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In-vitro Assay of Anti-proliferative Potential of *Amaranthus Cruentus* Aqueous Extract on Human Peripheral Blood Lymphocytes

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Abstract

Beneath the complexity and idiopathy of all cancers, lie a limited number of 'mission-critical' events that propel the tumor cells and their progeny into uncontrolled expansion and invasion. The goal of most current cancer therapies is to reduce the number of tumor cells and to prevent their further accumulation. To better accomplish this goal, a complex microenvironment must be provided which may inhibit the underlying mechanistic pathways of cell proliferation. The present study based on ethno-medical data approach, a first of its kind to evaluate an Indian plant used as a traditional herb, to assess its cytotoxic and anti-proliferative property on human peripheral blood lymphocytes. Also a parallel assessment with laboratory produced crude L-asparginase (an anti-proliferative agent) of bacterial origin indicated that *Amaranthus cruentus* aqueous extract can be used as an inexpensive, biocompatible alternate to other commercially available anti-proliferative therapeutics.

Key words: anti-proliferative, alternative therapeutic, anti-cancer, traditional medicine, L-asparaginase, microenvironment.

Introduction

Since medieval times, plants have been the source of medicines for treatment of diseases. Regardless of the availability of synthetic drugs, plants remain even in the 21st century, an integral part of the health care in different countries, especially the developing ones like India [1]. Ayurvedic medicine is a system of traditional medicine native to India and now recognized internationally after the formation of AYUSH (Department of Ayurveda, Yoga and Naturopathy, Unani, Siddha and Homoeopathy). Herbal remedies are often sought by patients with chronic and or non-curable diseases like Alzheimer's, cancer etc. [2] especially for symptomatic relief [3-5]. The advantage of using such plant derived agents is their relatively low toxicity and availability in consumable form [6]. A global increase in deaths associated with cancer [7], the callous side effects of some of the cancer chemotherapies [8] and the recurrence of drug-resistant tumors as well as the lack of selectivity of anticancer drugs [9], have triggered the search for more natural cancer fighting agents, particularly those derived from plants. Phyto-chemicals have been used for treating various human diseases since time immemorial. The use of complementary and alternative medicine such as herbal extracts is becoming increasingly popular among patients with cancer [10]. Typically, herbal medicine system emphasizes on the use of whole extract of a plant based on various documented evaluations implicating a synergistic mechanism of action of various bioactive components [11]. The need for novel natural products that can act as antiproliferative agents has triggered an increased interest in plant-derived bioactive compounds, with potential anticancer activity. A variety of edible plants and compounds have been reported to exert antiproliferative activities such as cabbage, red pepper, onion and broccoli [12] serving as useful dietary supplements for cancer patients.

The plant *Amaranthus cruentus* (*A. cruentus*) was chosen for the present study as no documented literature is available about the evaluation of cytotoxic and antiproliferative activity of leaves of this species on stimulated lymphocytes, while other plants of the genera having similar properties have been worked out. *A. cruentus* of family Amaranthaceae is a tall annual herb topped with clusters of dark pink flowers, having high nutritional value as it contains ascorbic acid, carbohydrate, protein, fiber and many other secondary metabolites. Phytochemical studies of *A. cruentus* account

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for the presence of alkaloids, saponins, tannins, inulins etc. *A. cruentus* is characterized by its ability to neutralize free radicals, because it contains flavonoids, phenolic acids, anthocyanins, vitamins and other antioxidants [13 &14], thus being anti-proliferative and anti-tumorigenic in nature.

L-asparaginase

The enzyme L-asparaginase which is known to possess antitumorgenic activity is used commercially as a chemotherapeutic agent. L-asparaginase production using bacteria such as Pseudomonas sps, *Bacillus sps*, *Escherichia coli*, *Enterobacter aerogenes*, Streptomyces gulbargensis, *Erwinia carotovora* [15-19] have proved to be beneficial source of this enzyme as well as cost effective. However, L-asparaginase has a major drawback in that; it is clinically used only for the treatment of acute lymphoblastic leukemia (ALL) and mast cell tumors [20]. Side effects such as fever, chills, nausea and vomiting allergic reaction, poor appetite, stomach cramping, neurotoxicity, sleepiness, depression and hallucinations due to L-asparaginase treatment are other concerns [21].

