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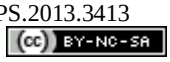
ISSN 2231-3354 

Modulatory effect of *Allium sativum*Ethanolic Extract on Cultured Human Lymphocytes

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ABSTRACT

Allium sativum (Garlic) have been known since from ancient years for its medicinal properties. It is widely used as antibacterial, antifungal, anticoagulant, anticancer, hypoglycaemicand hypocholesteromic. The aim of the present study was to assess the effect of different concentration ofethanolic extract of *Allium sativum* extract on cultured human lymphocytes. Cytotoxicity was assessed by tryphan blue dye exclusion assay, single strand DNA damage was studied by alkaline comet assay and apoptosis was assessed by DNA diffusion assay. The percentage of live and dead cells was counted in cell viability assay. In comet assay tail length, percentage tail DNA and olive tail movement were considered as parameters for DNA damage. In DNA diffusion assay number of apoptotic cells counted comparing the normal cell nucleus and apoptotic cell nucleus. The study was performed in 3 concentrations of *Allium sativum* extract, 10, 50 and 100µg/ml including untreated control group. The results showed that all the comet parameters was significantly ($p<0.05$) increased by the effect of *Allium sativum* extract, which was dose dependent. Percentage of apoptotic cells also increased with higher concentration of the garlic. These results conclude, the cytotoxicity induced by the garlic extract is directly proportional to the single strand DNA break. The increase in the DNA damage positively correlates to the number of apoptotic cells present in the culture medium.

INTRODUCTION

Allium sativum(Garlic) have been known since from ancient years for its medicinal properties (Banerjee *et al.*, 2002). It is widely used as antibacterial, antifungal (O'Gara *et al.*, 2000), anticoagulant (O'Gara *et al.*, 2000), anticancer, hypoglycaemic and hypocholesteromic (Bungu *et al.*, 2008). Garlic contain33 sulphur compounds, several enzymes, 17 amino acids, and minerals such as selenium (Newall*et al.*, 1996).

Since garlic is rich in many active compounds which possess the anticancer agents present in the garlic induces suppression of cell cycle proliferation, modification in DNA repair mechanism, upregulation of antioxidant defences (Sparreboom *et al.*, 2004). All herbal products carry the potential for contamination with other herbal products, pesticides, herbicides, heavy metals, and pharmaceuticals. Several acute and chronic toxicity has been reported. (Sumiyoshi *et al.*, 1984).

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However the cytotoxic effect of garlic on cultured lymphocytes has not been studied. So we have undertaken to study the cytotoxic and cytogenetic effect of different concentration of ethanolic extract of garlic on cultured lymphocytes.

MATERIALS AND METHODS

Preparation of garlic extract

The pods of *Allium sativum* were collected from the local market, Mangalore. 2 K.G bulbs were cleaned in tap water and in distilled water and outer coverings are removed and pod was peeled out chopped and shade dried for 15 days. The dried plant material was powdered subjected to soxhlet extraction with 99% ethanol for 72 hours. The mixture was evaporated to dry in a rotary flash evaporator and stored at 4^{o}C.

Lymphocyte isolation and culture

1 ml venous blood was collected from healthy, nonsmoking, non-alcoholic volunteer by venipuncture method. Peripheral blood mononuclear cells (PBMCs) were separated using

lymphocyte separation media (LSM-Himedia). 1 ml of fresh EDTA blood mixed with equal volume of phosphate buffered saline (PBS), which was overlaid on 1 ml of LSM. Lymphocytes were separated by density gradient centrifugation. Separated lymphocytes were washed twice with PBS and cells were suspended in minimum volume of RPMI-1640 (Himedia) and counted. 1 X 10^6 cells /ml cultured in RPMI medium supplemented with 6µg/ml Phytohemagglutinin and 15% Fetal Bovine Serum (FBS) at 37°C in a 5% CO2 incubator.

Experimental Design

The present study was divided into four groups.

Group-1: severed as control, Untreated.

Group-2: cultured human lymphocytes treated with 10 µg/ml garlic extract.

Group-3: cultured human lymphocytes treated with 50 µg/ml garlic extract.

Group-4: cultured lymphocytes treated with 100 µg/ml garlic extract.

Lymphocytes are treated and incubated for 2, 4 and 24 hour at 37°C supplemented with 5% CO_2. After the incubation period cells were subjected to tryphan blue dye exclusion assay, comet assay and DNA diffusion assay.

Cell viability assay

Cell viability was measured by trypan blue dye exclusion. Lymphocytes were mixed with equal volume of 0.4% Trypan Blue Dye for 3 minutes and cells were counted using haemocytometer. Viable and dead cells were scored under the microscope.

