



International Journal of Pharmacology

ISSN 1811-7775

science
alert

ansinet
Asian Network for Scientific Information

Methanolic Extract of *Nigella sativa* Seeds is Potent Clonogenic Inhibitor of PC3 Cells

¹Gowhar Shafi, ²Tarique Noorul Hasan and ³Naveed Ahmed Syed

¹Indo-American Cancer Institute and Research Centre, Road No. 14-Banjara Hills, Hyderabad, AP, India

²Department of Food Science and Nutrition, College of Food Science and Agriculture,
Post Box No. 2460, King Saud University, Riyadh-11421, Kingdom of Saudi Arabia

³Department of Biochemistry, Islamiah College, Vaniyambadi-635 752, TN, India

Abstract: We tried to find out the cytotoxic and anticancer property of methanolic extract of *N. sativa* seeds against prostate cancer cells (PC3). A concentration range of extract (100-0.01 $\mu\text{g mL}^{-1}$) in culture medium (DMEM) was tested. Inhibition of colony formation was studied and microscopic observations of stained colonies were performed. Colony inhibition at highest concentration (100 $\mu\text{g mL}^{-1}$) was 87.097% and at lowest concentration (0.01 $\mu\text{g mL}^{-1}$) it was 54.105. IC_{50} was $<0.01 \mu\text{g mL}^{-1}$. Microscopic observation confirmed karyolysis, karyorrhexis and picnosis in treated cells as well as presence of characteristic morphological changes associated with apoptotic cells like blebs, blisters and echinoid spikes etc. on cell membrane. These findings advocate that methanolic extract of *N. sativa* seeds is a potent clonogenic inhibitor of PC3 cell and cell deaths might have taken place through apoptosis. However exact mechanism for apoptosis can not find out from this study but study may help in searching new effective medicine for prostate cancer therapy.

Key words: Prostate cancer, *Nigella sativa* seed, methanolic extract, clonogenic inhibition assay, cytochemical detection of apoptosis

INTRODUCTION

Prostate cancer is the most frequently diagnosed cancer among men in the US, with an estimated 218890 new cases and 27,050 deaths in year 2007 (Jemal *et al.*, 2007). According to a WHO report, 36% of prostate cancer patients world wide in the year 2000 belonged to the US population (Wilkinson *et al.*, 2002). The incidence of prostate cancer has been increasing rapidly in recent years (Greenlee *et al.*, 2000). With increase in longevity it is also going to be a problem even in India (Rao *et al.*, 2004). The precise causes of cancers in general and prostate cancer in particular are not yet known. In the recent years, due to the development of the Prostate-Specific Antigen (PSA) test and awareness among the general population about the disease, prostate cancer diagnosis has become easier. Despite an extensive effort, the underlying mechanisms involved in the onset of prostate cancer and its progression are not well established. Nowadays for the treatment of prostate cancer in its early stages, available therapies are prostatectomy, chemotherapy, radiotherapy, hormone therapy and watchful waiting. Most efficient therapies normally, offered to men with a life expectancy of at least

10 years (Johansson *et al.*, 1997). From clinical trials it is reported that, adjuvant therapy such as androgen deprivation has been found to be able to improve survival when given during and for 3 years after, radiotherapy (Bolla *et al.*, 1997, 2002) and there are evidences that in case of prostate cancer patients with an intermediate or poor prognosis, hormone therapy results in delayed progression of disease (de Koning *et al.*, 2002; See *et al.*, 2002). However, till now there is no completely effective therapy for prostate cancer. Since the allopathic medical system to care for cancer becomes more complex, technical and expensive as well as fail to find a reliable and definitive cure for cancer. This fact leads researchers to focus into the traditional medicine system and herbal formulation to find out an effective cure of prostate cancer.

N. sativa (NS) is an annual herb belongs from family Ranunculaceae. NS is natural food additive in India and rest of the Asia. It has used for centuries in the Middle East, Northern Africa, Far East and Asia as a traditional medicine for the treatment of asthma (Kanter *et al.*, 2005). NS contain more than 30 lipids in fixed oil fraction and 0.40-0.45 w/w volatile oil. The volatile oil contain 18.4-24% thymoquinone and 46% many monoterpenes like p-cymene

Corresponding Author: Tarique Noorul Hasan, Department of Food Science and Nutrition,
College of Food Science and Agriculture, Post Box No. 2460, King Saud University, Riyadh-11421,
Kingdom of Saudi Arabia Tel: 00966-467-8286 Fax: 00966-467-8366

and α -pinene (El-Tahir *et al.*, 1993). Topical application of NS extract in mouse inhibits the proliferation of induced skin carcinogenesis (Salomi *et al.*, 1991). Ethyl acetate extract of NS seed had been reported as cytotoxic for different class of cancer viz. P388, Molt4, HepG2 and SW620 (Swamy and Tan, 2000) whereas ethanolic extract of NS seed shows anti tumor activity against intra peritoneally implanted LL/2 cells in BDFI mice (Kumara and Huat, 2001). In Farah and Begum (2003) had reported that NS alone or in combination with oxidative stress is effective in inactivating MCF-7 breast cancer cells.

