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EXTRACTION, CHARACTERIZATION AND ANTICANCER PROPERTY OF L-ASPARAGINASE FROM *SOLANUM NIGRUM* AND ENHANCEMENT OF ITS STABILITY USING NANOFORMS

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ABSTRACT

L-asparaginase (E. C. 3.5.1.1) an anticancer enzyme hydrolyzes L-asparagine to L-aspartate and ammonia and is used as a drug to treat ALL. The purpose of this investigation was to test for the production of L-asparaginase enzyme in Solanum nigrum. The enzyme assay by Nessler's method was carried out and the activity was determined usingstandard graph of Ammonium sulphate and was found to be 7.55 IU using spectrophotometer at 460nm absorbance and specific activity was calculated using standard graph of protein by Lowry's method and was found to be 4.833 µmoles/mg/min and hence these results confirmed the presence of this enzyme in Solanum nigrum. The optimum conditions of this enzyme were found to be similar to the ones reported earlier. Temperature, pH and time kinetics were carried out and the optimum conditions were determined. The optimum temperature was found to be 37˚C, optimum pH was 8, optimum incubation time was found to be 15 minutes and Km and Vmax was also determined using double reciprocal Lineweaver-Burk plot. The Km value was found to be 10 and Vmax to be 0.325 µmoles/ml/min. The study of enzyme stability was done using Scanning Electron Microscopy and it was reported that sodium nanoforms enhanced the stability of this enzyme. A549 cells (5x 10^3 cells/well) were cultured overnight and were treated with different dilutions of L-asparaginase enzyme from crude leaf extracts and incubated for 48h in a CO_2 incubator. MTT assay was carried out and confirmed its anticancer property. These findings report that Solanum nigrum is a good source of L-asparaginase enzyme.

KEY WORDS

L-asparaginase, Solanum Nigrum, MTT assay, Scanning Electron Microscopy, A549 cells.

INTRODUCTION:

The therapeutic enzyme L-asparaginase is intensively studied for its anticancer property and is used in the treatment of ALL. This enzyme has been derived from microbial sources and is commercially used as a drug to treat ALL and has found to have major side effects[1]. It has also been reported that the plant L-asparaginase is less toxic as compared to bacterial L-asparaginase[2,3]. Hence an attempt is made to screen for the sources of L-asparaginase from plants.

Solanum nigrum commonly called Black nightshade is native to Europe, Asia and North America. It is used as a traditional Indian medicine to treat wide range of ailments that include dysentery, ulcers, stomach complaints, skin diseases, laxative and asthma. It is also reported to be antitumorigenic, diuretic, antioxidant, hepatoprotective and antipyretic.

http://en.wikipedia.org/wiki/Solanum_nigrum

The present work focuses on extraction and characterization of the L-asparaginase enzyme from Solanum nigrum and determining its anticancer property. We also found that the stability of this enzyme could be enhanced using nanoforms.

MATERIALS AND METHEDOLOGY:

Extraction of crude enzyme: 1g of leaf sample was weighed, washed under tap water and rinsed with distilled water. Leaves were ground in a chilled mortar and pestle with two volumes of 0.1M phosphate buffer of pH7.5 at 3°C containing 5mM DTT and 1mM EDTA. Homoginization is carried out in chilled condition by placing ice bags below the mortar. The homogenate is passed through cheese cloth, centrifuged at 12,000 rpm for 30 minutes at 2-3°C.The supernatant serves as crude enzyme extract[4].

Estimation of total protein by Lowry's method: Working solution (200µg/ml) in aliquots of 0.2, 0.4, 0.6, 0.8 and 1mL was pippeted out into each test tube. The volume was made upto 1mL in each tube with 0.1N NaOH. 5mL of copper reagent was added to each tube. The tubes were incubated at 37°C for 10 minutes.0.8mL of F-C reagent was added and the tubes were incubated for 30 minutes at 37°C.Absorbance was read at 660nm.A graph was plot with the concentration of protein on X-axis and absorbance on Y-axis.

Standard graph of Ammonium sulphate by Nessler's Method: Ammonium sulphate working solution (50µg/ml) in aliquots of 0.2, 0.4, 0.6, 0.8 and 1mL was pipetted out into each test tube. The volume was made up to 3mL in each tube with distilled water.1mL of Nessler's reagent was added to each tube. Absorbance was read immediately at 460 nm using UV – spectrophotometer. A graph was plot with concentration of ammonium sulphate on X- axis and absorbance on Y-axis.

