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In vitro cytotoxic study for partially purified L-asparaginase from fresh leaves, unripe and ripe fruits of *Withania somnifera* plant

Saad M. Nada[1], Najwa S. Ahmed[1]*, Yasameen Ali Hadi[1], Zeina Saif Al-Dein Mohammed[1] and Zainib H. A. Al Rikabi[2]

[1]Biotechnology Research Center, Al-Nahrain University, Iraq.
[2]Genetic Engineering and Biotechnology Institute, University of Baghdad, Iraq.

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This work aimed to study the cytotoxic effect of L-asparaginase isolated from local *Withania somnifera* plant on lymphocyte leukemia cells. To achieve this goal, L-asparaginase was purified from *W. somnifera* fruits by two purification steps, ion-exchange chromatography using DEAE-cellulose and gel filtration chromatography using Sephadex G-150, and the study utilized an *in vitro* evaluation for the cytotoxic effect of the partially purified L-asparaginase with concentrations ranging (12.5 to 100 µg/ml) in a two fold serial dilutions on some cell suspension culture including, acute lymphocyte leukemia and chronic lymphocyte leukemia culture at different concentrations (12.5 to 100 µg/ml) and different exposure time of treatment (24, 48 and 72 h). This two purification steps raised the specific activity from 1.73 U/mg in crude extract to 2.29 U/mg after ion-exchange and 10.5 U/mg after gel filtration; the purification fold was 1.32 after ion-exchange and 6.06 after gel filtration, the enzyme recovery was 56% after two purification steps and the results, pointed that acute lymphocyte leukemia culture showed highest sensitivity toward the cytotoxic effect (62.3±0.9%) of the partial purified L-asparaginase (100 µg/ml) than other culture after 48 h in a dose dependent manner, and highest cytotoxic inhibitory effect (73.2±1.6%) after 48 h of exposure on chronic lymphocyte leukemia culture, while healthy lymphocyte culture showed novel behavior. The lowest concentration of cell treatment gave the most significant (P< 0.01) inhibitory effect. The conclusion is that there is enough evidence to support the claim that L-asparaginase from *W. somnifera* may be considered chemotherapeutic agent against cancer, such as acute lymphoblastic leukemia and lymphosarcoma.

Key words: Acute lymphocyte leukemia (ALL), chronic lymphocyte leukemia (CLL), L-asparaginase, cytotoxic assay.

INTRODUCTION

Leukemia is a cancer originating in any of hematopoietic cell that tends to proliferate as single cells within bone marrow and often circulate in the blood stream. Lymphocytic leukemia's are derived from B or T cell precursors. Four types of leukemia are classified, Chronic Lymphocytic Leukemia (CLL), chronic myelogenous leukemia (CML), acute lymphocytic leukemia (ALL) and acute myelogenous leukemia (AML). The development of a malignant cell clone is due to the dysregulation of the balance between cell proliferation and the programmed cell death apoptosis (Conter et al., 2005). L- asparaginase is an enzyme that destroys asparagines external to the cell.

*Corresponding author. E-mail: najwaun@yahoo.com.

Normal cells are able to make all the asparagine they need internally whereas tumor cells become depleted rapidly and die. The enzyme converts asparagine in the blood into aspartic acid by a deamination reaction; the leukemia cells are thus deprived of their supply of asparagine and will die (Grossman et al., 2004). Leukaemic cells lacking the mammalian asparagine-synthetase enzyme depend on exogenous sources of asparagine for protein synthesis and survival. Theoretically, the deamination of serum asparagines selectively kills leukaemic cells, leaving normal cells, which have the ability to synthesize asparagine intracellularly, unaffected (Broome, 1968). L-Asparaginase is known as a chemotherapeutic agent against cancer, such as acute lymphoblastic leukemia and lymphosarcoma, which is used mainly in the treatment of children (Borek and Jaskolski, 2001).

The current study was aimed at the *in vitro* cytotoxic study of the partial purified L-asparaginase on different types of suspension cell lines at different concentrations and exposure times.

MATERIALS AND METHODS

Sample collection of plant

The fresh leaves, unripe and ripe fruits of *W. somnifera* plant collected from the garden of plants in Baghdad University were included in this study. The plant parts were cleaned from the dust and other particles and stored in the freezer until use.

Extraction of L-asparaginase from plant tissues

After cleaning the plant tissues with distilled water, the plant tissues (leaves, unripe fruits and ripe fruits) were homogenized and approximately 3 g from each sample were ground with 2 m^3 of potassium phosphate buffer 0.1 M (pH 8.6) in a pestle and mortar, left on magnetic stirrer for 10 min, the extract was filtered to rid the cell debris, centrifuged at 12000 rpm for 10 min and the supernatant was taken to determine the L-asparaginase activity and protein concentration as explained in Ren et al. (2010) and Bradford (1976).

