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Anti cancer activity of *Trachyspermum ammi* against MCF-7 cell lines mediates by p53 and Bcl-2 mRNA levels

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ABSTRACT

Breast cancer is second most common in women and accounts for 23% of all occurring cancers in women. Patients with breast cancer have increasingly shown resistance and high toxicity to chemotherapeutic drugs. Plant-derived products have proved to be an important source of anti-cancer drugs. The present study was to investigate the anti cancer activity of ethanolic extract of *Trachyspermum ammi* against MCF-7 cell lines. The preliminary phytochemical studies of ethanolic extract of *Trachyspermum ammi* showed the presence of flavanoids, alkaloids, glycosides, steroids, carbohydrates, phenols, tannins and terpenes. The IC50 concentration of ethanolic extract of *Trachyspermum ammi* was determined by MTT assay. The results showed the greater degree of cytotoxicity at the dose of 25µg/ml of *Trachyspermum ammi* and it has been taken as IC50 value for our further study. Then, we also evaluated the apoptotic effect by measuring the morphological changes, cell viability rates using light and fluorescent microscopical studies and DNA fragmentation by using gel electrophoresis method. The ethanolic extract of *Trachyspermum ammi* showed significant signs of apoptosis such as cell shrinkage, membrane blebbing and nuclei DNA fragmentation. Further, we analyze the gene expression mRNA levels by using RT-PCR method, it showed the expression of p53 was significantly ($P<0.001$) increased when compared with normal MCF-7 cell line. The expression of anti apoptotic gene Bcl-2 was significantly ($P<0.01$) reduced when compared with MCF-7 cell line. From this study we conclude that ethanolic extract of *Trachyspermum ammi* having significant anticancer activity against MCF-7 cell lines and it might be good therapeutic value for further investigation to develop natural compounds as a anti tumor agents.

Keywords: *Trachyspermum ammi*, Apoptosis, Cytotoxicity, MCF-7 cell, DNA fragmentation.

INTRODUCTION

Cancer is a multi-step disease incorporating physical, environmental, metabolic, chemical and genetic factors [1]. Breast cancer is the most commonly occurring cancer in women, comprising almost one-third of all malignancies [2]. It accounts for approximately 25% of all female malignancies with a higher prevalence in developed countries. Breast cancer is the second leading cause of cancer-related death among females in the world [3].BRAC1, BRAC2 and p53 genes having tumor suppressing function. But mutation of BRAC1 and BRAC2 leads to activation of oncogenes. Inactivation of tumor suppressor gene, which control apoptosis leads to proliferation. Apoptosis is well-regulated physiological process of cell death. Normal breast growth is controlled by a balance between cell proliferation and apoptosis, and breast tumor growth not just as a result of uncontrolled proliferation but also due to reduced apoptosis [4]. Bcl-2 (B cell lymphoma 2) is an anti apoptotic gene plays important role in regulating apoptosis. Decrease in expression of Bcl-2 leads to apoptosis. p53 is a protein it can regulate the mutation of BRAC1, BRAC2 and it can activate apoptosis.

Development of breast cancer cell targeting drug without affecting the normal cells is a challenging task in the field of cancer drug discovery. The limited success of clinical therapies including radiation, chemotherapy, immunomodulation and surgery in treating cancer, as evident by the high morbidity and mortality rates, which also can produce severe side effects such as bone marrow depression, lecukopenia, anaemia, alopecia, and hyperuricaemia, teratogenicity, carcinogenicity, and also its reduced spermatogensis in men, amenoria in women because of this indication there is an imperative need of new cancer management. Herbal Plants have long been shown to be excellent and reliable sources for the development of novel anti-cancer drugs [5]. Use of natural products and its derivatives in the development of anticancer drugs are increasing all over the world because of lesser side effects as compared to synthetic drugs [6-8]. As more than 60% of the chemotherapeutic drugs are developed from plants and their derivatives. Medicinal plants are potential sources of natural products exhibiting anti-proliferation and anti-metastatic properties [9].