The present study was designed to evaluate the antiproliferative activity of *A. cruentus* plant extract as well as L- asparaginase crude extract of microbial origin, on cultured lymphocytes, with a view to promulgate usage of plant based anti-proliferative agent in lieu of its easy consumption as well as stability and marketability in lyophilized form.

Material and Methods

Plant material

The leaves of *A. cruentus were* collected from local market of Bhopal, Madhya Pradesh, India, in January 2011 and were identified by Dr. R. S. Yadav, senior horticulturist at the Central Institute of Agricultural Engineering (CIAE), Bhopal, and M.P. India.

Preparation of aqueous extracts of *A. cruentus* leaves

The leaves of *A. cruentus* were washed and shade dried for 1 week at room temperature (24 ± 2°C). The dried leaves were then ground into a coarse powder in sterile environment, weighed and extracted by hot soxhlation process with sterile distilled water for 6 hours. The crude extract was collected and filtered using whatmann filter paper 1 to obtain the aqueous supernatant. The aqueous extract of *A. cruentus* was lyophilized and stored at 4°C for further use. Just prior to use, the extract was dissolved in sterile distilled water and filter sterilized with 0.22 µm membrane syringe filter. The extract was not further characterized.

Screening of bacteria for L-Asparaginase production

Bacteria were isolated from local water bodies of Bhopal and identified on the basis of physiological and biochemical characteristics using the standard protocol [22]. L-asparaginase producing bacteria were screened using the substrate specificity protocol [23], then selectively enriched into the growth medium containing yeast extract (1.0%, wt/vol) and L-asparagine (1.5%, wt/vol). *The isolates positive for production were c*ultured in medium with yeast extract (5.0 g/L), dextrose (3.0 g/L), and K_2HPO_4 (5.0 g/L), pH adjusted to 8.0 and incubated at 37°C on a rotary shaker (60 rpm) for 24 hours. *For protein extraction,* cells were harvested, washed with neutral phosphate buffer and lysed. Determination of protein concentrations of extra- and intracellular fractions of crude enzyme preparations was done [24]. L-asparaginase activity was assayed by modified method of Mashburn and Wriston [25]. The active fractions were pooled and concentrated. All purification steps were carried out at 4°C unless stated otherwise. For purification of enzyme, finely powdered ammonium sulfate was added to the crude extract at a final concentration of 70% saturation and stirred for 1 hour at 10°C. The mixture was left for 12 hours at 4°C, which was followed by centrifugation at 9000 rpm for 30 minutes. The precipitate was dissolved in phosphate buffer (pH 8.5). 5 ml of crude enzyme extract fraction was applied to a Silica gel 60-120 mesh column (2.5x25cm) that was pre-equilibrated with a 0.02 M phosphate buffer pH 8.0. The mass of the crude protein was determined by native gel electrophoresis on 10% acrylamide gel in tris-glycine (pH 8.4) using Bio-Rad Mini Protean 3 cell 8x 10 vertical gel electrophoresis system (Bio-Rad, USA).

Study Design

Study for anti-proliferative activity was conducted in a dose dependent manner. Dose dependent response of aq. extract of *A. cruentus*, crude L-asparaginase and commercially available L-asparaginase (Leunase, Kyoma Hakko Kogyo Co. Ltd. Japan) on human peripheral blood lymphocytes was assessed with concentrations ranging from 12 µg/ 10 µl, 18 µg/ 10 µl and 24 µg/ 10 µl; crude L-asparaginase 12µg/10 µl 18µg/ 10 µl and 24 µg/10 µl and commercially available L-asparaginase ranging from 12 µg/10 µl, 18 µg/10 µl and 24 µg/10 µl,keeping stimulated lymphocytes and the ones non treated as control.

Lymphocyte culture

Informed consent was obtained from a healthy adult volunteer. 6 ml of heparinized venous blood was collected and allowed to stand for 20 minutes. The buffy coat was picked up and cultured in RPMI 1640 media (pH 7.4), supplemented with 10% fetal bovine serum and 1% of phytohemagglutinin-M (Sigma), followed by the incubation of cells at 37° C (Thermo Electron Co.,

Waltham, MA, USA) in 5% CO_2 atmosphere with 95% relative humidity. After 48 hours, cultured lymphocytes were examined and viability checked using trypan blue dye exclusion test.