Some of the other therapeutic properties attributed to NS oil are anticestodic, antinematodic, hepatoprotective (Mahmoud *et al.*, 2000), antioxidant and antiviral effect against murine cytomegalo virus infection (Salem and Hossain, 2000). The crude extract of NS seed exhibited spasmolytic and bronchiodilatory activities (Gilani *et al.*, 2001). There are some other known medicinal properties and many have to be investigated.

The objective of present *in vitro* study was to evaluate the potency of methanolic extract of NS seeds for killing of prostate cancer cells - PC3 cell line and detection of signs of apoptosis through cytochemical method and microscopy.

MATERIALS AND METHODS

Plant material: NS seeds were purchased from the local market of Tiruchirappalli in January 2007; voucher specimen was kept in Department of Botany, Jamal Mohamed College, Tiruchirappalli, Tamil Nadu, India for further references.

Preparation of methanolic extract: Air dried seeds of NS were powdered in a mixer grinder and extraction was performed in a Soxhlet apparatus with methanol. Extract had a little quantity of oil. Extract was concentrated by distilling off the solvent and then evaporated to dryness using vacuum drier.

Maintenance of PC-3 cell line: PC-3 cells were procured from the National Centre of Cell Sciences (NCCS), Pune, India. Cell line was maintained and propagated in 90% Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin streptomycin. Cells were cultured as adherent monolayer and maintained at 37°C in a humidified atmosphere of 5% CO₂, 95% air (Sowmyalakshmi *et al.*, 2005). Cells were harvested after a brief trypsinization.

Viability of cells: Cell viability was assayed by trypan blue exclusion test (James and Warburton, 1999; LaDuca *et al.*, 1989). The viability of cells was found to be 90-95%.

Clonogenic inhibition assay and detection of apoptosis:

Dried methanolic extract was dissolved in dimethyl sulphoxide (DMSO). Since it is reported in the published literature that DMSO below 0.1% of culture media is non toxic and does not alter the growth of cultured cells (Hasan *et al.*, 2007), the concentration of DMSO was maintained below 0.1% of test drug. Therefore, the data on cells treated with DMSO alone were not included. The colony inhibition assay was performed as described previously (Rao *et al.*, 2004) with some modification. In brief, sub confluent log phase PC-3 cells were harvested and 500 cells/flask were seeded in 25/75 cm² culture flask. After 24 h of seeding the cells, test drug were added to the culture flasks in 5 different concentrations of extract whereas control was left untreated. After incubation for 10 days, each flask was stained with crystal violet and colonies containing more than 50 cells were counted. Survival fractions (Table 1) of PC-3 cells in the presence of the various concentrations of methanolic extracts dissolved in DMSO were calculated as the ratio of the number of colonies formed to the product of the number of cells plated and the plating efficiency. Percentage inhibition of colony formation (Fig. 1) were obtained. The assays were repeated three times.

Stained flasks (control and treated) were screened microscopically for presence of morphological changes in PC-3 cells and their plasma membranes, for conformation of cell death through induction of apoptosis.

Table 1: Survival fraction of five different tests

Groups	Survival fraction (SF) \pm SD
Control	0.2583 \pm 0.0175
Tests (extract conc. μg mL⁻¹)	
100	0.0333 \pm 0.0022
10	0.0464 \pm 0.0024
1	0.0566 \pm 0.0037
0.1	0.0836 \pm 0.0041
0.01	0.1109 \pm 0.0053

*Only four digits after decimal had considered in each data point, p-value for all the test data against control found to be significant up to <0.05

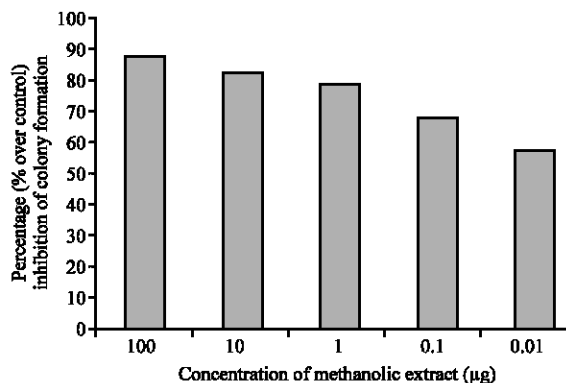


Fig. 1: Analysis of percentage inhibition of colony formation PC-3 cells in DMEM (10% FBS) in presence of methanolic extract at different concentrations

Statistical analysis: Statistical calculations were carried out, values were expressed as the mean±SD and significance was evaluated using student's t-test.