Alkaline comet assay

The alkaline comet assay was performed basically as described by Tice *et al.* 1991. Electrophoresis, which allowed for fragmented DNA migration was carried out for 20 min at 25 V and 300 mA. After electrophoresis, the slides were neutralized with 0.4 M Tris, pH 7.4, stained with 50µL of ethidium bromide (20 µg/mL) and analyzed with a fluorescence microscope (Olympus. 40x objective). The extent of DNA damage was assessed from the migration distance, which was derived by subtracting the nucleus from the total length of the comet. Fifty randomly selected cells were examined for each replicate, for each sample or subject. The quantification of the DNA strand breaks of the stored images was performed using Comet score software by which the percentage of DNA in the tail, tail length and OTM could be obtained directly.

DNA diffusion assay

To estimate the percentage of apoptotic cells, DNA diffusion assay was performed as described by Singh *et al.* 2000. The percentage of apoptosis measured by counting the apoptotic cells and normal cells. In brief,*slide preparation:* base layer 50µl of agarose smeared and air dried. 50µl of cell-agarose suspension was layered over the base layer and covered with coverslip. Slides were lysed by treating with alkaline lysing solution further slides were neutralized by treating with DNA precipitating solution for 30 minutes. This step repeated 2 times. Finally slides were stained with 20µg/ml ethidium bromide and slides were analyzed under florescent microscope (Olympus).

Statistical analysis

The results were expressed as mean ± standard deviation. Comparison between the control and treated groups were done by analysis of variance (ANOVA) followed by Tukey's test. The comparison between different time intervals was performed by One Way ANNOVA followed by Bonferroni test. In all these test criterion for statistical significance was $P<0.05$.

RESULTS AND DISCUSSION

Our present study suggests that at lower concentration (10µg/ml) there was no significant difference in cell viability, DNA damage and apoptosis when compared to the control (Table 1). But there was a significant difference in the percentage of cell viability, DNA damage and apoptosis at higher concentrations(50 and 100 µg/ml) when compared to control and 10 µg/ml concentration (Table1 and Table 2).

Table. 1: Determination of cytotoxicity by tryphan blue dye exclusion assay.

	2hr	4hr	24hr
Group1	97.00±2.0	96.70±2.0	98.04±4.0
Group 2	96.13±2.9	96.82±3.2	97.34±3.0
Group 3	84.81±1.6	80.33±4.9	73.01±3.5
Group 4	51.45±3.4	48.45±4.3	45.34±3.5

Results expressed in percentage of viable cells. Group 1: control, Group 2: lymphocytes treated 10µg/ml garlic extract, Group 3: lymphocytes treated with 50µg/ml garlic extract, Group 4: lymphocytes treated with 100µg/ml garlic extract.

Table. 2: Determination of apoptosis by DNA diffusion assay.

	2hr	4hr	24hr
Group 1	1.00±0.00	1.00±0.00	1.5±0.00
Group 2	3.22±0.90	3.46±0.45	5.53±0.07
Group 3	6.98±0.60	9.12±0.87	15.87±0.50
Group 4	22.45±1.40	25.00±1.92	34.35±3.50

Results expressed in percentage of apoptotic cells. Group 1: drug control, Group 2: lymphocytes treated 10µg/ml garlic extract, Group 3: lymphocytes treated with 50µg/ml garlic extract, Group 4: lymphocytes treated with 100µg/ml garlic extract.

At 100 µg/ml garlic extract treatment percentage of live cells was 51.45±3.4 and 45.34±3.5 at 2 and 24 hours respectively. Which was less than 50% when compare to control group and 10µg/ml concentration. So 100µg/ml garlic extract concentration inhibits significantly the normal lymphocyte multiplication (Table 1).

The result of the comet assay showed that when lymphocytes treated with 50 and 100µg/ml garlic extract will induce DNA migration (%DNA in tail) and OTM (Figure 1, 2, 3) significantly ($P<0.05$) when compare to control and 10 µg/ml and more in 100µg/ml at 24 hour duration.

Similar results were obtained in apoptotic DNA diffusion assay, the frequency of apoptotic cells significantly ($P<0.05$) increased according to the concentration of the garlic extract

increases. Cells treated with 100µg/ml showed 22.45±1.4 % at 2 post treatment and 34.35±3.5 % after 24 hour of garlic extract treatment (Table 2 and Figure 5).

