

## Antiproliferative effect of diallyl disulfide (DADS) on prostate cancer cell line LNCaP

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Garlic has been used throughout the world to treat coughs, toothache, earache, dandruff, hypertension, hysteria, diarrhoea, dysentery, diphtheria, vaginitis and many other conditions. Garlic contains a complex mixture of oil and water-soluble organosulfur compounds. Diallyl disulfide (DADS), an oil-soluble constituent of garlic seems to be effective in reducing tumour cells originating from colon, lung and skin. Hence our present study focuses on the dose-dependent effect of DADS on an androgen-dependent prostate cancer cell line. Various concentrations of DADS ranging from 25 to 100  $\mu\text{M}$  were given to LNCaP cells and the activity of lactate dehydrogenase (LDH) prostatic acid phosphatase (PacP) and the level of prostate specific antigen were studied. DADS reduced the secretory activity of LNCaP cells with the gradual increase in dosage. DADS was found to act as a good antiproliferative agent, which was confirmed by proliferation assay. DADS also induced apoptosis and nuclear segmentation in the higher doses. Copyright © 2005 John Wiley & Sons, Ltd.

KEY WORDS — apoptosis; diallyl disulfide; DNA fragmentation; garlic; prostate cancer cells; prostate-specific antigen

### INTRODUCTION

Prostate cancer has become one of the most common causes of death in males. In western countries it is the second leading cause of male deaths. The rate of prostate cancer growth is increasing by 1% per year in Asian countries.<sup>1</sup> Approximately 80–90% of prostate cancer is dependent on androgen in the initial stages. Endocrine therapy of prostate cancer is directed towards the reduction of serum androgens and inhibition of the androgen receptor.<sup>2</sup> At a certain stage androgen ablation therapy ultimately fails and prostate cancer progresses to a hormone refractory state. Radiation or chemotherapy both have potentially distressing side-effects including the possibility of impotence, incontinence or both. Despite improvements in therapy, mortality from prostate cancer has also increased. Therefore, there is an urgent need to

explore new therapies for managing this disease. There is some evidence that supplementation of diets with allium vegetables including garlic, onions, leeks, chives, scallions and shallots rich in flavonols and organosulfur compounds have tumour-inhibitory properties.<sup>3,4</sup> Garlic (*Allium sativum*) is a common plant used mainly as a food item and is deemed as a medicinal herb in many different cultures of the world. In general, garlic has been used throughout the world to treat coughs, toothache, earache, dandruff, hypertension, hysteria, diarrhoea, disentry, diphtheria, vaginitis and many other conditions.<sup>5–7</sup> Garlic contains a complex mixture of oil and water-soluble organosulfur compounds.<sup>7</sup> In *in vitro* experiments, oil-soluble compounds such as diallyl disulfide (DADS), diallyl sulfide (DAS) and diallyl trisulfide (DATS) are effective as antiproliferative agents against CMT-13 canine mammary tumour cells, while the water-soluble compounds S-allyl cysteine, S-ethyl cysteine and S-propyl cysteine have little or no inhibitory effect.<sup>8</sup>

Essential oils of garlic contain approximately 60% DADS.<sup>9</sup> Among the garlic constituents, DADS seems to be the most effective at reducing tumour cells

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originating from colon, lung and skin.<sup>10</sup> Knowles and Milner have reported that DADS was found to have an antiproliferative effect on human colon tumour cells.<sup>11</sup> There was found to be transient increases in cells in the G<sub>2</sub> to M phase of the cell cycle and a decrease in p34<sup>cdc2</sup> kinase activity. It has been reported that DADS induced apoptosis in human breast cancer cell line via the activation of caspase-3.<sup>12</sup> In our present investigation, we explored the antiproliferative impact of DADS on the androgen-dependent prostate cancer cell line LNCaP.

## MATERIALS AND METHODS

### *Cell culture*

The prostatic carcinoma cell line LNCaP was obtained from the National Centre for Cell Science (NCCS), Pune. The cells were grown in culture flasks containing RPMI 1640 medium supplemented with 15% FBS under 5% CO<sub>2</sub>, 95% O<sub>2</sub> at 37°C. Upon reaching confluence, the cells were trypsinized and about 1 × 10<sup>5</sup> cells were plated per well in 24-well plates and were incubated for 12 h for attachment. After 24 and 48 h of DADS treatments, the media were collected and the cells were lysed using 0.1% Triton X-100. The conditioned media and the cell lysates were then used for assay of the following parameters.