Extraction of L-asparaginase using liquid nitrogen

The plant tissues (leaves, unripe fruits and ripe fruits) were homogenized in liquid nitrogen, as the same as aforementioned.

Determination of L-asparaginase activity

0.5 ml of enzyme crude extract, 0.5 ml of 50 mM asparagine and 1 ml potassium phosphate buffer (0.02 M and pH 8.6) were mixed well, the mixture was incubated in water bath at 37°C for 15 min, after the incubation, 1 ml of 1.5 M trichloroacetic acid was added to the mixture to stop the reaction, then centrifuged at 12000 rpm for 10 min and the supernatant was collected (Ren et al., 2010). After that, the supernatant was transported to clear test tubes to determine the concentration of ammonia liberated from the enzyme action by the method of direct nesslerization, the concentration of ammonia was estimated for each sample by mixing 4 ml of distilled water with 0.5 ml of sample to be estimated and 0.5 ml of Nessler's reagent, the mixing was shaken well, incubated at 37°C for 15 min, and the absorbance was measured at wave length (450 nm). The blank was prepared by mixing 4.5 ml distilled water with 0.5 ml Nessler's reagent.

Purification of L-asparaginase

Preparation of ion exchange column (DEAE-cellulose)

The DEAE-cellulose column was prepared according to the method suggested by Whitaker (1972), the resin was packaged gently in glass column, the dimensions of resin was 1 × 24 cm, and the equilibration was achieved by the same potassium phosphate buffer at flow rate approximately 30 ml/h to next day.

Separation through ion exchange resin (DEAE-cellulose)

10 ml from enzyme crude extract was loaded onto ion exchange column, the separated fractions was collected at flow rate of 30 ml/h approximately (2 ml for each fraction), the wash was achieved by using potassium phosphate buffer, the same buffer used in equilibration, and the elution was achieved by the same buffer used in equilibrium with graduated concentrations of potassium chloride (0.1, 0.05, 0.02 and 0.01 M) respectively; the flow rate of elution was 30 ml/h too, the protein concentration of the fractions was measured at wave length 280 nm and enzymatic activity was estimated for fractions as in Ren et al. (2010) and the fractions which give higher activity was collected, lyophilized (freeze dried), and stored in the freezer until use.

Gel filtration chromatography

Preparation of sephadex G-150 column

Preparation of gel was achieved as recommended by the supply company (Sigma, USA). 5 g from gel sephadex G-150 was suspended in 1 L Tris-HCl buffer (0.1 M) and pH 8.6, then the suspension was left in water bath at 90°C for 5 h to ensure the swelling of gel beads with gentle agitation from time to time, after that, the gel was transferred to graduated cylinder, left to stagnate for 20 min, then the supernatant was removed, the gel was resuspended in 600 ml of Tris-HCl buffer, then the gel was degassed by using vacuum, then, packaged gently in glass column with dimensions (1 × 28) cm. The column was equilibrated using same buffer used in gel suspension at flow rate of 20 ml/h approximately until the next day.

Separation through sephadex G-150 column

The lyophilized extract produced from ion exchange step was suspended in 5 ml Tris-HCl buffer, the suspension was added gently on the surface of gel, the elution was done by using the same buffer (Tris-HCl) at flow rate of 20 ml/h (2 ml for each fraction), the protein concentrations for fractions was measured at wave length of 280 nm, the enzyme activity was estimated according to method mentioned in Ren et al. (2010), then the fractions with higher enzyme activity was collected, lyophilized and stored in freeze for other steps.

Preparation of L-asparginase dilutions

Pure L-asparaginase stock solution was prepared by dissolving 5 m with 1 ml PBS, then filtered through millipore 0.22 µm filter and stored

at -20°C until used. Serial dilutions were made starting from concentration 100, 50, 25 and 12.5 µg/ml. The dilutions were done in a sterile laminar cabinet using a sterile PBS and sterile stoppard tubes.

Sample collection of blood

5 ml of blood was collected by vein puncture from 10 (ALL and CLL) cases for each, and 5 healthy persons (control) who were admitted to the National Center of Hematology Diseases / Al Mustanisyria University. The patients were diagnosed by the consultant medical staff at the centre.