Enzyme assay of L-asparaginase in Solanum Nigrum**:** Four clean and dry test tubes were taken. The first tube was marked as "Blank", "Test 1 and 2" and a "control".0.5mL of substrate was added to each test tube.0.5mL of 50mM potassium phosphate buffer of pH 8.0 was added to each tube.0.25mL of 20%TCA was added only to the control tube.0.5mL of crude enzyme extract (Solanum Nigrum**)** was added to all the tubes except blank. The tubes were incubated for 15minutes at 37°C. 0.25mL of 20% TCA is added to all tubes except control. Tubes were centrifuged at 8000rpm for 5minutes.The supernatant was collected, and the pellet was discarded.

To the supernatant 3mL of distilled water was added followed by 1mL of Nesseler's reagent.

Absorbance was read at 460nm using UV spectrophotometer. By using ammonium sulphate standard graph, the amount of ammonia liberated was determined.

Enzyme Kinetics:

Temperature Kinetics:

9 clean and dry test tubes were taken. The first tube was marked as 'blank', 'test' and 'control'. The tubes were labeled with various temperature 4, 27, 37,100. 0.5mL of L-asparaginase substrate, followed by 0.5mL of 50mM potassium phosphate buffer of pH 8 was added to each tube.0.25mL of 20% TCA was added to control tube.0.5mLof crude enzyme extract (Solanum nigrum***)*** was added to each tube. The tubes were incubated for 15 minutes in their respective temperatures.0.25mL of 20% TCA was added to the tubes marked as 'Test'. The tubes were centrifuged at 8000rpm for 5minutes, the supernatant was collected. To the supernatant 3mL of distilled water and 1mL of nesseler's reagent was added. Absorbance was read at 460nm using UV spectrophotometer. A graph was plotted with the temperature on X axis and absorbance on Y axis.

pH Kinetics:

10 clean and dry test tubes were taken and labeled as pH 7.0- pH 9.0.0.5mL of L-asparaginase substrate was added to all tubes.0.5mL of 50mM potassium phosphate buffer was added to all tubes. The tubes had different pH of 7,7.5,8.0,8.5, 9.0 and blank was maintained at pH8.0.25mL of 20% TCA was added to control. 0.5mL of crude enzyme extract (Solanum nigrum) was added to all the tubes. The tubes were incubated at 37°C for 15minutes and then the reaction was stopped by adding 0.25mL of 20%TCA to the test tubes. The tubes were centrifuged at 8000rpm for 5minutes, the supernatant solution was collected. To the supernatant 3mL of distilled water and 1mL of Nessler's reagent was added. Absorbance was read at 460nm using UV spectrophotometer. A graph was plotted with different pH values along x-axis and absorbance on y-axis.

Time Kinetics:

15 clean and dry test tubes were taken. The first tube was marked as 'blank', 'test' and 'control'. 0.5mL of L-asparaginase substrate, followed by 0.5mL of 50mM potassium phosphate buffer of pH 8 was added to each tube.0.25mL of 20% TCA was added to control tube. 0.5mLof crude enzyme extract (Solanum nigrum***)*** was added to each tube. The tubes were incubated at 37°C for respectiv time 0,5,10,15,20,25, 30 minutes. 0.25mL of 20% TCA was added to the tubes marked as 'Test'.

The tubes were centrifuged at 8000rpm for 5minutes, the supernatant was collected and 3mL of distilled water and 1mL of nesseler's reagent was added. Absorbance was read at 460nm using UV spectrophotometer. A graph was plotted with the time on X axis and absorbance on Y axis.

Km and Vmax:

Different aliquots (0.1-0.5 mL) of Lasparaginase substrate was pipetted into different test tubes. The volume was made up to 0.5mLwith 50mM potassium phosphate buffer of pH 8.0. 0.5mL of crude enzyme (Solanum nigrum**)** was added to all the tubes. The tubes were incubated at 37°C for 15 minutes. 0.25mL of20%TCA was added to all tubes. Tubes were centrifuged at 8000 rpm for 5 minutes. Supernatant was collected and 3mL of distilled water and 1mL of Nesseler's reagent was added. Absorbance was read at 460nm using UV spectrophotometer. A graph was plotted using double reciprocal Lineweaver-Burk plot[5].

