In vitro Anticancer Activity of *Pisum sativum* Seed against Breast Cancer Cell Line (MCF-7)

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Abstract: The present study investigated the presence of secondary metabolites in the ethylacetate extract of *Pisum sativum* seeds and their cytotoxic effects against MCF-7 cell lines. *P. sativum* is commonly known as green pea comes under Family Fabaceae. The plant has been shown to possess many biological activities including anticancer properties among others. The phytochemical analysis was carried out following standard procedures and GC-MS studies. The cytotoxic activity of the ethylacetate extract was assessed against human breast adenocarcinoma (MCF-7) cell lines by using the MTT assay. The phytochemical screening revealed the presence of alkaloids, cardiac glycosides, flavonoids, phenols, saponins, steroids, tannins, and terpenoids. The maximum percentage of growth inhibition value obtained was 79.1± 3.93% at 3mg/ml concentration. Thus it is concluded that the *P. sativum* seeds possesses anticancer activity. The anticancer effects of the ethylacetate extract of *P. sativum* seed may be related to their content of secondary metabolites. This study validates the traditional use of the plant in the management of cancer.

Keywords: Breast cancer, Cytotoxicity, Phytochemistry, MCF-7 cell line, *Pisum sativum*, Secondary metabolites.

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1 INTRODUCTION

CANCER is a multistep disease incorporating physical, environ-mental, metabolic, chemical, and genetic factors, which play a direct and/or indirect role in the induction and deterioration of cancers.Cancer is a major public health burden in both developed and developing countries. The limited success of clinical therapies including radiation, chemotherapy, immune-modulation, and surgery in treating cancer, as evident by the high morbidity and mortality rates, indicates that there is an imperative need of new cancer management (Dai and Mumper, 2010). Medicinal plants besides therapeutic agents are also a big source of information for a wide variety of chemical constituents which could be developed as drugs with precise selectively. These are the reservoirs of potentially useful chemical compounds which could serve as newer leads and clues for modern drug design (Vijayalakshmi and Ravindran, 2012**)**. Drug discovery from medicinal plants has played an important role in the treatment of cancer and, indeed, most new clinical applications of plant secondary metabolites and their derivatives over the last half century have been applied towards combating cancer (Balunas and Kinghorn, 2005**)**. Medicinal plants and natural products of plant origin are a rich source of cancer chemotherapy drugs, and exhibited low (or) almost no toxicity to normal tissues. Hence, more attention is being paid to searching for new antitumor agents from natural products (Desai *et al.*, 2008; Sigstedt *et al.*, 2008; George *et al.*, 2010; Dai *et al.*, 2011). Pea (*Pisum sativum)*, is an annual herbaceous legume in the family Fabaceae grown for its edible seeds and seedpods. The oil from ripened pea seeds reduced pregnancy rate in women by 60% in a 2 year. The oil of pea seeds has antisex harmonic effects; produces sterility and antagonizes effect of male hormones (Duke, 1981). It has been reported that seeds contain trypsin and chymotrypsin which could be used for contraceptive, ecbolic, fungistatic and spermicide (Duke, 1981). Smart (1990) reported that no toxicity or anti-metabolites in peas. Rehman and Khanum (2011) reported that the isolated peptide from *Pisum sativum* has antibacterial activity. However, no studies on anticancer activity of *P. sativum* seeds against breast cancer. Therefore the present study was investigated the anticancer activity of *P. sativum* seed extract against human breast cancer cell lines (MCF-7).

2. MATERIALS AND METHODS

2.1 Collection of Plant Material

P. sativum (Pea) seeds were purchased from grocery shop, Namakkal. They were thoroughly washed with tap water and dried under shadow. Then they were ground well using domestic grinder.

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2.2 Preparation of Extracts

About 250 gm of dried, fine seed powder of *P. sativum* was loaded into the Soxhlet column connected with the round bottom flask constituted 500 ml of ethylacetate solvent and extracted at the appropriate temperature of solvent. The Soxhlet apparatus was continued for extraction up to 2 cycles for 48 hrs. Then, extract was concentrated to dryness and the residues were obtained. The residues were stored into a pre-weighed sample container for further use.

2.3 Phytochemical Screening of *P. sativum* seed Extract

The ethyl acetate extract of *P. sativum* seed were used for qualitative screening of phytochemicals such as alkaloids, carbohydrates, flavonoids, cardiac glycosides proteins, phenols, saponins, steroids, tannins and terpenoids by standard biochemical procedure (Kokate, 1997).