Trachyspermum ammi belongs to the family Apiaceae commonly known as Ajowan is widely used for curing various diseases in both humans and animals. *Trachyspermum ammi* is a profusely branched

annual herb mainly used as digestive aid. From the pharmacological investigation it has been proved to possess anti-hypertensive, antispasmodic and bronchodilating activity hepatoprotective, anti-inflammatory effects [10]. In *in vivo* study, anticancer activity is done by inducing tumor in animals by various methods. *In vitro* anticancer evaluation is universal model for evaluation of cancer activity. *In vitro* methods have an edge over come *in vivo* methods since they are less time consuming, more cost effective, large number of compounds can be tested with small amount of sample, easier to manage. Because of these advantages, the *in vitro* methods have been preferred to assess the anticancer activity [11]. Based on the literature survey there is no anticancer activity of *Trachyspermum ammi* using MCF-7 cell lines was scientifically validated. With this background the main aim of present study has been taken to investigate the *in vitro* anticancer activity of ethanolic extracts of *Trachyspermumammi* using MCF-7 cell lines.

MATERIALS AND METHODS

Plant materials collection and ethanolic extraction

The seeds of *Trachyspermum ammi* were used for extraction which was collected at Tirunelveli. It was authenticated by Mr. Dr. V. Chelladurai, (scientist retd.), department of Botany, Central Council for Research in Ayurveda & Siddha, chennai. 50 gram of seeds were packed into soxhlet apparatus and was subjected to extraction with 500ml of ethanol. The extraction was continued until the colour of the solvent in the siphon tube became colourless. Extract of ethanol were subjected to evaporation at room temperature till a semisolid mass was obtained.

Phytochemical analysis

The freshly prepared extracts were subjected to phytochemical evaluation of chemical constituents [12-14].

Cell lines and culture

The Human Breast Cancer cell line, MCF-7 was procured from King Institute of Preventive Medicine, Chennai. The cells were grown in culture flask using Minimum Essential Medium supplemented with 3% L- Glutamine, 10% Foetal bovine serum, Penicillin (100 IU/ml), Streptomycin(100 µg/ml) and Amphotericin B along with 7.5% sodium bicarbonate in a T_{25} ml cultured vented flask and incubated at 37°C in 5% CO_2 incubator. After 3 days, about 80-90% confluent monolayer (adherent) formation was confirmed by inverted microscope. Then it was sub cultured by using TPVG solution along with minimum essential medium and used for further study [15].

Preparation of stock solution

The dried seed extract was dissolved in 1ml of DMSO (0.1% v/v) and made up to 10 ml with complete media (MEM) to give stock solution of extract 10 mg/ml.

Extract dilution

Stock solution was diluted with complete media to obtain the concentrations of 1, 10, 25, 50, 100 µg/ml. All were stored in air tight container until tested.

Cytotoxicity study

MTT Assay [16]

Growth inhibition of MCF-7 cells by *Trachyspermum ammi* was determined by MTT assay. It is a universally accepted *in vitro* method for screening the drugs having cytotoxic activity. This assay is used to determine the IC_{50} concentration of extract. The cells were harvested and seeded at a density of 10000 cells/ well in a 96 well plates and the plates were incubated for 24 hours at 37°c in 5% CO_2 for attachment of cells. After 24 hours various concentrations of *Trachyspermum ammi* such as1 µg/ml,10 µg/ml, 25 µg/ml,50 µg/ml,100 µg/ml and standard 5-fluoro uracil (5FU) were added to the cells and incubated for 48 hours. Each concentration was tested in triplicates. After incubation, the medium was replaced with phenol red and FBS free medium and 15 µl of MTT (5 mg/ml) dye was added per well and wrapped with aluminium foil and the plate was incubated again for 4 hours. After incubation without disturbing MTT crystals, medium was removed carefully and 100 µl of DMSO were added to each well to solubilize the formazan crystals. The optical density (OD) was measured at the wavelength of 570 nm. The percentage of cell inhibition was determined by following formula

$$\%\ of\ cell\ viability = 100 - \frac{OD\ of\ test}{OD\ of\ control}\ \text{x}\ 100$$

Apoptosis studies

Microscopic analysis [16, 17]

Light microscopic studies

Morphological changes in the cancer cells before and after drug treatment can be studied with the help of light microscope. Sub cultured flask containing MCF-7 taken as control and observed under inverted microscope. The medium inside the flask was decanted and the cells were treated with IC_{50} concentration of 5FU, ethanolic extract and the flask was incubated for 48 hours at 5% $CO_2$and 37°C. The flask was taken and observed under inverted light microscope.