### *Assay of prostate specific antigen (PSA)*

The Can Ag PSA EIA kit is intended for the quantitative determination of total PSA (Prostate-specific antigen). This kit was obtained from Can Ag diagnostics, AB Majnabbe Terminal, SE 41455, Gothenburg, Sweden. PSA was quantified by the method of Nilson *et al.*<sup>13</sup> The Can Ag PSA EIA is a solid-phase, non-competitive immunoassay based upon the direct sandwich technique. Calibrators, controls and samples were incubated together with biotinylated anti-PSA monoclonal antibody and horseradish peroxidase (HRP) labelled anti-PSA monoclonal antibody in streptavidin-coated microtiter stripes. After washing, buffered substrate (hydrogen peroxide) was added and the enzyme reaction was allowed to proceed. Then chromogen reagent (3,3',5,5' methyl benzidine) was added to each well. The colour intensity was determined in the microtiter plate spectrophotometer at 620 nm and also optionally at 450 nm after addition of stop solution. Calibration curves were constructed for each assay by plotting absorbance versus the concentration of each calibrator. The PSA concentration of samples was then read from the calibration curve.

### *DNA agarose gel electrophoresis*

Agarose gel electrophoresis was performed by the method of Chaudary *et al.*<sup>14</sup> After the DADS treatment for 24 and 48 h, the cells were washed with PBS. The cells were centrifuged at 1000 g and resuspended in lysis buffer (0.1 M Tris +0.02 M EDTA +0.8% SDS). To this phenol:chloroform:isoamyl alcohol (25:24:1) was added, mixed and centrifuged at 9500 × g for 15 min to separate the DNA-containing upper aqueous phase. This phenol–chloroform extraction was repeated twice, finally the pellet was washed with 70% ethanol.

The pellet was allowed to dry at room temperature for approximately 30 min and resuspended in 50 µl of Tris-EDTA (TE) buffer. The DNA was quantified by UV–visible spectroscopy and 10 µg of DNA was electrophoresed in a 1.2% agarose gel containing ethidium bromide in a mini gel tank containing Tris boric acid EDTA (TBE) buffer for 2 h under 90 V. Then the gel was examined under the HERO LAB (Germany) gel documentation system.

### *Ethidium bromide/acridine orange (EB/AO) staining*

Ethidium bromide/acridine orange (EB/AO) Staining was carried out by the method of Spector *et al.*<sup>15</sup>

Twenty-five µl of cell suspension (0.5–2.0 × 10<sup>6</sup> ml<sup>-1</sup>) were incubated with 1 µl of AO/EB solution (1 part of 100 µg ml<sup>-1</sup> AO in PBS; 1 part 100 µg ml<sup>-1</sup> EB in PBS), mixed gently and each sample was evaluated immediately. Of the cell suspension 10 µl was placed onto a microscope using a fluorescein filter of 60 × objective and was viewed.

### *Proliferation assay*

Cell proliferation was assessed by the Crystal violet method.<sup>16</sup> Cells (5 × 10<sup>4</sup>) were plated in 24-well multi-well plates with RPMI medium containing 10% FBS. The cells were incubated for 12 h under 5% CO<sub>2</sub>, 95% O<sub>2</sub> at 37°C. The control plates received 0.01% DMSO containing medium and treatment plates received 25, 50, 75 and 100 µM concentrations of DADS-containing medium. After 24 and 48 h the cells in the monolayer were washed twice with PBS. Then 0.1% crystal violet in PBS was added to the plates. After 10 min the excess dye was removed by washing thrice with distilled water and the cells were air dried for 20 h.

The incorporated dye was solubilized in 0.1 M sodium citrate solution in 50% ethanol. In order to determine cell number in each sample, the absorbance was measured at 550 nm in an Ultraspec 4000 spectrophotometer.