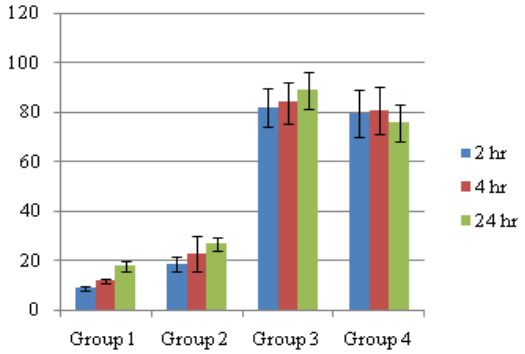

Fig. 1: Degree of DNA damage expressed in tail length in control group and treated groups.
Group 1: drug control, Group 2: lymphocytes treated 10µg/ml garlic extract, Group 3: lymphocytes treated with 50µg/ml garlic extract, Group 4: lymphocytes treated with 100µg/ml garlic extract.

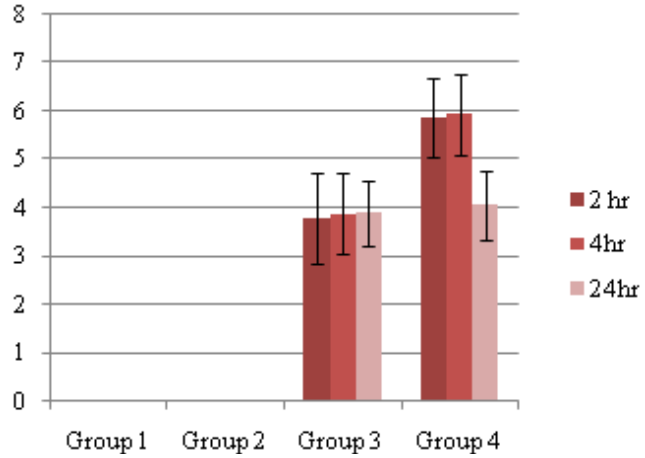

Fig. 2: Degree of DNA damage expressed in percentage tail DNA in control group and treated groups.
Group 1: drug control, Group 2: lymphocytes treated 10µg/ml garlic extract, Group 3: lymphocytes treated with 50µg/ml garlic extract, Group 4: lymphocytes treated with 100µg/ml garlic extract.

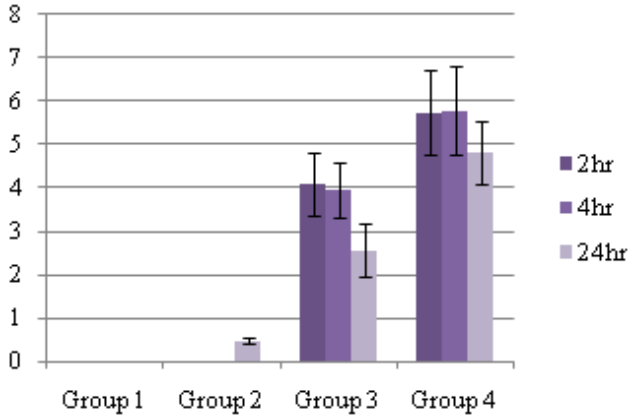

Fig. 3: Degree of DNA damage expressed in OTM in control group and treated groups. Group 1: drug control, Group 2: lymphocytes treated 10µg/ml garlic extract, Group 3: lymphocytes treated with 50µg/ml garlic extract, Group 4: lymphocytes treated with 100µg/ml garlic extract.

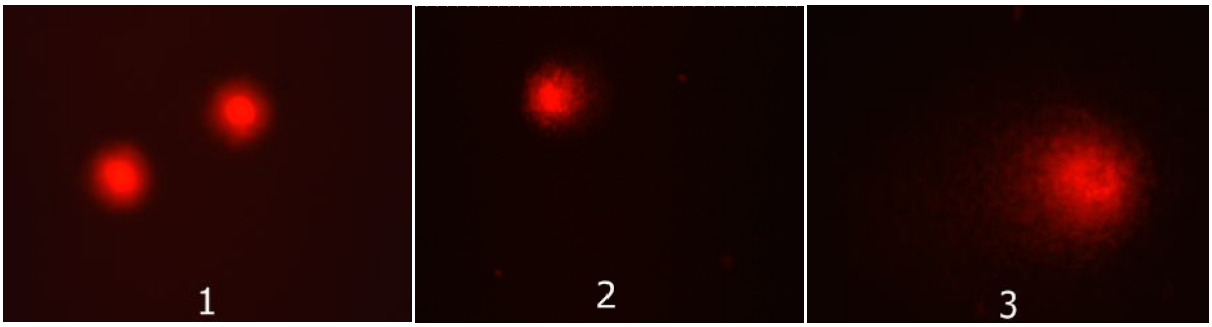

Fig. 4: Comet images 1: Control lymphocyte, 2: Slightly damaged lymphocytes treated with 50µg/ml garlic extract 3: Sever damaged lymphocytes treated with 100µg/ml garlic extract.