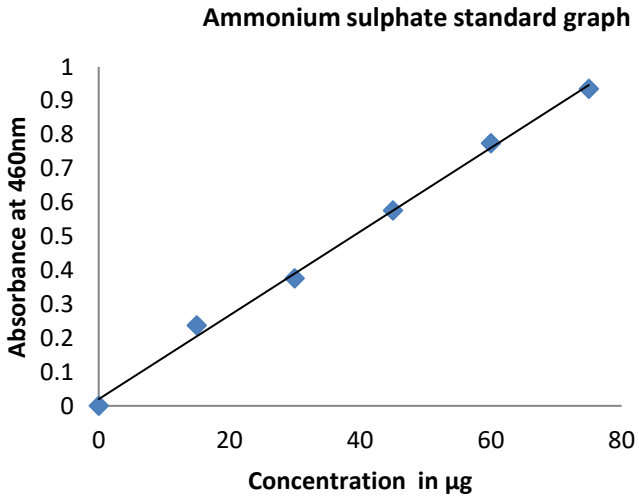
Stability check of L-asparaginase enzyme by various nanoforms:

- Nanoparticles of Na with concentration of 2.1µg/ml and 14.7µg/ml were prepared, incubated with the enzyme for about 5hrs with constant stirring. This nanoparticle incubated enzyme sample was used to perform enzyme assay on first and seventh day to find out the enzyme activity in order to check the stability of the nano-enzyme complex.
- Nanoparticles of Mg with concentration of 2.1µg/ml and 14.7µg/ml were prepared, incubated with the enzyme for about 5hrs with constant stirring. This nanoparticle incubated enzyme sample was used to perform enzyme assay on first and seventh day to find out the enzyme activity in order to check the stability of the nano-enzyme complex.
- Nanoparticles of $AgNO_3$ with concentration of 2.1µg/ml and 14.7µg/ml were prepared, incubated with the enzyme for about 5hrs with constant stirring. This nanoparticle incubated enzyme sample was used to perform enzyme assay on first and seventh day to find out the enzyme activity in order to check the stability of the nano-enzyme complex[6].

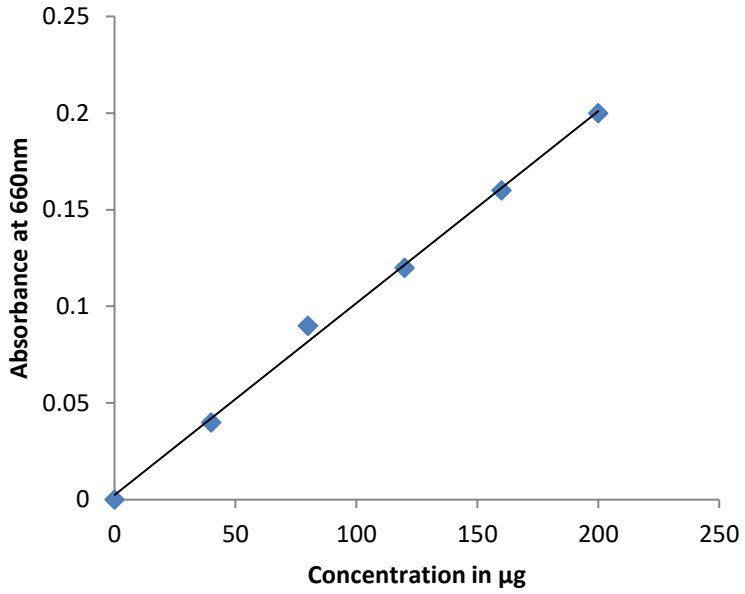
Cell Viability (MTT) assay:

Cell viability, MTT (3(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was carried out as per the protocol described by Baba *et al* 2013. The MTT assay involves the reduction of soluble yellow dye (MTT) to an insoluble purple formazan salt. Cells were cultured in a sterile 96 well plate (5x 10^3cells/well) in a 100µl media and incubated overnight for attachment. The cells were treated with different dilutions of L-asparaginase crude extract for 48h and at the end of the reaction 50 µl of MTT dye (5mg/ml) was added at each well and incubated further for 4h at 37˚C in a CO_2 incubator. The formazan product formed in cells were dissolved in DMSO (100 µl) and the absorbance was measured at 540nm using multimode plate reader (Perkin Elmer) [7].

