (3-(4, 5-dimethyl-2-thiazolyl)-2, 5- diphenyl tetrazolium bromide) to formazan dyes, giving a purple color. This two are commonly used to determine cytotoxicity of potential medicinal agents and toxic materials, since these types of materials are expected to stimulate or inhibit cell viability and growth. The main difference between MTS and MTT assay is the MTS assay used for only suspension type cell lines like leukemia cancer cell lines and MTT assay is used for the solid type cancers (Breast and ovarian cancer cell lines).

Cytotoxic effect of L-Asparaginase on Jurkat J6

Fifty (50) µl cell suspension was seeded in a 96-well plate at required cell density (20,000 cells per well), along with the 50 ul test agent in required concentrations (2 to 10%). Plates were incubated for 24 hrs at **37°C** in a 5% CO_2 atmosphere. After the incubation period, plates were taken from incubator, and 20 µL of MTS reagent was added according to the manufacturer's instructions. MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] is a colored formazone product that is soluble in cell culture media. Plates were wrapped with aluminium foil to avoid exposure to light and replaced in incubator for 3 hours. Incubation time should be kept constant while making comparisons. Gentle stirring in a gyratory shaker is performed to enhance dissolution of the MTS reagent. Absorbance was read on a spectrophotometer or an ELISA reader in between450nm and 630nm is used as reference wavelength [13]. The IC_{50} value was determined by using linear regression equation i.e. $Y = Mx + C$ Here, Y = 50, M and C values were derived from the viability graph. The viability of the cells is indicated by the intensity of the purple colour which can be read at 570 nm. Viability % and anti cancer activity of purified enzyme was represented in (Table 2) & (Figure 6, 7, 8 and 9). Cell viability was calculated using the following formula-

$$\text{Cell proliferation \%} = \frac{\text{OD of sample}}{\text{OD of control}} \times 100$$

Cytotoxic effect of L-Asparaginase on Pa_1 cells

The cytotoxic activity of the L-Asparaginase was assessed by MTT assay [14]. PA_1 cell lines were procured from NCCS (Pune) and routinely maintained in MEM 90%+10% FBS with 1% pen- strep solution at 5% CO_2 and 37^0C in a humidifying incubator, as performed in Jurkat J6 cell lines.PA_1 cells were seeded in 96-well plate and incubated in CO_2 incubator (5% CO_2 and 37^0C). After 24 hours the medium was removed and fresh medium without FBS was added. The organic extract/compound was dissolved in DMSO was filtered using PVDF syringe filters and added to the wells in different concentrations (10, 20 and 30µl). Final volume in all wells was made equal (100µl). Incubated at 37^0C, 5% CO_2 in humidifying CO_2 incubator. 20 µl of MTT solution [5mg/ml in warm PBS was added to each well. Incubation was extended for another 4 hours. MTT solution was removed and formazan crystals were dissolved using DMSO (for 20 minutes). The absorbance was read at 570nm. Cell proliferation was estimated as described in Jurkat J6 cells. The % of cell proliferation and activity of enzyme on pa_1cell lines was represented in (Table 3) and (Figure 10 & 11).

RESULTS

Isolation of Marine fungi

From the two marine water samples, 10 isolates were isolated. Among 10 isolates, one potent isolate was selected for asparaginase production given in Table.1

Table1
Colony Characterization of Potent isolate.

Isolate	Colony Characterization
BKJM $_2$	Size-Large Shape-Filamentous Color- off white, Reverse plate appeared yellowish. Margin-Filamentous Elevation-Convex and whole colony filamentous.

Figure 1
Colony morphology

Morphological characterization

The potent isolate on lacto phenol cotton shows filamentous fungi and conidia.

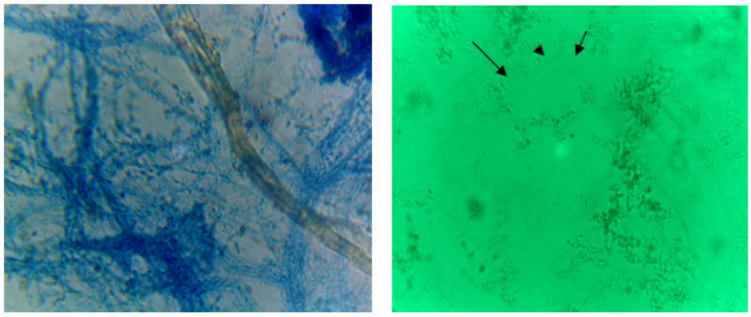


Figure 2&3
Lacto phenol cotton blue staining

Screening of L-asparaginase producing marine isolate

All the isolates were screened for L-Asparaginase production. The isolated strains were inoculated in glycerol asparagines agar supplemented with 0.3 ml of 2.5% phenol red dye at P^H 6.5(P. Sivasankar *etal*., 2013) .The formation of pink colored zone around the colony shows the positive results for L-Asparaginase production.