Fluorescent microscopic studies

Fluorescent microscopy was used to study the viability of cells as well as nuclei and chromatin condensation with the help of fluorescent binding dye.

Three culture flasks with fully grown or 90% confluence reached MCF- 7 cells were taken, one serving as control and the others for 5FU, plant extract treatment. The medium was decanted and treated with IC_{50} concentration of 5FU, ethanolic extract and incubated for 48 hours at 5% CO_2 and 37°C. Cells were trypsinised from control, 5FU and ethanolic extract treated flask and subjected to centrifugation. Pellet of cells were resuspended in phosphate buffer saline of pH 7.4. 100 µl of this cell suspension was introduced into microscopic slide along with equal mixture of acridine orange and ethidium bromide for staining. The cells were then viewed under fluorescent microscope. The viable cells (green color) and dead cells (red color) were identified by differential uptake of these two fluorescent DNA binding dyes.

Extraction of DNA and RNA

The Cells treated with IC_{50} concentration of ethanolic extract and 5-FU for 48 hours were collected and subjected to extraction of RNA, DNA and protein using GeNei TM TRIsoln according to manufacturer's instructions. TRIsoln is a monophasic solution in which the samples were lysed, addition of chloroform results in three phase separation, aqueous phase predominantly of RNA, an interphase, predominantly of DNA and organic phase, predominantly of protein. The extracted RNA was used for real time RT-PCR, the extracted DNA was used for DNA fragmentation study.

DNA fragmentation study

A distinctive feature of apoptosis at the biochemical level is DNA fragmentation [18]. This method is used as a semi-quantitative method for measuring apoptosis [19]. The DNA were precipitated from an interphase obtained from phase separation. DNA fragmentation was carried by agarose Gel electrophoresis method. Electrophoresis is a method of separating substances based on the rate of movement under the influence of electric field. 1.2g of agarose gel was dissolved in 100ml of TAE buffer. Agarose was melted until the solution become clear, then the 3µl of ethidium bromide was added to the solution and mixed well. After that the gel was allowed to solidify without disturbing the wells. The gel was then transferred to 5 x TAE buffer filled electrophoresis tank. 2µl of 6x loading dye was added to 10µl of sample DNA and mixed well. So that a total of 12µl of samples were loaded to electrophoresis chamber. The power card terminals were connected to their respective positions and the gel was run at 80 V till the gel loading dye migrated to more than half of the length of gel. The unit was switched off and the separated sample DNA was visualized with a DNA ladder marker under a UV Trans-illuminator.

Gene expression

RNA extraction

An 0.5ml of isopropanol added to the aqueous phase obtained from phase separation. Then it was centrifuged at 12000 rpm for 10 minutes at 2-8˚C. The RNA precipitated at gel like pellet. Then the obtained pellet was washed with 75% ethanol and centrifuged at 10000 rpm for 10 minutes at 2-8˚C. After that the pellet was air dried and resuspended with 100µl of RNase free water. The resultant RNA was quantified and used for RT-PCR.

Reverse transcriptase PCR (RT-PCR)

It is a technique where expression of RNA is studied by converting it into cDNA with the help of enzyme reverse transcriptase and quantitatively measuring the amount of amplified target sequence from entire cDNA using fluorescent dye SYBR green in real time. 15µl of extracted RNA was added to the 5µl of 5X buffer, 2µl of 10mm DNTPs and 1.5µl of hexamer primer and kept in thermal cycler at 70°c for 5 minutes to separate the false double stranded, after that it was immediately cooled with ice. Then1.5µl of Reverse transcriptase and 1µl of DTT was added and spun for few seconds. It was then placed in thermal cycler , at 25°c for 5 minutes for binding of hexamer, followed by at 42°c for 45 minutes for cDNA synthesis, followed by at 85°c for 5 minutes for denaturation of remaining unconverted RNA's and finally at 4°c for 5 minutes . The primers synthesized were P53, Bcl2, along with housekeeping gene GAPDH. The primers were synthesized by Geno Rime with the help of Primer express software with the available primer sequence (Table I).