2.4 GC-MS Analysis

GC-MS analysis was performed to identify the bioactive compounds in the *P. sativum* ethyl acetate extract. GCMS analysis was performed using a JEOL GC MATE II instrument employing the following conditions: Front inet temperature 220°C; Column HP 5Ms; Helium gas (99.99%) was utilized as carrier gas at a constant flow rate of 1ml/min. In the case of oven temperature it was 50 to 250°C @ 10°C/min. The ion chamber temperature and GC interface temperature was 250°C. Mass analysis was done with the help of Quadruple Double Focusing Analyzer: For detection, the Photon Multiplier tube was used. At 70eV, mass spectra were taken. The necessary data were gathered by the full-scan spectra within the scan range of 50-600 amu. Percentage peak area was nothing but the percent composition of constituents of the extract.

2.4.1 Identification of Components

On the basis of GC retention time, chemical compounds in ethyl acetate extract were identified. The mass spectra were matched with the standard mass spectra available in libraries. By employing National Institute Standard and Technology's data base having more than pattern and Wiley spectra libraries, mass spectrum GC-MS interpretation was made. Components were compared with the known spectrum compounds which were stored in NIST library. The compound name, weight of molecule and its formula and compound structure of the test materials were ascertained from NIST and Pubchem libraries.

2.5 Evaluation of Anticancer Activity

2.5.1 Procurement of Cell Lines

Cell lines of human breast cancer (MCF-7) were obtained from National Center for Cell Science (NCCS), Pune, India. These cells were stored in DMEM medium supplemented with 10 % FBS (Sigma-Aldrich, St.Louis, Mo, USA). Penicillin at 100 μg/mL and streptomycin at 100 μg/mL were used as antibiotics (Himedia, Mumbai, India). At humidified atmosphere the culture was kept at 5% CO2 level in a CO_2 incubator at 37°C (Forma, Thermo Scientific, USA).

2.5.2 Cell Viability Assay

The MTT tetrazolium salt colorimetric assay, described by Mosmann (1983) was performed to measure the cytotoxicity of ethyl acetate extract of *P. sativum*. The plant extract were dissolved in 100% DMSO to prepare a stock. The stock solution was diluted separately with fresh medium to get various concentrations from 0.3 to 3.0mg. 96-well plates at plating density of 5000 cells/well were seeded after 24 hrs. Exactly 100μl of sample was added to wells. DMSO (0.02 %) was used as the solvent control. After 24 hrs, 20 μl of MTT solution (5mg/mL in PBS) was added to each well and the plate was wrapped with aluminum foil and incubated for 3 hrs at 37°C. By adding 100 μL of DMSO to each cell the purple formazan product was dissolved. The absorbance was observed at 570 nm (measurement) and 630 nm (reference) using a 96-well plate reader (Bio-Rad, iMark, USA). In order to calculate the respective mean values, data were collected for triplicates each. The following formula was used to calculate percentage of growth inhibition.

$$\text{Growth inhibition} = \frac{\text{Mean OD of control cells} - \text{Mean OD of treated cells}}{\text{Mean OD of Control}} \times 100$$

From the obtained values, IC_{50} for 24 hrs for MCF-7 cells were computed by probit analysis using SPSS windows based software.

2.5.3 Acridine Orange (AO)/ Ethidium Bromide (EB) (AO/EB) Staining fluorescent Assay for Cell Death

Apoptotic morphology was investigated by AO/EB double staining technique as described by (Spector *et al.,* 1998). MCF-7 cells were cultured separately in 6-well plates and treated with IC_{50} concentration of the plant extracts for 24 hrs, when DMSO (0.02%) was used as solvent control. The cells of treated and untreated (25μl of suspension containing 5000 cells) were incubated with the solution of acridine orange and ethidium bromide (1 part of 100 μg/mL each of acridine orange and ethidium bromide in PBS) and examined in a fluorescent microscope (Carl Zeiss, Jena, Germany) using a UV filter (450 - 490 nm). For each sample, three hundred cells were counted, in triplicate, for each time point and scored as viable or dead cells by apoptosis

or necrosis as judged from the nuclear morphology and cytoplasmic organization and percentages were calculated for apoptotic and necrotic cells. Morphological features of dead cells were photographed (Kumar *et al.,* 2008).

2.5.4 Assessment of Mitochondrial Potential (JC1 Staining)

Mitochondrial membrane potential ($\Delta\psi m$) was assessed using the fluorescent probe JC-1, which produces orange-red fluorescence when accumulated in the mitochondria of healthy cells but fluoresces green when leached out into the cytosol due to loss of $\Delta\psi m$ resulting in a negative internal potential (Reers *et al.,* 1991). The MCF-7 cells were grown in glass cover slips (22 × 22 mm) placed in the wells of 6-well plates and treated with the ethyl acetate extract of *P. sativum* at 24 hr IC_{50} concentration, and 0.02% DMSO was used as solvent control. The cells were stained with JC-1 dye after 12 hr exposure. In the fluorescent microscope, the cells' mitochondrial depolarization was observed and the pathological changes in the cells were also observed and recorded. The mean and standard deviation were calculated from the collected data of the three replicates each.