RESULTS:



Graph 1: Ammonium sulphate standard graph to calculate the enzyme activity.



Graph 2: Lowry's standard graph to calculate the specific activity

Enzyme activity:

Sl.no	Plant description	Absorbance at 460nm	Enzyme activity: IU
1	Solanum Nigrum	0.321	7.55

Table1: Enzyme activity

Specific activity:

Sl.no	Plant description	Total protein (mg)	Specification(µmoles/mg/min)
1	Solanum Nigrum	1.562	4.8335

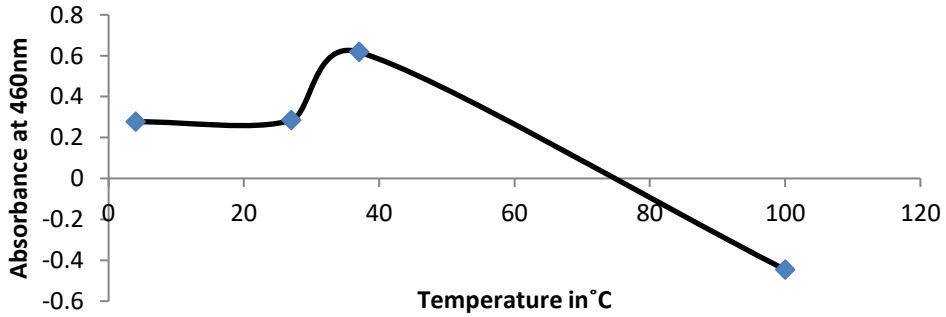
Table2: Specific activity

Enzyme Kinetics:

Temperature Kinetics:

Temperature in ˚C	Absorbance at 460nm
4	0.277
27	0.285
37	0.618
100	-0.446

Table3: Temperature Kinetics

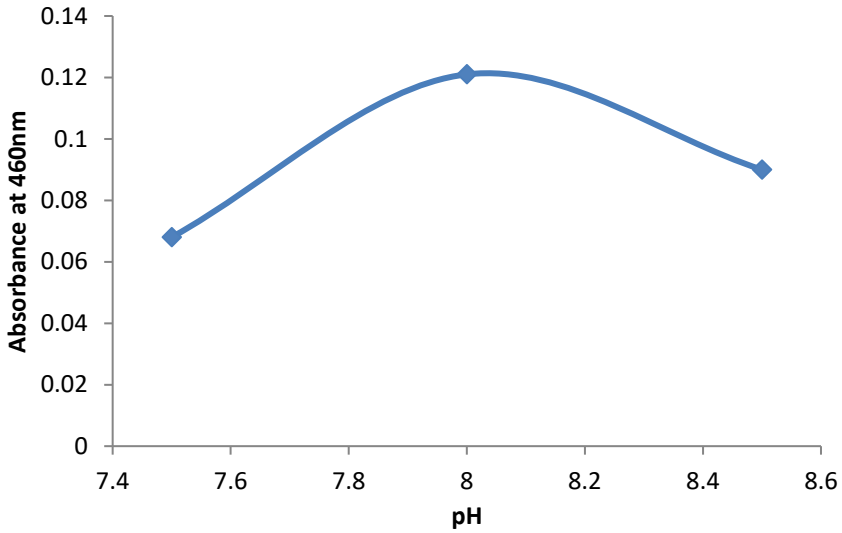


Graph 3: Temperature Kinetics

pH Kinetics:

pH	Absorbance at 460nm
7.5	0.068
8	0.121
8.5	0.090

Table4: pH Kinetics

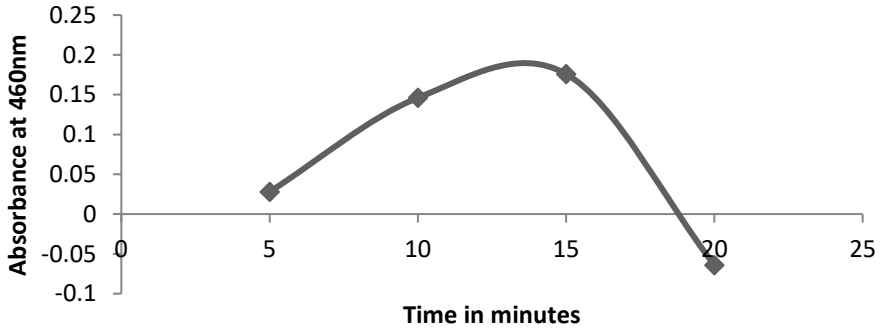


Graph 4: pH Kinetics

Time Kinetics:

Time in minutes	Absorbance at 460nm
5	0.028
10	0.146
15	0.176
20	-0.064

Table5: Time Kinetics

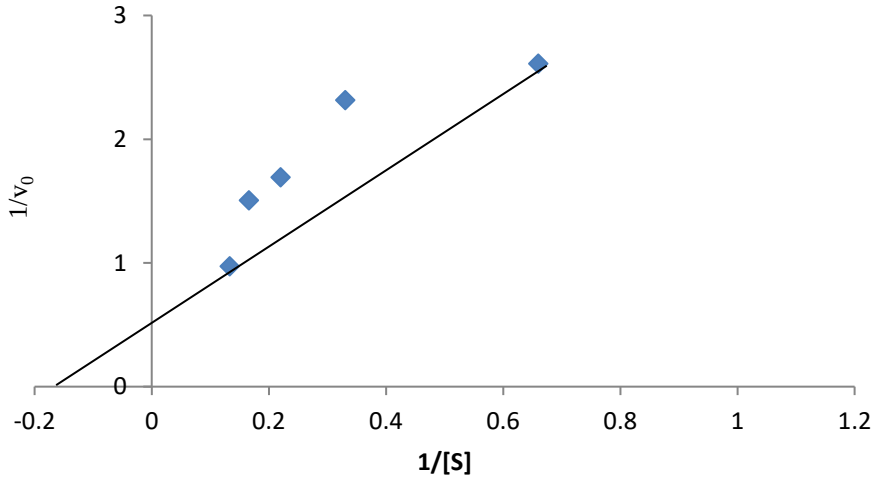


Graph 5: Time Kinetics

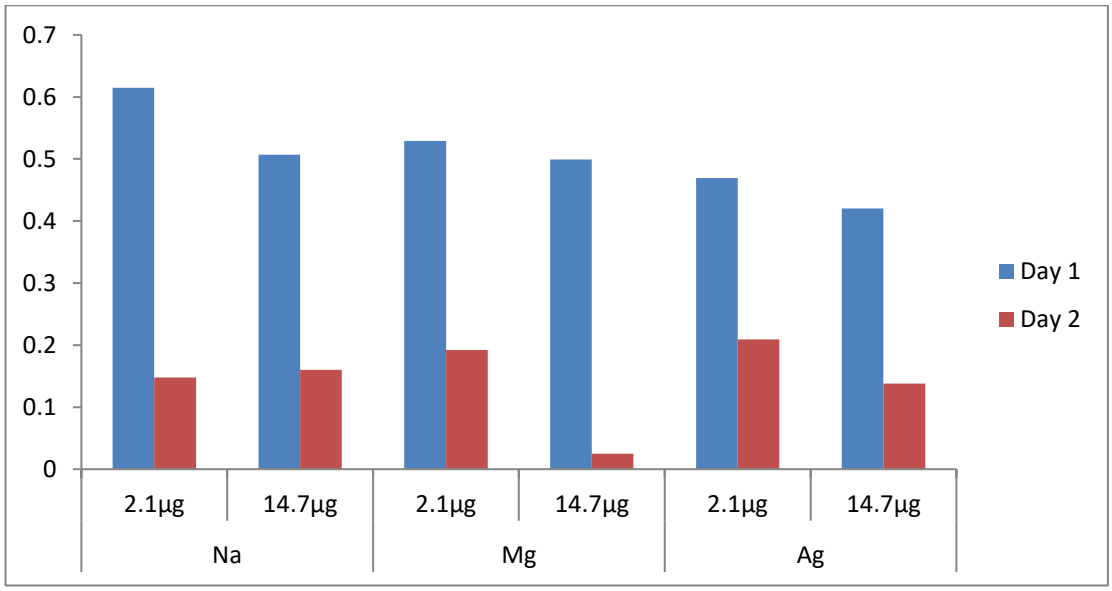
Km and Vmax:

[S] in mg	1/[S]	V_o	1/ V_o
1.5	0.66	0.383	2.610
3	0.33	0.432	2.314
4.5	0.22	0.591	1.692
6	0.166	0.664	1.506
7.5	0.133	1.028	0.972