Real time PCR

The cDNA was synthesized from RT-PCR. The real time reaction consist of 5µl of cDNA,2µl of 25 pM/µl forward primer ,2µl of 25 pM/µl reverse primer, RT mix, 16µl PCR grade water and 25µl of SYBR green. Place the plate in real time PCR and set the program as follows pre denaturation at 95˚C for 1 minute, denaturation at 95˚C for 15 seconds.; 40 cycles of 95˚C for 15 seconds, annealing at 60˚C for 15 seconds and extension at 72˚C for 45 seconds, The relative expression of genes was analyzed and interpreted by Applied Biosystem Software.

Statistical analysis

All the data expressed as mean±SEM were analysed by one-way analysis of variance (ANOVA), followed by Dunnett's test using Prism Graph Pad and values of $P<0.05$ were considered as statistically significant.

RESULTS

Phytochemical analysis

The ethanolic extract of *Trachyspermum ammi* showed the presence of Flavanoids, Alkaloids, Glycosides, Steriods, Carbohydrates, Phenols, Tannins and Terpenes.

Determination of IC_{50} concentration using MTT assay

The IC_{50} concentration of ethanolic extract of *Trachyspermum ammi* in MCF-7 cells was determined by MTT assay. The inhibition activity of ethanolic extract of *Trachyspermum ammi* on MCF-7 cells were plated and treated with different concentration such as 1, 10, 25, 50, 100 µg/ml. The IC_{50} value was determined based on cell viability rates. The 50.79% inhibition was observed in concentration of 25µg/ml which has been taken as IC_{50} value and fixed for our further study (Fig.1).

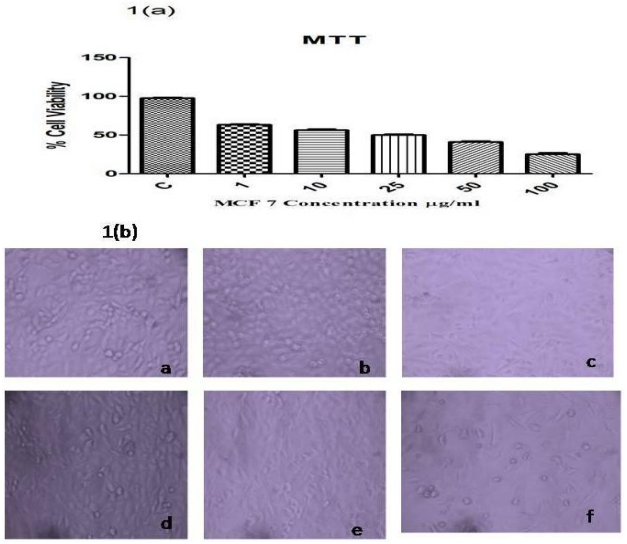

Figure 1: a. Dose dependent cytotoxicity effect of *Tranchyspermum ammi* over cell viablity. **b.** Phase contrast image of MCF -7 cell lines at 10X magnification. (a) Normal MCF -7 cells (b) 1µg/ml (c) 10µg/ml (d) 25µg/ml (e) 50 µg/ml (f) 100µg/ml.

Effect of *Trachyspermum ammi* on MCF-7 cell lines in Light microscopical studies

Apoptosis activity of ethanolic extract *Trachyspermum ammi* was determined by light microscopical studies. The morphological features of apoptosis light destruction of cells, reduction of MCF-7 cell population, reduction of cell volume, loss of integrity of membrane which resulted in crooked and vesicle shape of the membrane and chromatin condensation compared with normal MCF-7 cell lines. When compared with 5-FU, the ethanolic extract of *Trachyspermum ammi* shows significantly decreased in apoptosis activity (Fig 2).