### Lactate dehydrogenase (LDH) assay

Lactate dehydrogenase activity was measured in both cell lysate and in the conditioned medium. After 24 and 48 h incubation the cultured medium was taken separately and the attached cells were lysed by adding 0.1% Triton X-100 and subjecting them to two cycles of freezing and thawing. The activity of LDH was measured by the method of King.<sup>17</sup> Briefly, the substrate reaction buffer (0.5 mM lactic acid +0.1 N NaOH +0.1 M glycine buffer) was added to the cell lysate and medium. Dinitro-phenylhydrazine (0.02%) was added as chromogenic agent and the absorbance values at 460 nm were read in an Ultraspec 4000 spectrophotometer.

### Prostatic acid phosphatase (PACp) assay

Prostatic acid phosphatase (PACp) activity was measured in cell lysates. After 24 and 48 h incubation, the cultured medium was taken separately and the attached cells were lysed by adding 0.1% Triton X-100 and subjecting them to two cycles of freezing and thawing. The activity of PACp was measured by the method of Tenniswood *et al.*<sup>18</sup> Briefly, the substrate reaction buffer (0.5 ml of 0.1 M citrate buffer (pH 4.85) +0.5 ml of 0.4% p-nitrophenyl phosphate substrate) was added to the 200  $\mu$ l of cell lysate. The reaction was arrested by the addition of 3.8 ml of 0.1 N NaOH. Nitrophenol released was measured at 410 nm in an Ultraspec 4000 spectrophotometer.

### Statistical analysis

*One-way analysis of variance (ANOVA).* The data were subjected to statistical analysis using one-way analysis of variance (ANOVA) as adopted by Zar.<sup>19</sup>

## RESULTS

Figure 1 represents the effect of DADS on the crystal violet staining for the cell proliferation assay in the LNCaP cell line for both 24 and 48 h. There was a significant decrease in the cell number at 50, 75 and 100  $\mu$ M concentrations of DADS for the 24-h treatment. With 48 h treatment, a significant decrease in cell number was observed in all of the concentrations, ranging from 25 to 100  $\mu$ M, of DADS-treated cells.

The data obtained for the effect of DADS on the activity of PACp in cell lysates for both 24 and 48 h treatments in the LNCaP cell line is shown in Figure 2. A significant decrease in the activity of PACp was observed at 48 h in cells treated with DADS at concentrations ranging from 25 to 100  $\mu$ M. For 24 h

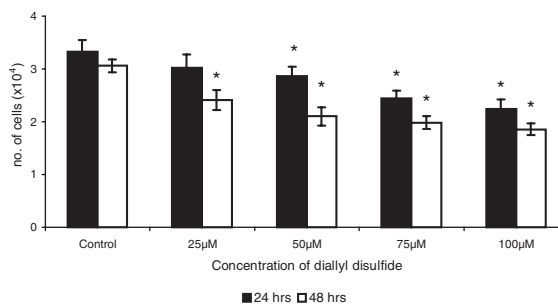


Figure 1. Effect of diallyl disulfide on cell proliferation. Each bar represents the mean value and the vertical line above denotes  $\pm$  SEM of five observations; \*significance at  $p < 0.05$

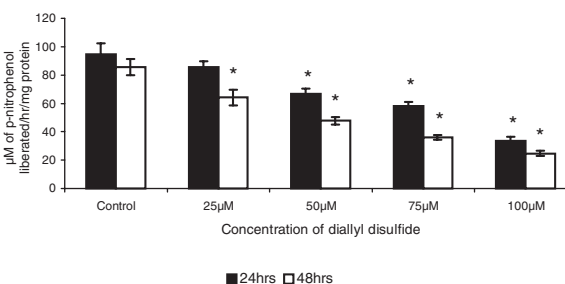


Figure 2. Effect of diallyl disulfide on the activity of prostatic acid phosphatase. Each bar represents the mean value and the vertical line above denotes  $\pm$  SEM of five observations; \*significance at  $p < 0.05$

of DADS treatment, a significant decrease in the activity of PACp was observed at 50, 75 and 100  $\mu$ M concentrations of DADS.