RESULTS

***N. sativa*-induced clonogenic inhibition of PC-3 cells:** Methanolic extract of NS produce significant colonogenic inhibition of PC-3 cells. Survival fraction at highest tested concentration ($100 \mu\text{g mL}^{-1}$) was 0.0333 ± 0.0022 and at lowest tested concentration ($0.01 \mu\text{g mL}^{-1}$) was 0.1109 ± 0.0053 in comparison of control (no extract) which was 0.2583 ± 0.0175 (Table 1) Among the five doses of methanolic extract treated 100 and $10 \mu\text{g mL}^{-1}$ produced

87.096 and 54.105% inhibition of colony, respectively (Fig. 1). IC_{50} was found to be less than $0.01 \mu\text{g mL}^{-1}$.

Identification of apoptosis through morphological changes: Microscopic observation of stained colony shown the cells with unique morphological changes in the cells associated with apoptosis, such as presence of condensed chromatin (Pycnosis), dispersed chromatin mass (karyorrhexin), Dissolution of chromatin resulting in disappearance of nuclei and faintly stain nuclear ghosts (karyolysis) (Fig. 2b.) were found, in addition of that, cell surface morphology associated with apoptosis like blebs, echinoid spikes and blister (Willingham, 1999) were also found (Fig. 2c, d) in comparison of control (Fig. 2a).

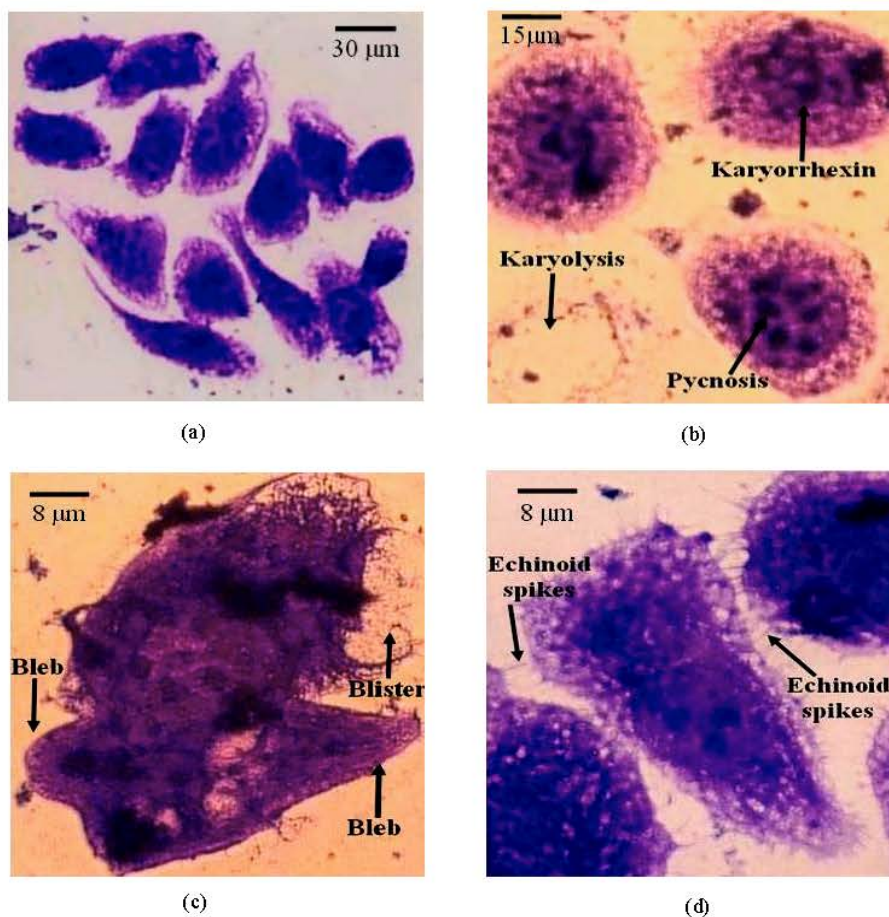


Fig. 2: (a) A colony of PC-3 cells stained with crystal violet (original magnification X 200), (b) some unique morphological changes in the cells associated with apoptosis, viz. presence of condensed chromatin (Pycnosis), dispersed chromatin mass (karyorrhexin), Dissolution of chromatin resulting in disappearance of nuclei and faintly stain nuclear ghosts (karyolysis) (original magnification X 400), (c) cell surface morphology associated with apoptosis like blebs and blister (original magnification X 1000) and (d) cell surface morphology associated with apoptosis like echinoid spikes can be visualized (original magnification X 1000)