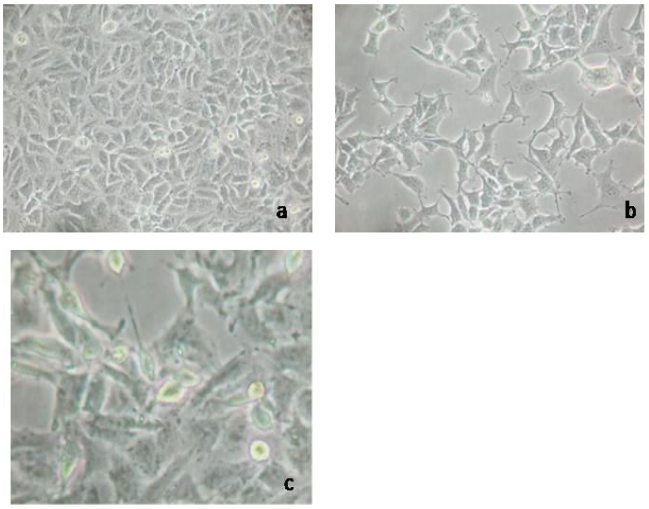

Figure 2: Morphological evidence of apoptosis by light microscopical method. (a) Normal MCF -7 cell lines.(b). MCF-7 cell lines treated with 5-FU (7.8 µg/ml). (c). MCF -7 cell lines treated with ethanolic extract of *T. ammi* (25 µg/ml).

Effect of *Trachyspermum ammi* on MCF-7 cell lines in Fluorescence microscopic studies

Apoptotic effect of ethanolic extract of *Trachyspermum ammi* further confirmed with fluorescence microscopical studies. Cells treated with IC_{50} concentration of ethanolic extract of *Trachyspermum ammi* showed apoptotic activity such as chromatin condensation and nuclear fragmentation when compared with normal MCF-7 cell lines. The presence of apoptotic activity shows ethanolic extract of *Trachyspermum ammi* may have anticancer activity (Fig 3).

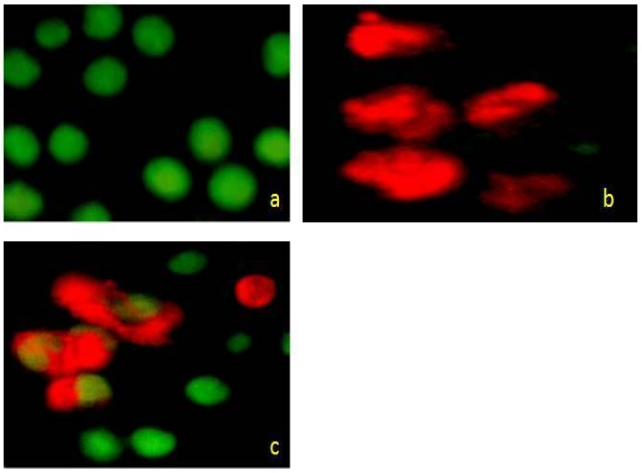

Figure 3: Morphological evidence of apoptosis by fluoresecent microscopical analysis using AO/EB staining. (a) MCF -7 cell lines. (b). MCF-7 treated with 5 FU 7.8 µg/ml. (c). MCF-7 treated with *T. ammi* 25 µg/ml.

Effect of *Trachyspermum ammi* on MCF-7 cell lines in DNA fragmentation analysis

DNA fragmentation study was performed by Agarose Gel Electrophoresis. The DNA migrated as discrete bands which were compared to DNA markers, giving a ladder of approximately 100 base pairs. Such DNA ladders are considered to be a hall mark of apoptosis. In the present study, DNA ladders appeared in both standard 5 FU and ethanolic extract of *Trachyspermum ammi* treated cells but not in control cells which proves that the apoptosis induced ethanolic extract of *Trachyspermum ammi* was by fragmenting the DNA (Fig 4).

Figure 4: DNA fragmentation assay. Lane M: 100 base pair DNA marker Lane 1: MCF-7 cells treated with 5 FU (7.8 µg/ml), Lane 2: MCF-7 cells treated with ethanolic extract of *T. Ammi* (25 µg/ml).Lane 3: MCF-7 cell lines.