Figures 3 and 4 illustrate the activity of total LDH in cell lysates as well as in conditioned media of the LNCaP cell line for both 24 and 48 h treatments. At 50, 75, 100  $\mu$ M concentrations, there was a significant decrease in the activity of LDH in cell lysates for both

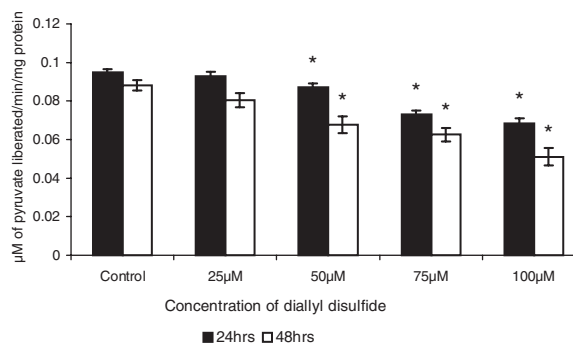


Figure 3. Effect of diallyl disulfide on the activity of lactate dehydrogenase in cell lysate. Each bar represents the mean value and the vertical line above denotes  $\pm$  SEM of five observations; \*significance at  $p < 0.05$

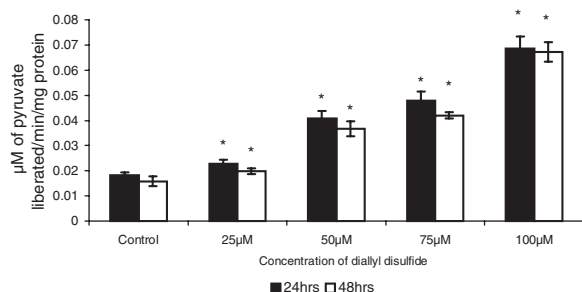


Figure 4. Effect of diallyl disulfide on lactate dehydrogenase specific activity in the conditioned medium. Each bar represents the mean value and the vertical line above denotes  $\pm$  SEM of five observations; \*significance at  $p < 0.05$

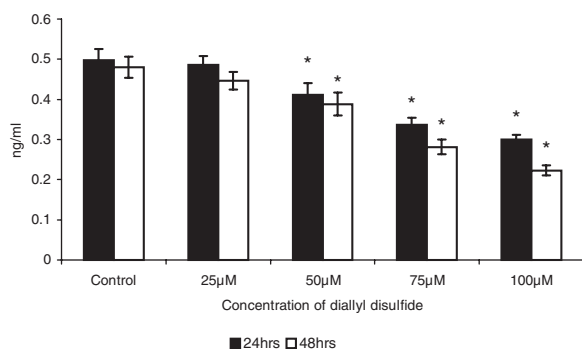


Figure 5. Effect of diallyl disulfide on the level of prostate-specific antigen. Each bar represents the mean value and the vertical line above denotes  $\pm$  SEM of five observations; \*significance at  $p < 0.05$

24 and 48 h treatments. However, in conditioned media the LDH activity was increased at all the concentrations of DADS for both 24 and 48 h treated cells.

Figure 5 depicts the effect of diallyl disulfide on the level of prostate-specific antigen in the LNCaP cell line. At 50, 75 and 100  $\mu$ M concentrations of DADS, there was a significant decrease in the PSA level when compared to the control for both 24 and 48 h treatments.

Table 1. Effect of diallyl disulfide in induction of apoptosis in LNCaP cells

	Control	Concentration of DADS			
		25 $\mu$ M	50 $\mu$ M	75 $\mu$ M	100 $\mu$ M
Viable cells	93 $\pm$ 7	85 $\pm$ 6*	78 $\pm$ 3*	68 $\pm$ 5*	62 $\pm$ 4*
Apoptotic cells	8 $\pm$ 0.6	12 $\pm$ 0.8*	19 $\pm$ 0.6*	30 $\pm$ 0.7*	36 $\pm$ 0.5*

Each value represents mean  $\pm$  SEM of five independent observations of 100 cells in 24 h of diallyl disulfide treatment.

\* $p < 0.05$  control versus treated cells.