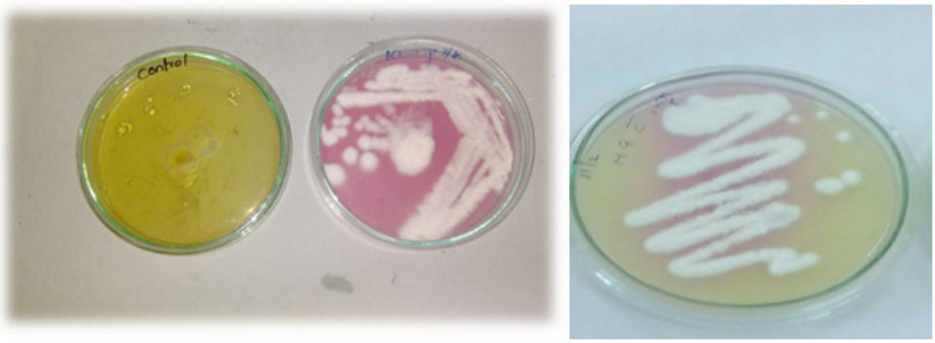


Figure 4&5
Screening for L-Asparaginase producing potent isolate.

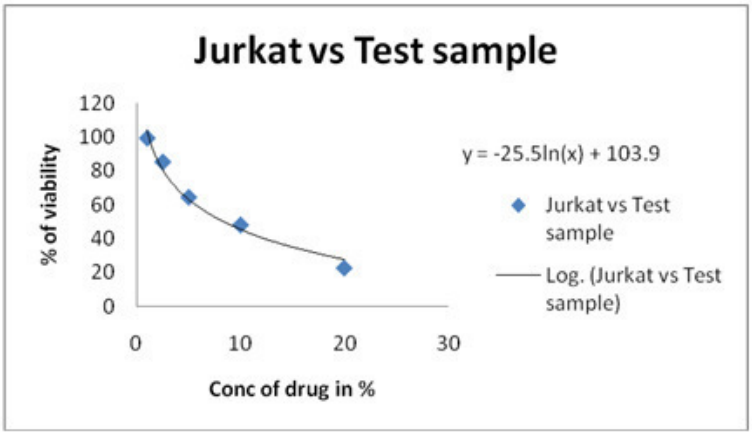
Cytotoxic activity of L-Asparaginase by MTS assay on Jurkat J6 and on MTT assay for Pa_1 cell lines

In MTS Assay the activity of purified enzyme shows its effect is dose dependent. On Jurkat j6 cell line specific for acute lymphoblastic leukaemia, the IC_{50} (Inhibitory concentration 50) was calculated as 8.27% of the purified enzyme. [13]Reported anti cancerous activity of purified L-Asparaginase against three different cell lines Jurkar, MCF-7 and K 562 The IC_{50} of purified L-asparaginase from *Bacillus licheniformis* RAM-8 was found to highly effective against the leukemic cell lines viz. Jurkat clone E6-1 cell lines and K-562 cell lines with IC_{50} of 0.22 IU and 0.15 IU, respectively. Jurkat cell lines specific for Acute lymphoblastic leukemia and pa_1 cell lines are specific for ovarian cancer.

Table 2
Cytotoxic Activity of L-Asparaginase enzyme sample on Jurkat J6 cell line.

Concentration (%)	OD values at 570nm (Duplicates)		% of viability	Mean ± SD
Blank	0.011,	0.015	NA	NA
Untreated	0.675,	0.658	100	0.65± 0.01
6MP (62µmol)	0.362,	0.339	51.64	0.33 ± 0.01
1	0.649,	0.674	99.23	0.64 ± 0.01
2.5	0.573,	0.567	85.23	0.55 ± 0.004
5	0.423,	0.446	64.49	0.42± 0.01
10	0.322,	0.334	48.20	0.31 ± 0.008
20	0.169,	0.159	22.87	0.14 ± 0.004

IC 50 = 8.27 %
Each value is the mean of three readings ± SD.



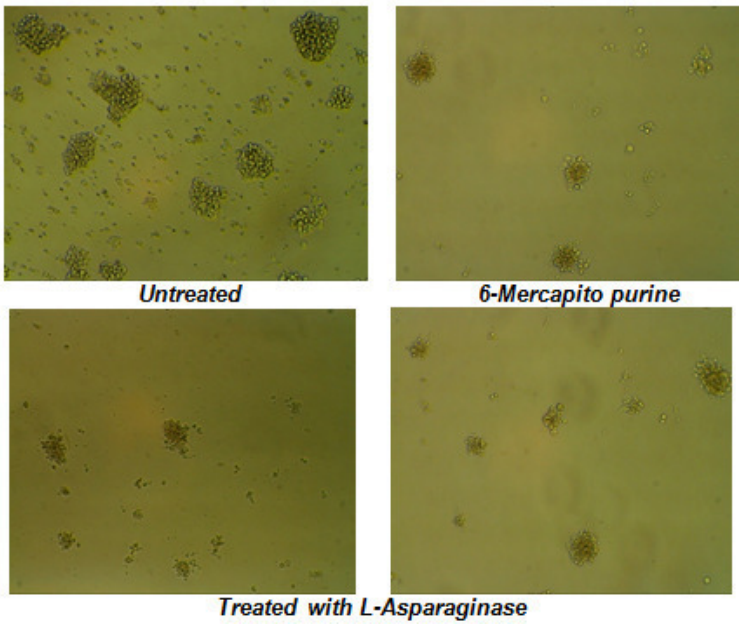


Figure 6, 7, 8 & 9
Cytotoxic Activity of L-Asparaginase enzyme sample on Jurkat J6 cell line