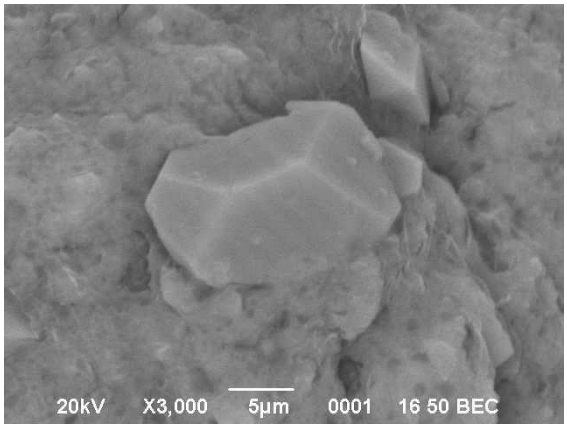
Table6: Km and Vmax



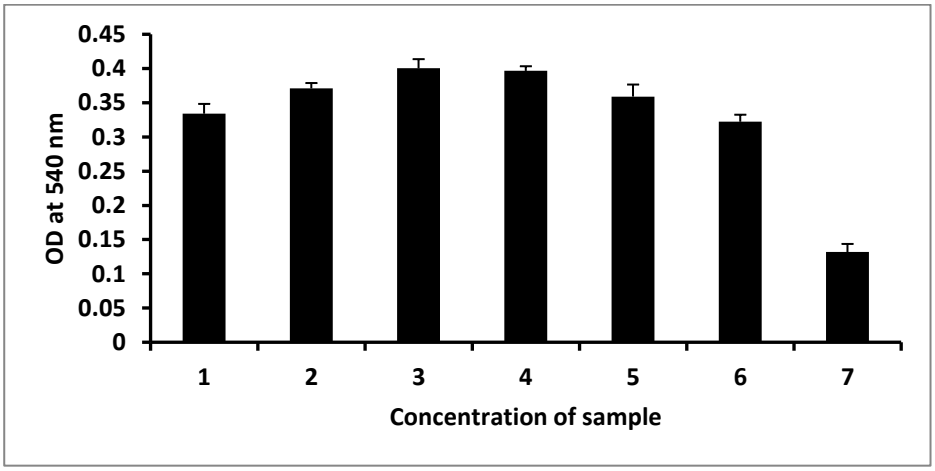
Graph 6: Km and Vmax



Graph 7: Graphical representation of stability of nanoforms of L-asparaginase enzyme on its enzyme activity. High stability was observed in Na nanoform.



Picture 1: Scanning electron microscopic photograph of highly stable Na nanoform of L-asparaginase enzyme.



Graph 8: Effect of L-asparaginase enzyme on the viability of A549 cells. A549 cells (5x 10^3 cells/well) cultured overnight were treated with different dilutions of L-asparaginase enzyme and incubated for 48h in a CO_2 incubator. MTT assay was carried out and absorbance was measured at 540nm. Results were expressed as % of viability of cells compared by taking control as 100%.

DISCUSSION:

The crude leaf extract of Solanum nigrum showed L-asparaginase enzyme activity of 7.55IU and specific activity of 4.833µmoles/mg/min. These finding confirmed the production of L-asparaginase in Solanum. The kinetic parameters were similar to the L-asparaginase reported till date. The optimum conditions of the L-asparaginase enzyme with respect to temperature were 37˚C, pH was 8 and incubation time was 15min. The Km value of L-asparaginase enzyme was found to be 10 and Vmax was 0.325µmoles/ml/min, as per the double reciprocal Lineweaver- Burk plot. The major drawback of enzymes is their stability. In this investigation an attempt was made to increase the stability of L-asparaginase enzyme using nanoforms and this enzyme showed high stability with Na nanoforms. Increase in absorbance in the MTT assay inferred increased cytotoxic effect of the enzyme in crude extracts of leaves of Solanum indicating very good anticancer properties. IC 50 values were not possible to determine as crude extracts of the sample was used.

CONCLUSION:

Enzyme assay and biochemical characterization confirmed the production of L-asparaginase enzyme in Solanum nigrum. The crude extracts of Solanum showed excellent anticancer properties. Hence further purification and molecular characterization of this enzyme from Solanum nigrum will definitely count this medicinal herb to fight acute lymphoblastic leukemia more effectively.

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