2.5.5 Single Cell Gel Electrophoresis (Comet Assay)

DNA damage was detected using the comet assay of (Singh *et al.,* 1988). The MCF-7 cells were treated with the complex at its 24 hr IC_{50} concentration for 12 hrs, with 0.02% DMSO as the solvent control. The harvested cells were suspended in low melting point agarose in PBS and pipette out to microscope slides pre-coated with a layer of normal melting point agarose. The chilled slides on ice for ten minutes and then immersed in lysis solution (2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris, 0.2 mM NaOH [pH 10.01], and Triton X-100) and was incubated overnight at 48˚C in order to lyse the cells and permit DNA unfolding. Thereafter, the slides were exposed to alkaline buffer (300 mM NaOH, 1 mM Na2EDTA, [pH >13]) for 20 minutes to allow DNA unwinding. The slides were washed with buffer (0.4 M Tris, pH 7.5) to neutralize the excess alkali and to remove the detergents before staining with ethidium bromide (20 mL in 50 mg/mL). Photomicrographs were obtained using the fluorescent microscope. 150 cells from each treatment group were digitized and analysed using CASP software. The images were used to determine the DNA content of individual nuclei and to evaluate the degree of DNA damage representing the fraction of DNA in the tail, so as to assign the cells among the five categories: dead, highly damaged, damaged, slightly damaged and intact. Data were gathered for three replicates each and used to compute the values for means and the standard deviations.

2.6 Statistical Analysis

Data from each of the three experiments are expressed as mean ± standard deviation (SD). IC_{50} values were calculated by probit analysis using windows based SPSS statistical software.

3. RESULTS AND DISCUSSION

3.1 Preliminary Phytochemical Screening

The Results showed the presence of secondary metabolites such as alkaloids, cardiac glycosides, flavonoids, phenols, saponin, phytosterols, tannin and triterpenoids in ethylacetate extract of *P. sativum* (Table 1). Flavonoids are potent water soluble antioxidants and free radical scavengers, which prevent oxidative cell damage, have strong anticancer activity (Del-Rio *et al.,* 1997; Okwu, 2004).

Table 1: Phytochemical screening of ethyl acetate extract of *P. sativum*

S. No	Phytocompounds	Test Name	Results
1	Alkaloids	a. Hagers test	Positive
		b. Mayers test	Positive
		c. Wagners test	Positive
2.	Carbohydrates	a. Barfoeds test	Negative
		b. Benedict test	Negative
		c. Fehlings test	Negative
3.	Cardiac glycosides	a. Bromine water test	Positive
4.	Flavonoids	a. Alkaline test	Positive
		b. Ferric chloride test	Positive
		c. Lead acetate test	Positive
		d. Shinoda test	Positive
		e. Mg turning test	Positive
		f. Zinc test	Positive
5.	Phenols	a. Alkaline test	Positive
		b. Ferric chloride test	Positive
		c. Lead acetate test	Positive
6.	Protein	a. Millions test	Negative
		b. Xanthoproteic test	Negative
7.	Saponins	a. Bubble test	Positive
		b. Emulsion test	Positive
		c. Foam test	Positive
8.	Steroids	a. Liberman – Burchards test	Positive
9.	Tannins	a. Ferric chloride test	Positive
		b. Lead acetate test	Positive
10	Terpenoids	a. Con H_2SO_4 test	Positive
		b. Liberman – Burchards test	Positive

3.2 GC-MS analysis of ethanolic extract of *P. sativum* seed

Totally eleven major phytochemical compounds were identified in ethylacetate extract of *P. sativum* seed through GC-MS spectrum profile (figure 1). Identified compounds were Tetradecane, Flavone, Hexadecane, Hexadecane, 2 methyl-, Octadecane, 4H-1-Benzopyran-4-one, 7-hydroxy-3-[4-methoxyphenyl]-, n-Hexadecanoic acid, 4H-1-Benzopyran-4-one, 5,7-dihydroxy-2-[4-hydroxyphenyl]-6-methoxy-, 4'-Methoxy-5,7-dihydroxy isoflavone, Docosane and Tetracosane and their retention time were 10.88, 12.82, 13.38, 15.12, 15.7, 17.27, 17.78, 18.8, 19.5, 19.67and 21.43 respectively. The compound n-Hexadecanoic acid exhibited highest peak area (20.37%). The molecular structure, molecular formula and molecular weight of compounds are illustrated in table 2.