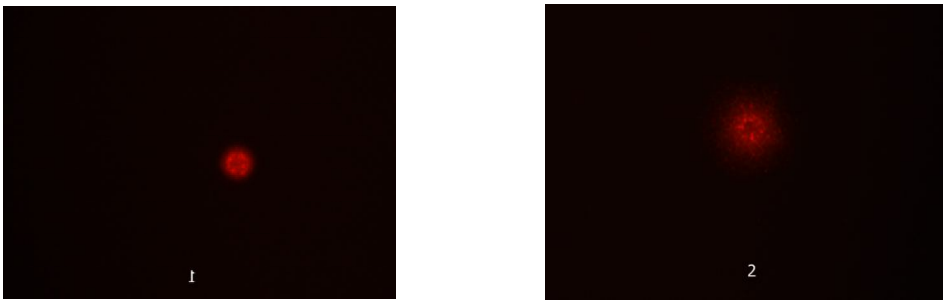

Fig. 5: DNA diffusion assay images 1: Non apoptotic lymphocyte, 2: apoptotic lymphocyte .

Garlic is a phytomedicine and widely used as antimicrobial, hypolipidimic, antioxidant, antithrobotic and anticancerous (Amagase *et al.*, 2001). The organosulfur compound present inthe garlic are responcible for the multiple effects (Boreck, 2001). Higher concentration of garlic extract has been shown to be clastogenic in mice which was appreciably reduced at lower concentration (Das *et al.,* 1996). Several adverse effects were reported in the litrature are anaemia, weight loss, failure to grow, increase in urea, D-aspartate amino transferase .the toxicity also reported with garlic power and garlic oil (Nakagawa *et al.,* 1980).

It is well known that garlic and its components possessed good anticancer property. In vitro cytotoxic effect of garlic extract and Diallyl trisulphide (DTS) on human gastric cancer cell lines. It has also been proved that, DTS is three times more potent than mytomycin C in killing cancer cells (Pan *et al.,* 1985*).* However there is no report on normal tissue toxicity of garlic extracts. In mouse macrophages, an aqueous garlic extract and a protein fraction isolated from the extract demonstrated significant dose-related augmentation of oxidativeburst and enhanced T nlymphocyteblastogenesis (Lau *et al.,* 1991).Alliin significantly increased pokeweed mitogen-induced peripheral bloodmononuclear cell (PBMC) proliferation, increased IL-1-beta and TNF-alpha production,and enhanced the engulfing capacity of phagocyting cells; Con-A induced cell proliferation and IL-6 production decreased following incubation with alliin, whereas PHA-induced cell proliferation, IL-2 and superoxide anion generation remainedUnchanged (Salman *et al.,* 1999).

Aged garlic extract significantly enhanced the cytotoxicity of human peripheralblood lymphocytes (PBL) against both natural-killer (NK)-sensitive K562 and NKresistantnM14 cell lines. This effect was enhanced synergistically by concurrent treatmentwith interleukin 2 (IL-2), suggesting that garlic extracts serve as efficient immunostimulant (Morioka *et al.,* 1993). Some of the acute toxic effects reported in the literature were heartburn, nausea, vomiting, diarrhea, flatulence, bloating, mild orthostatic hypotension, flushing, tachycardia, headache, insomnia, sweating and dizziness as well as offensive body odour (Holzgartner *et al*., 1992). Prolonged topical use (garlic compresses left in place for six hours or more) hasalso led to irritant burns(Farrell *et al.,* 1996). Rats fed up to 2 grams/kg of aged garlic extract for five out of seven days every week for six months demonstrated no serious toxicity (Sumiyoshi H *et al*., 1984).

Intraperitoneal and oral administration of high doses (5 mL/kg of pure garlic juice) led to weight loss, hepatic and pulmonary toxicity in rats (Alnaqeeb *et al*., 1996). However, in one study,hypertensive rats given garlic supplements four times daily developed erratic pulses,dehydration, weight loss and lethargy (Ruffin *et al.,* 1983). There is a case report of platelet dysfunction in an 87-year-old patient whochronically took 2 grams daily of garlic cloves (McGuffin *et al*., 1997). However the cytotoxic effect of garlic on cultured lymphocytes has not been reported so far.Finding of our study suggests that fresh garlic extract has cytotoxic effect at higher concentration which may be due to presence of several active cytotoxic agents.

CONCLUSION

The present study concludes that, garlic is cytotoxic at higher concentration on normal cultured lymphocytes.The toxicity induced by the garlic extract is directly proportional to single stranded DNA break. Increase in the DNA damage positively correlates to the number of apoptotic cells present in the culture medium. To the best of our knowledge, it is first of its kind of study so it needs to be evaluated further.

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