DISCUSSION

Seeds of *Nigella sativa* have been used by mankind for thousands of years, as spice and food preservative. In ancient, Greek *N. sativa* seeds were used for treatment of headache, nasal congestion, intestinal worms as well as to promote menstrual and increase lactation. Nowadays their anti oxidant, immunomodulatory, anti microbial, anti viral, anti helminthic property in general and anti tumor properties against many cell lines, in particular are well established facts (Salem, 2005). In this *in vitro* study, it is found that *N. sativa* seeds methanolic extract is potent colonogenic inhibitor, even at lowest tested concentration $0.01 \mu\text{g mL}^{-1}$ survival fraction was 0.1109 ± 0.0053 . IC_{50} of methanolic extract for PC-3 cells is less than $0.01 \mu\text{g mL}^{-1}$ as well as all other concentrations examined were also found to be effectively inhibiting the colony formation. This finding conforms that the seeds of *N. sativa* have principles which are cytotoxic and so anticancerous against PC3 cell lines even at very low concentration.

After treatment with different concentration of methenolic extract, evidences for apoptosis were much more prominent. Several morphological changes like blebs and spikes (Fig. 2c, d) and change in the cellular structures like condensation of nucleus and chromatin, disappeared chromatin mass and dissolved chromatin resulting in disappearance of nuclei (Fig. 2b), etc. were also observed. So it can be speculated that the apoptosis might have been taken place (Willingham, 1999).

These findings can be used for characterization and purification of active principles from *N. sativa* seeds which are cytotoxic and colonogenic inhibitor and for PC3 cell lines, which may leads to formulation of novel medicines against prostate cancer.

Although, the cell surface morphological changes, changes in nuclear content and morphology of nucleus add probability for the activities of proteases and DNAses (Willingham, 1999) but, from present investigation, a definite mechanism for apoptosis can not be brought out, exploration into the molecular mechanism in future investigation might disclose the exact apoptotic signaling cascade in case of treatment of PC-3 cells with methanolic extract of *N. sativa*.

ACKNOWLEDGMENTS

Authors acknowledge financial support from Mohd. Shafi Masoodi, encouragement and help from Dr. Shasjahan A., Mohd. Tariq and technical assistance from Taha Shafi.

REFERENCES

- Bolla, M., D. Gonzalez, P. Warde, J.B. Dubois and R.O. Mirimanoff, 1997. Improved survival in patients with locally advanced prostate cancer treated with radiotherapy and goserelin. *N Eng. J. Med.*, 337: 295-300.
- Bolla, M., L. Collette, L. Blank, P. Warde and J.B. Dubois, 2002. Long-term results with immediate androgen suppression and external irradiation in patients with locally advanced prostate cancer (an EORTC study): A phase III randomised trial. *Lancet*, 360: 103-106.
- de Koning, H.J., A. Auvinen, A. Berenguer Sanchez, F. Calais da Silva and S. Ciatto, 2002. Large-scale randomized prostate cancer screening trials: Program performances in the European randomized screening for prostate cancer trial and the prostate, lung, colorectal and ovary cancer trial. *Int. J. Cancer*, 97: 237-244.
- El Tahir, K.E., M.M. Ashour and M.M. Al-Harbi, 1993. The respiratory effect of volatile oil the black seed (*Nigella sativa*) in guinea-pigs: Elucidation of the mechanism(s) of action. *Gen. Pharmacol.*, 24: 1115-1122.
- Farah, I.O. and R.A. Begum, 2003. Effect of *N. sativa* and oxidative stress on the survival pattern of MCF-7 breast cancer cells. *Biomed. Sci. Instrum.*, 39: 359-364.
- Gilani, A.H., N. Aziz, I.M. Khuram, K.S. Chaudhary and A. Iqbal, 2001. Bronchodilator, spasmolytic and calcium antagonistic activities of *N. sativa* seeds (Kalongi). A traditional herbal product with multiple medicinal uses. *J. Pak. Med. Assoc.*, 51: 115-120.
- Greenlee R.T., T. Murray, S. Bolden and P.A. Wingo, 2000. Cancer statistics 2000, CA. *Cancer J. Clin.*, 50: 7-33.
- Hasan, T.N., S.N. Ahmed, S.M.M. Aalam, C. Kumar and G. Shafi, 2007. Evaluation of cichorium extract for the growth supporting property in rat hepatocyte primary culture. *Asian J. Plant Sci.*, 6: 431-433.
- James, R. and S. Warburton, 1999. Hemocytometer Cell Counts and Viability Studies: Cell Quantification. In: *Cell and Tissue Culture: Laboratory Procedures in Biotechnology*, Doyle, A. and J. Bryan Griffith (Eds.). 1st Edn., John Wiley & Sons, England, pp: 57-61.
- Jemal, A., R. Siegel, E. Ward, T. Murray, J. Xu and M.J. Thun, 2007. Cancer statistics 2007. CA. *Cancer J. Clin.*, 57: 43-66.
- Johansson, J.E., L. Holmberg, S. Johansson, R. Bergstrom and H.O. Adami, 1997. Fifteen-year survival in prostate cancer: A prospective, population-based study in Sweden. *J. Am. Med. Assoc.*, 277: 467-471.