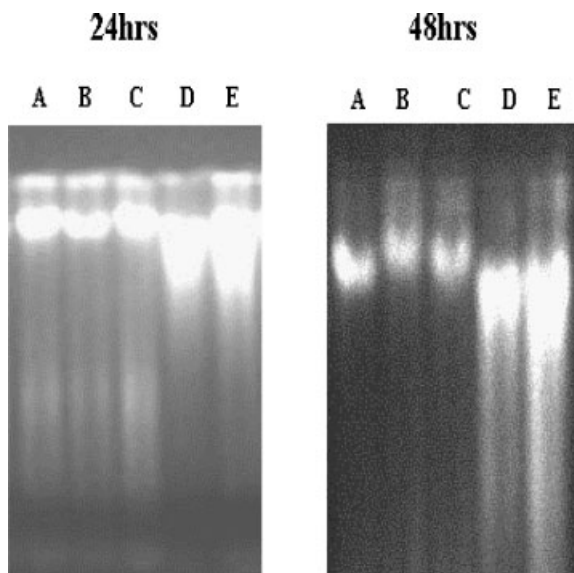


Figure 6. Agarose gel electrophoretic pattern of DNA of LNCaP cells treated with diallyl disulfide. Lane A, control; B, 25  $\mu$ M; C, 50  $\mu$ M; D, 75  $\mu$ M; E, 100  $\mu$ M diallyl disulfide for 24 h and 48 h treatments, respectively

Figure 6 shows agarose gel electrophoretic pattern of LNCaP cells treated with DADS.

Apoptotic cells often produce nuclear fragments that can be observed by DNA agarose gel electrophoresis. After 24 and 48 h treatment 75 and 100  $\mu$ M doses caused DNA damage in LNCaP cells.

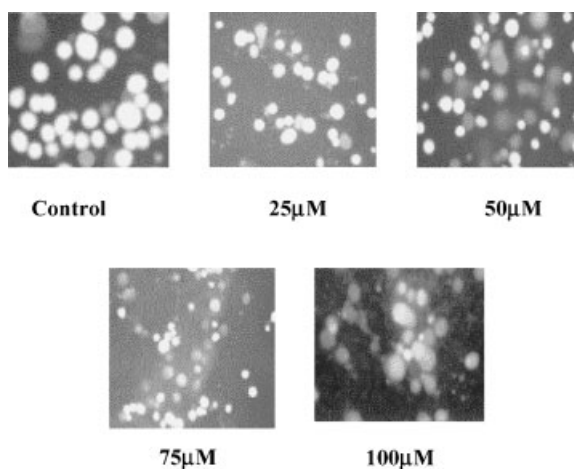


Figure 7. Ethidium bromide and acridine orange staining of LNCaP cells treated with different concentrations of diallyl disulfide for 24 h. The viable cells are shown as bright light colour, early apoptotic cells as grey colour and late apoptotic cells are shown as dark colour

Table 1 and Figure 7 show the ethidium bromide/acridine orange staining for the conformation of apoptosis. After 24 h of DADS treatment, as the concentration of DADS increased from 25 to 100  $\mu\text{M}$ , the number of dead cells also increased. Table 1 gives the number of viable and apoptotic cells for different concentration of DADS. The number of viable cells was found to decrease significantly whereas there was a significant increase in the number of apoptotic cells.