Anti-proliferative assay

The cytotoxicity of plant and microbial extracts was tested on lymphocytes cultured in 12 well plates. For experiment, lymphocytes were plated in 12 well plates. After 48 h, the aqueous extracts of *A. cruentus* and L-asparaginase at different concentration were added to respective wells and incubated for 1 hour. Control group cells received no treatment. Commercially available L-asparaginase served as positive control. Growth of lymphocytes was quantified by the ability of living cells to reduce the yellow dye 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) to a blue formazan product by addition of 1 mg/mL of MTT per well and incubated for 3h at 37°C. The formazan product of MTT reduction was dissolved in DMSO, and absorbance was measured using ELISA plate reader (Bio-Rad, USA). Drug effect was quantified as the percentage of control absorbance of reduced dye at 590 nm [26]. The % cell viability was calculated with the following formula: Cell viability % = Mean OD sample/ Mean OD of control x 100.

Results and Discussion:

The antiproliferative activity of two natural compounds of different origins at varying concentrations was evaluated on stimulated human peripheral blood lymphocytes, for their efficacy.

Screening and assessment of antiproliferative activity of L-Asparaginase

13 bacterial isolates were tested for L-asparaginase production of which two gave good results. Extracellularly, no appreciable levels of L-asparaginase were detected as also observed by Kumar *et al* [27] which led to the inference that the enzyme was secreted as an intracellular product. There are many reports on the production of intracellular L-asparaginase from *E. coli*, *E. aerogenes*, and *P. aeruginosa* [28-31]. *In our study, m*aximum L-asparaginase activity was assessed using modified method of Mashburn and Wriston [25] and was found to be 12.6 U/mL for *Psedomonas spp.* and 4.3 U/mL for *Enterobacter spp.* Using MTT assay, the *in vitro* cytotoxicity effect of *Psedomonas spp.* produced L-asparaginase crude enzyme on stimulated peripheral blood lymphocytes was studied. The incubation of human peripheral blood lymphocytes with gradual doses of *Psedomonas spp.* L-asparaginase enzyme lead to a dose dependent inhibition of cell proliferation when compared to proliferation of untreated control cells (Fig. 1).