- Kanter, M., O. Coskun and M. Budancamanak, 2005. Hepatoprotective effect of *Nigella sativa* L. and *Utricularia dioica* L. on lipid peroxidation antioxidant enzyme system and liver enzyme in carbon tetrachloride-treated rats. *World J. Gastroenterol.*, 11: 6684-6688.
- Kumara, S.S.M. and B.T. Huat, 2001. Extraction, isolation and characterization of antitumour principle, a-hederin from seeds of *N. sativa*. *Planta Med.*, 67: 29-32.
- LaDuca, F.M., L.A. Tinsley, C.V. Dang and W.R. Bell, 1989. Stimulation of fibrinogen synthesis in cultured rat hepatocytes by fibrinogen degradation product fragment D. *Proc. Natl. Acad. Sci. USA.*, 86: 8788-8792.
- Mahmoud, M.R., H.S. Akber and S. Saleh, 2000. The effect of *N. sativa* oil against the liver damage induced by *Schistosoma mansoni* infection in mice. *J. Ethnopharmacol.*, 79: 1-11.
- Rao, K.V.K., S.A. Schwartz, H.K. Nair, R. Aalinkeel and S. Mahazan, 2004. Plant derived products as a source of cellular growth inhibitory phytochemicals on PC-3M, DU-145 and LNCaP prostate cancer cell lines. *Curr. Sci. India*, 87: 1585-1588.
- Salem, M.L. and M.S. Hossain, 2000. Protective effect of bank seed oil from *N. sativa* against murine cytomegalovirus infection. *Int. Immunopharmacol.*, 22: 729-740.
- Salem, M.L., 2005. Immunomodulatory and therapeutic properties of the *Nigella sativa* L. seed. *Int. Immunopharmacol.*, 5: 1729-1770.
- Salomi, M.J., S.C. Nair and K.R. Panikkar, 1991. Inhibitory effects of *Nigella sativa* and saffron (*Crocus sativus*) on chemical carcinogenesis in mice. *Nutr. Cancer*, 16: 67-72.
- See, W.A., M.P. Wirth, D.G. McLeod, P. Iversen and I. Klimberg, 2002. Bicalutamide as immediate therapy either alone or as adjuvant to standard care of patients with localized or locally advanced prostate cancer: First analysis of the early prostate cancer program. *J. Urol.*, 168: 429-435.
- Sowmyalakshmi, S., M. Nur-e-Alam, M.A. Akbarsha, S. Thirugnanam and J. Rohr, 2005. Investigation of semecarpus lehyam-A siddha medicine for breast cancer. *Planta*, 220: 910-918.
- Swamy, S.M. and B.K. Tan, 2000. Cytotoxicity and immunopotentiating effects of ethanolic extract of *N. sativa* seeds. *J. Ethnopharmacol.*, 70: 1-7.
- Wilkinson, S., L.G. Gomella, J.A. Smith, M.K. Brawer and N.A. Dawson, 2002. Attitudes and use of complementary medicine in men with prostate cancer. *J. Urol.*, 168: 2505-2509.
- Willingham, M.C., 1999. Cytochemical method for detection of apoptosis. *J. Histochem. Cytochem.*, 47: 1101-1109.