## DISCUSSION

In the present study, we have investigated the effects of DADS on cell proliferation and apoptosis. DADS was found to decrease the cell number with increasing concentrations ranging from 25 to 100  $\mu\text{M}$  after both 24 and 48 h of treatment. It is suggested that, DADS increases the p21<sup>waf1/cip1</sup> protein which is involved in cell cycle arrest in G<sub>1</sub> or G<sub>2</sub> phases in both CaCo-2 and HT-29 cells.<sup>20</sup> The DADS effect in increase of p21<sup>waf1/cip1</sup> is due to lysine 14 acetylation of histone H3, in these two cell lines. DADS induces histone hyperacetylation through inhibiting histone deacetylases (HDAC) activity.<sup>20</sup> This mechanism could be involved in the reduction of cell number in the LNCaP cell line. In the present study, we observed that DADS induced apoptosis, which was confirmed by the DNA damage caused by 75 and 100  $\mu\text{M}$  concentrations of DADS for both 24 and 48 h treatments. Apoptosis is a cell death program originally characterized by specific morphological and biochemical modifications in higher eukaryotic cells. These structural changes such as plasma and nuclear membrane blebblings, chromatin condensation, protease activation and DNA fragmentation are considered to be landmarks of the apoptotic process.<sup>21,22</sup> The mechanism by which DADS causes growth inhibition remains unclear while previous studies suggest that DADS is an effective inhibitor of both ER-positive and ER-negative human breast cancer cells. The growth inhibitory properties of DADS were attributed to the upregulation of the apoptotic protein Bax and the downregulation of antiapoptotic Bcl-X<sub>L</sub>. DADS also induces apoptosis via activation of caspase-3.<sup>12</sup> In work undertaken by Sundaram and Milner, it was shown that DADS caused a sustained and dose-dependent increase in intracellular Ca<sup>2+</sup> in HCT-15 cells.<sup>10</sup> Excessive intracellular calcium is frequently associated with the activation of Ca<sup>2+</sup>-dependent endonucleases. Activation of these enzymes is known to induce apoptosis in several *in vitro* models.<sup>23–26</sup>

In the present investigation, it was observed that DADS significantly increased the leakage of LDH

assayed in both 24 and 48 h treatments. LDH leakage monitors the integrity and permeability of the plasma membrane and is sensitive and easy to perform.<sup>27</sup> It seems that DADS increases the permeability of the plasma membrane and hence there is a leakage of LDH from the cells into the medium and as a result an increase in the activity of LDH was observed in conditioned media and decreased LDH activity in the cell lysate. These results suggest that DADS is cytotoxic to the LNCaP cell line. However in primary rat hepatocytes, DADS in 0.5 to 1 mM concentration, increased the cell viability.<sup>28</sup> Human prostate acid phosphatase (PACp) and prostate-specific antigen (PSA) represent an androgen-responsive enzyme and protein respectively, and are used as markers for diagnosing prostate cancer. In our present work it has been observed that DADS significantly reduced the level of PACp and PSA in cell lysate and in conditioned media, respectively. This effect could be due to the reduction in the cell number. To conclude, DADS significantly inhibited the growth of the prostate cancer cells *in vitro* possibly by either upregulation of apoptotic Bax and downregulation of antiapoptotic Bcl-X<sub>L</sub> or by increasing the p21<sup>waf1/cip1</sup> protein.

## REFERENCES

- Parkin DM, Whelan SL, Ferlay J, Raymond L, Young J. *Cancer Incidence in Five Continents*, vol. VII. IARC Scientific Publications No. 143, Lyon, 1997.
- Denis LJ, Griffiths K. Endocrine treatment in prostate cancer. *Sem Surg Oncol* 2000; **18**: 52–74.
- Fleischauer AT, Poole C, Arab L. Garlic consumption and cancer prevention: Meta analyses of colorectal and stomach cancers. *Am J Clin Nutr* 2000; **72**: 1047–1052.
- Ali M, Thomson M, Afzal M. Garlic and onion: their effect on eicosanoid metabolism and its clinical relevance. *Prostaglandins Leukot Essent Fatty acids* 2000; **62**: 55–73.
- Raj KP, Parmar RM. Garlic-condiment and medicine. *Indian Drugs* 1977; **15**: 205–210.
- Leung A. *Encyclopedia of Common Natural Ingredients Used in Food, Drugs and Cosmetics*. John Wiley & Sons: New York, 1980; 176–178.
- Block E. The chemistry of garlic and onions. *Sci Am* 1985; **252**: 114–119.
- Sundaram SG, Milner JA. Impact of organosulfur compounds in garlic on canine mammary tumor cells in culture. *Cancer Lett* 1993; **74**: 85–90.
- Dausch JG, Nixon DW. Garlic: a review of its relationship to malignant disease. *Prev Med* 1990; **19**: 346–361.
- Sundaram SG, Milner JA. Diallyldisulfide suppresses the growth of human colon tumor cell xenografts in athymic nude mice. *J Nutr* 1996; **126**: 1355–1361.
- Knowles LM, Milner JA. Depressed p34<sup>Cdc2</sup> kinase activity and G<sub>2</sub>/M phase arrest induced by diallyl disulfide in HCT-15 cells. *Nutr Cancer* 1998; **30**: 169–174.
- Nakagawa H, Tsuta K, Kiuchi K, *et al.* Growth inhibitory effects of diallyl disulfide on human breast cancer cell lines. *Carcinogenesis* 2001; **22**: 891–897.