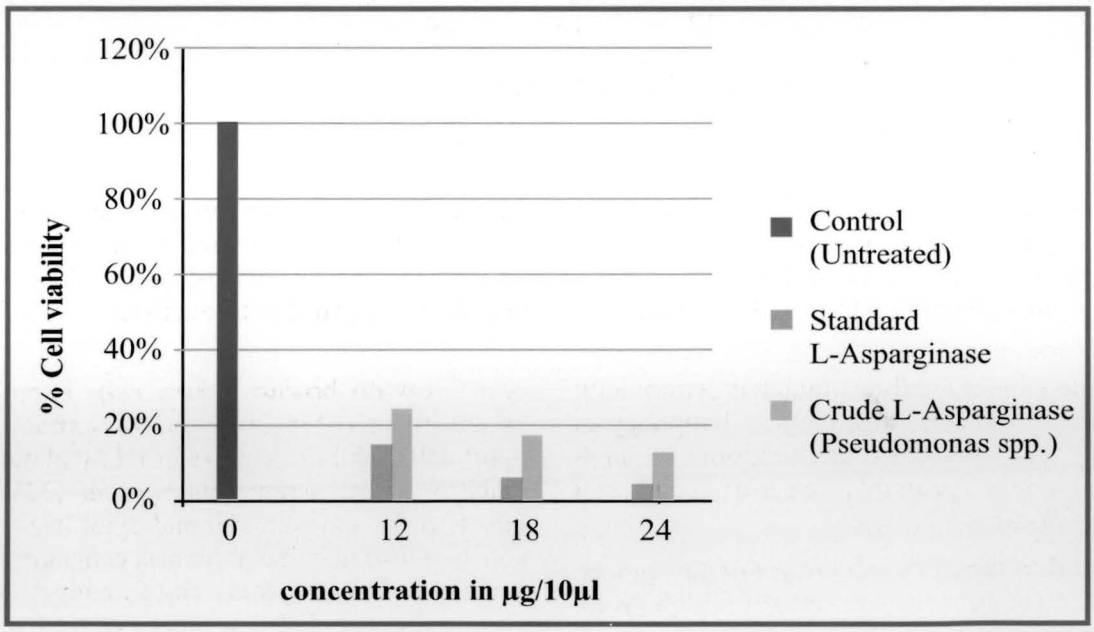

Fig. 1

Effect of commercially available and crude L-Asparaginase on viability of cultured lymphocytes .% cell viability of lymphocytes treated with crude L-asparaginase at concentrations of 12µg/10 µl, 18µg/10 µl and 24 µg/10 µl and exposure time of 60 min. 24 µg/10 µl concentration of crude L-asparaginase showed maximum antiproliferative activity.

Significant cytotoxic activity was recorded at all concentrations implicating that inhibition efficacy of L-asparaginase was dose and purity dependent. Commercially available L-asparaginase was used as positive control and results obtained presented a similar pattern. Moharam *et al* [16] studied the antitumor and antioxidant activities L-Asparaginase isolated from *Bacillus sp* R36. The enzyme inhibited the growth of two human cell lines hepatocellular carcinoma and colon carcinoma. Scotti *et al* [32] have reported using MTT assay that L-asparaginase isolated from *H. pylori* is a novel antigen that functions as a cell-cycle inhibitor of fibroblasts and gastric cell lines.

Screening and assessment of antiproliferative activity of crude extract of *A.cruentus*

The results of MTT assay indicated that all concentrations of the aqueous extract of *A. cruentus* inhibited the proliferation of stimulated lymphocytes. The efficacy of extract increased in a dose dependant manner and 24 µg/10µl of concentration showed marked cytotoxicity against cultured lymphocytes as shown in (Fig. 2).

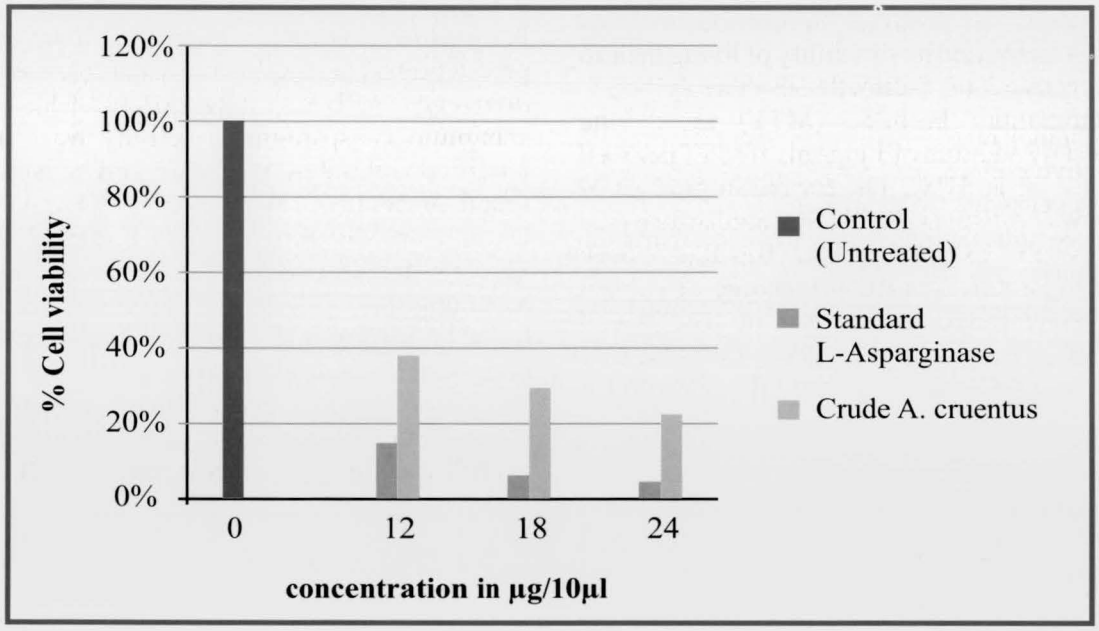

Fig. 2

Effect of commercially available L-asparaginaseand crude *A. cruentus* on cell viability of cultured lymphocytes. % cell viability of lymphocytes treated with crude extract of *A. cruentus* at concentrations of 12µg/10 µl, 18µg/ 10 µl and 24 µg/10 µl and exposure time of 60 min. 24 µg/10 µl concentration of crude extract of *A. cruentus* showed maximum antiproliferative activity.