Table 3
Cytotoxic Activity of L-Asparaginase enzyme sample on Pa_1 cells

Concentration	OD value	%cell proliferation	Mean ± SD
Control	0.600	100	0.603 ± 0.006
DMSO	0.600	100	0.601 ± 0.002
10 µg	0.001	0	0.001 ± 0.001
20 µg	0.001	0	0.003 ± 0.002
30µg	0.001	0	0.001 ± 0.002

Each value is the mean of three readings ± SD.

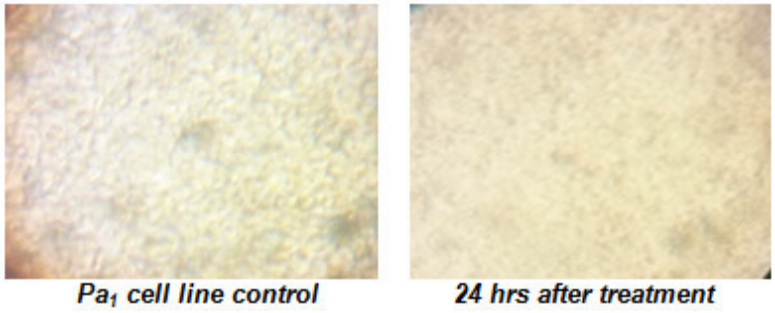


Figure 10 & 11
Cytotoxic Activity of L-Asparaginase enzyme sample on Pa_1 cells

DISCUSSION

Anti-tumour activity of the L-asparaginase was evaluated in in vitro conditions on two different human cancer cell lines namely Jurkat J6 and Pa_1, which induce acute lymphoblastic leukemia and ovarian cancer by MTS and MTT assay. The results were incorporated in table 2,3 & figures 5, 6, 7, 8, 9 & 10. The viability of tumor cells was measured by their ability to reduce metabolically tetrazolium salt to purple color formazone crystals after 24 hrs treatment with enzyme sample at various concentrations such as 1, 2.5, 5, 10, and 20% respectively. IC50 value is the concentration at which the L-Asparaginase causes 50% inhibition of cancer cell growth and it is 8.27% with respect to Jurkat cell line. No, significance was noticed at 1% concentration compared to the untreated control. Even at 2.5% concentration very little change in viability was observed. Sixty four percentage viability was found at 5% concentration of the purified enzyme on Jurkat cell line. There is a significant difference in percentage of viability at 10 & 20 concentrations expressed in percentage. At 20% concentration only 22% viability was observed at the end of incubation period. Manish Bhat & Thankamani reported 98% of viability even after

48 hrs incubation with *Salinococcus sps*. With respect to the positive control 6- Mercapito purine, 51% viability was detected. In case of Pa_1cell lines tested with the enzymatic sample there was a significant difference between the control and treated sample as illustrated on figures 9 and 10 and table 3. When compared to Jurkat cell lines strong inhibitory effect was recorded. The cytotoxic effect was evaluated at three different concentrations (10, 20 and 30 µg/ well) using MTT reduction method. After treatment with tetrazolium salt no purple color formazone formation was observed with purified L-asparaginase in contrast to deep purple color control sample indicated that there is no viability of the cells even at the starting concentration of 10 µg/well. Ali Mohamed Elshafei *et al*.,(2012) demonstrated 65.5% viability at 10 µmol with the crude enzyme extract of *Pencillium brevicompactum* on HEPG-2 tumor cell lines. But they were no previous evidences showing 100% cell death with the purified L-Asparaginase enzyme sample. This is the first report of marine fungi $BKJM_2$ shows 100% cell death of Ovarian cancer cell lines. From the experimental evidence, it is clears that the isolated enzymatic sample is effective not only against ALL cell line, but also against the ovarian cancer cell line mostly prevalent in woman. Moreover it has selective cytotoxicity on cancer cell lines. Thus it can be used as a potent broad spectrum chemotherapeutic drug.

CONCLUSION

Finally we Conclude that L-Asparaginase isolated from Veli costal region of Kerala possess good potential anti-cancer activity against Jurkat j6 (acute lymphoblastic leukemia) and Pa_1 (ovarian cancer cell lines. The potency of Asparaginase is used for the development of acute lympho blastic leukemia chemotherapeutic agent, which can induce death of cancer cells while protecting the non cancerous cells.

CONFLICT OF INTEREST

Conflict of Interest declared none.

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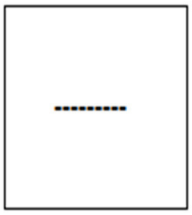

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