13. Nilson O, Peter A, Andersson I, Nilsson K, Grundstrom B, Karlsson B. Antigenic determinants of prostate specific antigen (PSA) and development of assays specific for different forms of PSA. *Br J Cancer* 1997; **75**: 789–797.
14. Chaudhary KS, Abel PD, Stamp GWH, Lalani E. Differential expression of cell death regulators in response to thapsigargin and adriamycin in Bcl-2 transfected DU 145 prostatic cancer cells. *J Pathol* 2001; **193**: 522–529.
15. Spector DL, Goldman RD, Leinwand LA (1998). Cell culture analysis; Apoptosis analysis. In *Cells a Laboratory Manual*. Cold Spring Harbour Laboratory Press: Cold Spring Harbor, New York, USA, 2001; 15.6–15.7.
16. Wosikowski K, Hasmann M, Loser R, Eppenberger U. Inhibition of growth factor antiproliferation by anti-estrogens and effects on early gene expression of Mef.7 cells. *Int J Cancer* 1993; **53**: 290–297.
17. King J. Lactate dehydrogenase. In *Practical Clinical Enzymology*, King J (ed.). D. Van Norstrand Co.: London, 1965; 83–93.
18. Tenniswood M, Bird CE, Clark AF. Acid phosphatases: androgen dependent markers of rat prostate. *Can J Biochem* 1976; **54**: 350–357.
19. Zar JH. In *Biostatistical Analysis*. Zar JH (ed.). New Jersey. Prentice Hall Inc.: Englewood Cliffs, NJ, USA, 1974; 151–181.
20. Drusene N, Pagnicz A, Mayeur C, et al. Diallyl disulfide (DADS) increases histone acetylation and p21<sup>waf1/cip1</sup> expression in human colon tumor cell lines. *Carcinogenesis* 2004; **25**: 1227–1236.
21. Jacobson MD, Weil M, Raff MC. Programmed cell death in animal development. *Cell* 1997; **88**: 347–354.
22. Fadeel B, Orrenius S, Zhivotovsky B. Apoptosis in human disease: a new skin for the old ceremony? *Biochim Biophys Res Commun* 1999; **266**: 699–717.
23. Joseph R, Li W, Han E. Neuronal death, cytoplasmic calcium and internucleosomal DNA fragmentation evidence for DNA fragment being released from cells. *Mol Brain Res* 1993; **17**: 70–76.
24. Matsubara K, Kubota M, Adacht S, et al. Different mode of cell death induced by calcium ionophore in human leukemia cell lines: possible role of constitutive endonuclease. *Exp Cell Res* 1994; **210**: 19–25.
25. Ruruya Y, Lundmo P, Short AD, Gill DL, Isaacs JT. The role of calcium pH and cell proliferation in the programmed apoptotic death of androgen dependent prostatic cancer cells induced by thapsigargin. *Cancer Res* 1994; **54**: 6167–6175.
26. Sinha BK, Yamazaki H, Eliot HM, Schneider E, Borner MM, O'Connor PM. Relationships between proto-oncogene expression and apoptosis induced by anticancer drugs in human prostate tumor cell. *Biochim Biophys Acta* 1995; **1270**: 12–18.
27. Moldeus P, Hogberg J, Orrenius S. Isolation and use of liver cells. In *Methods in Enzymology* (vol. 52), Fleischer S, Packer L (eds). Academic Press: New York, 1978; 60–71.
28. Sheen LY, Wu CC, Tasi SJ. Effect of diallyl sulfide and diallyl disulfide, The active principles of garlic on the aflatoxin B<sub>1</sub> induced DNA damage in primary rat hepatocytes. *Toxicol Lett* 2001; **122**: 45–52.