Isolation of lymphocytes

Preparation of solutions and media were done according to the methods described by Marlise (1997) and Bottran and Vetvicka (2001). The lymphocytes were isolated from the peripheral heaprinized whole blood as follows: 3 ml of blood was centrifuged at 1000 rpm for 15 min, buffy coat was collected in a 10 ml centrifuge tubes and diluted with 5 ml RPMI 1640 (cell suspension), 5 ml of the diluted cell suspension was layered on 3 ml of ficoll-isopaque separation fluid, and the tubes were centrifuged at 2000 rpm for 30 min in a cooled centrifuge at 4°C. After centrifugation, the mononuclear cells were visible as cloudy band between the RPMI1640 and lymphoprep layers. The band was collected in a 10 ml test tube and the cells were suspended in 5 ml RPMI 1640. The tube was centrifuged at 2000 rpm for 5 min, the supernatant was discarded and the cells were resuspended in 5 ml RPMI 1640 (repeated twice). The suspension was centrifuged at 1000 rpm for 10 min, and the supernatant was discarded. The precipitated cells were resuspended in 1 ml RPMI media Boyum (1968) and viable count were performed (Porakishvili et al., 2004), the numbers of lymphocytes were counted by improved neuber chamber and the cells concentration was adjusted to 1×10^6 cell/ml. The isolated cells were grown in a flask containing 10 ml RPMI 1640 medium supplemented with bovine serum albumin (BSA) and incubated at 37°C for 48 h in CO_2 incubator (Boyum, 1968).

3 (dimethylthiazol-2-yl) -2,5- (diphenyltetrazoliumbromide) MTT

A 50 mg MTT dye was dissolved in 100 ml distilled water. The solution was filtered through 0.22 µm millipore filter to remove any blue formazan product and stored in a sterile, dark screw–capped bottles at 4°C.

Cytotoxicity assay

It is also called a cell growth inhibition assay. The *in vitro* method was used to investigate the effect of pure L-asparaginase on two types of leukemia culture (ALL- acute lymphocyte leukemia; CLL-chronic lymphocyte leukemia) at different concentrations (100, 50, 25 and 12.5 µg/ml) and exposure times (24, 48 and 72 h) (Masters, 2000). Using a microtiteration plate (96 wells) and cell culture technique, 4×10^5 cells/ml were exposed to serial dilutions of L-asparaginase in the concentrations range mentioned earlier. At the end of the exposure times, 10 µl of MTT solution was added to all wells and incubated for 4 h at 37°C, during this period, formazon crystals were formed at the bottom of each well. The spent medium was pipetted out along with suspension of culture and 100 µl of acid isopropanol (100 µl of 0.04N HCL in anhydrous isopropanol) was added to all wells and mixed thoroughly to dissolve the dark blue crystals and incubated in shaker at room temperature for 30 min. The plates of different cell culture at the end of the assay were examined by ELISA reader at 540 nm transmitting wave length (Skehan et al., 1990; Mosmann, 1983). The inhibition was calculated according to the following equation:

$$\% \text{ inhibition} = 100 - \frac{\text{Correct sample}}{\text{Correct solvent}} \times 100$$

Correct sample = Growth cell GC – negative control, correct solvent = solvent control (SC) – negative control, RPMI 1640 + PBS (negative control), RPMI + cell [cell growth control (GC)], RPMI 1640 + cell + PBS [solvent control (SC)] (Bottran and Vetvicka, 2001).

Statistical analysis

The statistical analysis is a very important final step in the research to analyse and evaluate the obtained results. Medical statistics of this study was conducted via computer based statistical program which was: 1) SPSS for windows computer package (programmer 11.5; 2) Microsoft excel 2003.

The statistical analysis system (SAS, 2004) was applied for all results to show effect of different concentration and other factors in studied parameters. The least significant difference (LSD) test and Duncan test are the comparative between means in this study. It is a well used test for the medical statistics; P value <0.01 is considered a significant correlation.

RESULTS AND DISCUSSION

Cytotoxic effect growth inhibitory assays of the partial purified L-aspariginase

L-asparaginase has been purified from *W. somnifera* fruits by two purification steps, ion-exchange chromategraphy using DEAE-cellulose and gel filtration chromategraphy using sephadex G-150; using these two steps, the specific activity was raised from 1.73 U/mg in crude extract to 2.29 U/mg after ion-exchange and 10.5 U/mg after gel filtration; the purification fold was 1.32 after ion-exchange and 6.06 after gel filtration; the enzyme recovery was 56% after using the two purification steps. Three cell lines were studied (ALL, CLL, Healthy) at three times of exposure (24, 48 and 72 h), using a two fold dilutions to get concentration from 100 to 12.5 µg /ml of partial purified L-aspariginase. The cytotoxic effect on acute lymphocyte leukemia culture (Table 1) revealed that the high concentrations gave a significantly ($P<0.01$) high inhibition rate of cells while being low gradually with low concentrations. After 48 h of exposure, a high cytotoxic inhibitory effect (5%) of the extract was started at the concentration (100 µg/ml). The extracted partial purified L-aspariginase was very sensitive and liable through many environmental changes even with suitable protection which led to the best results at 48 h of exposure than after 72 h of cells treatments. This might explain the more significant inhibition rate at different concentration for 48 h than 72 h of exposure.