A. cruentus crude extract exhibited targeted cytotoxicity against triggered human peripheral blood lymphocytes thus indicating that continued consumption in high concentrations could probably exhibit sustained antiproliferative efficacy.

Sani *et al* [33] studied the anti cancer effect of red spinach (*Amaranthus gangeticus* Linn) *in vitro* through MTT assay and found that aqueous extract of *A gangeticus* inhibited the proliferation of liver and breast cancer cell lines thus corroborating our results for the anti-proliferative activity of crude extract of leaves of *A. cruentus* as both belong to same genera.

Researchers have [34] also reported the cytotoxic effect of another species of *Amaranthus* i.e. *Amaranthus retroflexus* on bovine kidney cells exposed to various concentrations of the *A. retroflexus* extracts (100 ppm-0.1 ppm),cell viability being reduced by about 35% in MTT viability assay. Mpountoukas *et al* [35] evaluated the genotoxicity, cytostaticity and cytotoxicity of Amaranth and demonstrated that at highest concentration (8 mM), it showed potential cytotoxicity in human lymphocytes *in vitro* and concluded that plant extract bound directly to DNA and caused perturbation in nucleotide sequences.

Literature reports [36] indicate that *A. cruentus* contains lectins which possess anticancer property as they can bind to ribosomes and inhibit protein synthesis. They also modify the cell cycle by inducing non-apoptotic G1-phase accumulation mechanisms, G2/M phase cell cycle arrest and apoptosis, and can activate the caspase cascade.

Lectins can also downregulate telomerase activity and inhibit angiogenesis. *A.cruentus* is an excellent source of the pro-vitamin A carotenoid, β- carotene. The β-carotene is split into vitamin A molecules during absorption from the intestine. Carotenoids have important antiageing and anticancer properties, such lutein, which protects the tissues of the retina, while canthaxanthin has been demonstrated to inhibit cancer cell proliferation [37]. Pasko *et al* [13] reported that *A. cruentus* seeds and sprouts had antioxidant activity attributed to anthocyanins. Anthocyanins exhibit anti-proliferative activity towards multiple cancer cell types *in vitro* [38-43]. Cell proliferation was inhibited by the ability of anthocyanins to block various stages of the cell cycle via effects on cell cycle regulator proteins (e.g., p53, p21, p27, cyclin D1, cyclin A, etc.).

Studies on medicinal plants with high phenolic content and other bioactive components have gained importance over the past few years due to the high antioxidant activity, scavenging of free radicals; regulation of gene expression in cell proliferation, cell differentiation, oncogenes and tumor suppressor genes; induction of cell-cycle arrest and apoptosis; modulation of enzyme activities in detoxification, oxidation, and reduction, anti-inflammatory and anti-carcinogenic activities [44-48]. The plant *A. cruentus*, in addition to being a part of normal Indian diet, has been also been documented as non-toxic for regular consumption of upto 0.41 (0.84) servings/person/week [49]. However, percentage absorption of the active biologicals through the GI tract may not be high, as is the case with curcumin [50]. Accumulating evidence hence clearly indicates that apoptosis may be a critical molecular event that can be targeted by this dietary bioactive agent for the management of cancer.

Conclusion

Experimental studies have provided evidence of biological activity of *Amaranthus* extracts or active compounds including antioxidant, anti-inflammatory, free radical-scavenging actions. The current study adds to the growing literature database that *A. cruentus* too demonstrates anti-proliferative activity on human peripheral blood lymphocytes *in vitro* and presents easy consumability in stable form. Further research is needed to elucidate the *in vivo* activities of this plant extract in comparison to its commercially available chemotherapeutic counterpart; L-asparaginase, since all concentrations of the extract showed activity in potential range for further investigation on cancer cells lines and as a probable alternative to current chemotherapeutic agents used in the treatment of ALL and other tumor types like L-asparaginase which being a foreign protein, often generates anaphylactic response.

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