Effect of *Trachyspermum ammi* on MCF-7 cell lines in Real time reverse transcriptase polymerisation chain reaction

To investigate the molecular mechanism of ethanolic extract of *Trachyspermum ammi* induced apoptosis in MCF-7 cell lines. The expression levels of apoptosis related genes such as Bcl-2, p53 were determined and it is major mRNA involved in apoptosis. mRNA levels were performed using one step RT-PCR SYBR green mix quantitative real time reverse transcription PCR using CF96 real time system. The mRNA level of p53 of *Trachyspermum ammi* was found to be significantly ($p<0.01$) up regulated when compared with normal MCF-7 cell lines. The expression of Bcl-2 of *Trachyspermum ammi* is significantly ($p<0.001$) decreased when compared with normal MCF-7 cell lines (Fig 5, 6).

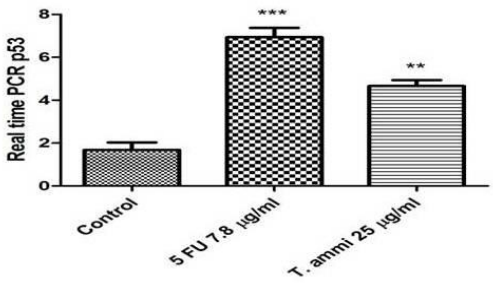

Figure 5: Effect of *T. ammi* on p53 mRNA levels on MCF -7 cell lines. Each column represents mean ± SEM. The symbol denotes the significance level: ***P<0.001, **P<0.01 and *P<0.05 compared with control MCF-7 cell lines.

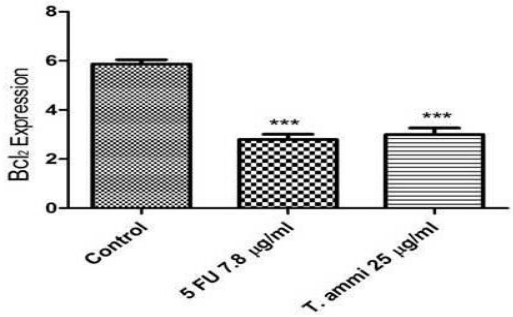

Figure 6: Effect of *T. ammi* on Bcl-2 mRNA levels on MCF -7 cell lines. Each column represents mean ± SEM. The symbol denotes the significance level: ***P<0.001, **P<0.01 and *P<0.05 compared with control MCF-7 cell lines.

DISCUSSION

Medicinal plants constituents a common alternative for cancer prevention and treatment in many countries around the world [20, 21 & 22]. The preliminary phytochemical studies of ethanolic extract of *Trachyspermum ammi* showed the presence of Flavanoids, Alkaloids, Glycosides, Steriods, Carbohydrates, Phenols, Tannins and Terpenes. Previous studies which can supports for our present study that polyphenolic compounds might inhibit cancer cells by xenobiotic metabolizing enzymes that alter metabolic activation of potential carcinogens, while some flavonoids also alter the hormone production and inhibit aromatase to prevent the development of cancer cells. The mechanism of action of anti-cancer activity of phenols is by disturbing the cellular division during mitosis at the telophase stage. It was also reported that phenols reduce the amount of cellular protein and mitotic index and colony formation during cell proliferation of cancer cells [23]. So they are regarded as promising anticancer agents against most human cancers [24, 25].

MTT assay is a universally accepted *in vitro* method for screening the drugs having cytotoxic activity. *In vitro* cytotoxic activity against MCF-7 cell line at different concentrations of ethanolic extracts of *Trachyspermum ammi* was evaluated. In our study, we showed that ethanolic extract of *Trachyspermum ammi* inhibited the growth of MCF-7 breast cancer cell lines and had strong cytotoxicity in a concentration-dependent manner. Cytotoxic effect against the MCF-7 cell line is considered as a prognostic anticancer activity. IC_{50} value of ethanolic extract of *Trachyspermum ammi* is 25µg/ml.