Table 1 shows the results of the significant effect at $P < 0.01$ level after 48 h of exposure. The highest concentration

Table 1. The cytotoxic effect of extract pure L-asparaginase on three types of suspension leukemia culture (at 24, 48 and 72 h) of exposure.

Treatment	Concentration (µg/ml)	Time (h)		
		24	48	72
ALL	100	33.6 ± 0.6[a]	62.3 ± 0.9[a]	20.1 ± 0.2[a]
	50	30.7 ± 1.01[b]	58.6 ± 0.5[b]	20.6 ± 0.7[a]
	25	20.5 ± 0.3[c]	50.3 ± 0.7[c]	19.5 ± 0.3[a]
	12.5	19.8 ± 0.4[d]	40.1 ± 0.006[d]	15.1 ± 0.3[b]
CLL	100	55.3 ± 2.6[a]	73.2 ± 1.6[a]	26.3 ± 0.6[a]
	50	31.7 ± 0.6[b]	54.9 ± 0.9[b]	15.8 ± 3.1[b]
	25	25.2 ± 1.1[c]	51.1 ± 2.7[b]	-17.5 ± 1.1[c]
	12.5	6.8 ± 0.5[d]	48.4 ± 0.6[c]	-39.6 ± 0.5[d]
Healthy	100	2.6 ± 0.7[a]	2.9 ± 0.5[a]	2.9 ± 0.5[a]
	50	2.5 ± 0.3[a]	2.3 ± 0.2[a]	-10.1 ± 0.09[b]
	25	2.2 ± 0.04[a]	2.7 ± 0.3[a]	-13.3 ± 0.2[b]
	12.5	1.8 ± 0.6[a]	2.2 ± 0.4[a]	-16.9 ± 0.3[b]

Mean having different small letters (a, b and c) at the same column are significantly different at $P < 0.01$.

(100 µg/ml) of L-aspariginase, showed the highest cytotoxic inhibitory effect (62.3 ± 0.9%); the inhibitory effect decreased (33.6 ± 0.6, 20.1 ± 0.2%) after 24 and 72 h of exposure respectively on acute lymphocyte leukemia culture and highest cytotoxic inhibitory effect (73.2 ± 1.6%) after 48 h of exposure on chronic lymphocyte leukemia culture. The L-asparaginase was an antiproliferative effect without a significant inhibitory effect (2.6 ± 0.7, 2.5 ± 0.3, 2.2 ± 0.04 and 1.8 ± 0.6%) at concentrations (100, 50, 25 and 12.5 µg/ml) respectively; after 24 h of exposure, healthy lymphocyte culture and the cytotoxic inhibitory effect decrease to reach -16.9 ± 0.3% after 72 h of exposure for 12.5 µg/ml concentration then reached (2.9 ± 0.5, 2.3 ± 0.2, 2.7 ± 0.3 and 2.2 ± 0.4%) respectively for all concentration in descending manner respectively for 48 h of exposure. Among the three types of cell suspension (ALL, CLL and Healthy) at all concentration and different intervals of exposure (24, 48 and 72 h), the extracted purified L-aspariginase had the best efficiency in inhibiting CLL culture within (100 µg/ml) 48 h which was about 73.2 ± 1.6%.

Oza et al. (2009) showed the enzyme was purified and characterized from *W. somnifera*, a popular medicinal plant in South East Asia and Southern Europe and a traditionally used Indian medicinal plant (Oza et al., 2009). L-asparaginase (E.C. 3.5.1.1) is used as a therapeutic agent in the treatment of acute childhood lymphoblastic leukemia (Oza et al., 2011). L-aspara-ginases are known chemotherapeutic agents against cancer, such as acute lymphoblastic leukemia and lymphosarcoma. Several recent reviews are available concerning the use of L-asparaginase in cancer therapy (Muller and Boos, 1998; Oza et al., 2011). Leukaemic cells lacking the mammalian asparagine-synthetase enzyme depend on exogenous sources of asparagine for protein synthesis and survival. Theoretically, the deami-nation of serum asparagines selectively kills leukaemic cells, leaving normal cells, which have the ability to synthesize asparagine intracellularly and unaffected (Broome, 1968). Asparaginase is an enzyme which produces its anti-cancer effects by “breaking down” asparagine, a substance normally found in the body that is involved in biological processes essential for cells to maintain life. Healthy cells are able to create asparagine for themselves; however, cancer cells are not able to create asparagine. Therefore, the depletion of asparagine by asparaginase kills cancer cells, while healthy cells are not as affected (Muller and Boos, 1998; Chumchalova and Smarda, 2003).

Conclusions

L-asparaginase purified from *W. somnifera* fruits may be considered as a chemotherapeutic agent against acute lymphoblastic leukemia and chronic lymphoblastic leukemia culture.

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