Apoptosis is a programmed cell death characterized by cleavage of chromosomal DNA into oligonucleosomal fragments. Apoptosis is an active form of cell death that occurs in response to several agents, including chemotherapeutic drugs. Apoptosis was initially described by its morphological characteristics, including cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation [26, 27]. Analysis of nuclear morphology is highly essential to examine the induction of cell death. The MCF-7 cells treated with ethanolic extract of *Trachyspermum ammi* and 5-FU showed cell shrinkage, membrane blebbing and disorganized cell structures but not in control cells. Further study was done to observe the changes at nuclear level such as chromatin condensation and nuclear fragmentation under fluorescent microscopy after the cells stained with acridine orange (AO) and ethidium bromide (EtBr) dyes. Acridine orange (AO) is a membrane permeable cationic dye that binds to nucleic acids of viable cells and causes a green fluorescence. Ethidium bromide (EtB) is impermeable to intact membranes but readily penetrates the membranes of non-viable cells and binds to DNA or RNA, causing orange fluorescence [28]. In our study, ethanolic extract of *Trachyspermum ammi* and 5-FU treated groups induced apoptosis which was evidenced by the formation of nuclear changes and nuclear fragmentation.

Cleavage of chromosomal DNA into oligonucleosomal fragments is a hallmark of apoptosis. This cleavage of DNA or its fragmentation can be visualized by DNA Laddering assay [29 & 30]. In present study, DNA fragmentation occurred in both standard 5 FU and ethanolic extract of *Trachyspermum ammi* treated cells but not in control cells. The results indicate that the ethanolic extract of *Trachyspermum ammi* induced significant internucleosomal DNA fragmentation in MCF-7 cell line. This activity may be contributed by the extract constituents. The fragmentation was not found in distinct, but increased DNA damage was observed, which provide evidence for apoptotic cell death. No substantial DNA fragment was observed in control cells.

p53 is the most commonly mutated gene associated with cancer[31], which helps to regulate the cell cycle and has a key role in ensuring that damaged cells are destroyed by apoptosis. Bcl-2 is an anti-apoptotic protein belonging to the B-cell lymphoma-2 (BCL-2) family. Bcl-2 opposes apoptosis by binding to the proapoptotic members and neutralizing their activity [32].

p53 acts as a guardian of the genome and is one of the major factors controlling cell proliferation, growth and transformation. The p53 tumor suppressor gene is mutated in over 50% of human cancers, and the oncogenetic activity of p53 mutation is due to its ability to interfere with p53-dependent apoptosis by a dominant negative mechanism [33]. Activation of p53 is often deducted with natural chemotherapeutic agents.

In the present study, IC_{50} concentration of ethanolic extract of *Trachyspermum ammi* showed upstream regulation of p53 protein expression when compared with control, but slightly lesser than 5FU. Hence, ethanolic extract of *Trachyspermum ammi* may possibly enhance the susceptibility of MCF-7 cells to apoptosis by attenuating the tumor suppressor protein.

Bcl-2 (B cell lymphoma 2) is a anti apoptotic gene plays an important role in regulating apoptosis. Mutation in Bcl-2 gene decreases the propensity of the cells for undergoing apoptosis [34, 35 &36]. Bcl-2 plays a major role in cancer and its resistance thereby interfering with therapeutic action of chemotherapeutic drugs. Decrease in expression of Bcl-2 leads to apoptosis. The expression of Bcl-2 was found to be down regulated in ethanolic extract of *Trachyspermum ammi* treated cells which was slightly lowered than 5-FU treated MCF-7 cell, while upstream regulation was found in control MCF-7 cell.

CONCLUSION

The medicinal plant constitutes a common alternative for cancer prevention and treatment in many countries around the world. The *Trachyspermum ammi* have been commonly used in traditional Indian medicine for the treatment of various human ailments for many years. Results of anticancer activity of ethanolic extract of *Trachyspermum ammi* showed a potent cytotoxic activity against MCF-7 breast cancer cell lines. Thus, we can assume that the possible mechanism of its anti cancer activity may be apoptosis induced by DNA fragmentation and

this is contributed by active phytochemicals such as alkaloids, phenols and flavonoids found in the extracts. It also induces apoptosis by modulating the expression levels of p53 and Bcl-2. Our current investigation supports the ethanolic extract of *Trachyspermum ammi* as an anti cancer agent in the traditional medicine system.

Conflict of interest

Authors declare that there is no conflict of interest to reveal.

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