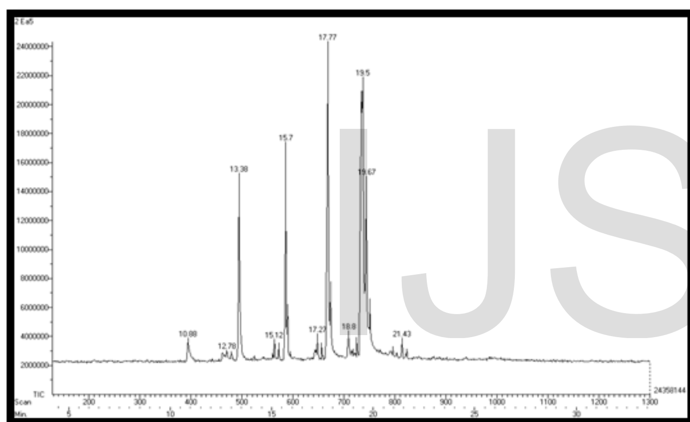

Fig. 1: GC-MS chromatogram of ethyl acetate extract of *P. sativum* seed powder.

Table 2: Bioactive compounds identified in ethyl acetate extract of *P. sativum* seed powder by GC-MS analysis.

S. No	RT	Compound Name	MF	MS	MW	PA (%)
1	11	Tetradecane	$C_{14}H_{30}$		198	3
2	13	Flavone	$C_{15}H_{10}O_2$		222	2
3	13	Hexadecane	$C_{16}H_{34}$		226	13
4	15	Hexadecane, 2 methyl-	$C_{17}H_{36}$		240	3
5	16	Octadecane	$C_{18}H_{38}$		254	15
6	17	4H-1-Benzopyran-4-one, 7-hydroxy-3-[4-methoxyphenyl]-	$C_{16}H_{12}O_4$		268	4
7	18	n-Hexadecanoic acid	$C_{16}H_{32}O_2$		256	20
8	19	4H-1-Benzopyran-4-one, 5,7-dihydroxy-2-[4-hydroxyphenyl]-6-methoxy-	$C_{16}H_{12}O_6$		300	4
9	20	4'-Methoxy-5,7-dihydroxy isoflavone	$C_{16}H_{12}O_5$		284	19
10	20	Docosane	$C_{22}H_{46}$		311	13
11	21	Tetracosane	$C_{24}H_{50}$		339	3

MF - Molecular Formula; MS - Molecular Structure; MW - Molecular Weight; PA - Peak Area.

3.3 Effect of ethanolic extract of *P. sativum* treatment on the viability of breast cancer cell line (MCF-7)

The inhibitory effect of the ethyl acetate extract of *P. sativum* at different concentrations for 24 hrs on MCF-7 cells was investigated adopting MTT assay. The assay was aimed at determining the integrity of mitochondria so as to reflect on the viability. The higher concentration of 3.0 mg/ml was exhibited 79.1 ± 3.93% when compared to the other extracts of *P. sativum* and very minimum inhibitory concentration was obtained at 0.3 mg/ml. The significant difference was obtained in the percentage of cell inhibition ($f_{10,11}$= 129.795; $p<0.005$; Table 3) among the different concentrations of ethyl acetate extract of *P. sativum*. SNK results showed that increasing cell inhibition rate when increases the concentration of ethyl acetate extract of *P. sativum*. Probit analysis results exhibited an IC_{50} value (1.914 mg/ml; Fig. 2). The reduction of OD value during MTT assay has a direct correlation with the growth inhibitory rate and inverse relation with proliferative rate (Andrew *et al.*, 2002).

Table 3: Ethyl acetate extract of *P. sativum* MTT assay

Concentration	Cell Inhibition (%) (Mean ± SD)	Cell Viability (%) (Mean ± SD)
Control	0.0 ± 0.00	100.0 ± 0.00

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	(0.0 - 0.0)	(100.0 - 100.0)
0.3	2.6 ± 3.71 (0.0 - 5.2)	97.4 ± 3.71 (94.8 - 100.0)
0.6	17.7 ± 6.73 (12.9 - 22.5)	82.3 ± 6.73 (77.5 - 87.1)
0.9	21.0 ± 2.11 (19.6 - 22.5)	79.0 ± 2.11 (77.5 - 80.5)
1.2	31.2 ± 0.41 (30.9 - 31.5)	68.8 ± 0.41 (68.5- 69.1)
1.5	47.0 ± 1.36 (46.1 - 48.0)	53.0 ± 1.36 (52.0 - 53.9)
1.8	52.3 ± 1.56 (51.2 - 53.4)	47.7 ± 1.56 (46.6 - 48.8)
2.1	53.6 ± 0.85 (53.0 - 54.2)	46.4 ± 0.85 (45.8 - 47.0)
2.4	55.6 ± 5.58 (51.7- 59.6)	44.4 ± 5.58 (40.4 - 48.3)
2.7	74.5 ± 0.09 (74.5 - 74.6)	25.5 ± 0.09 (25.4 - 25.5)
3.0	79.1 ± 3.93 (76.3 - 81.9)	20.9 ± 3.93 (18.1 - 23.7)

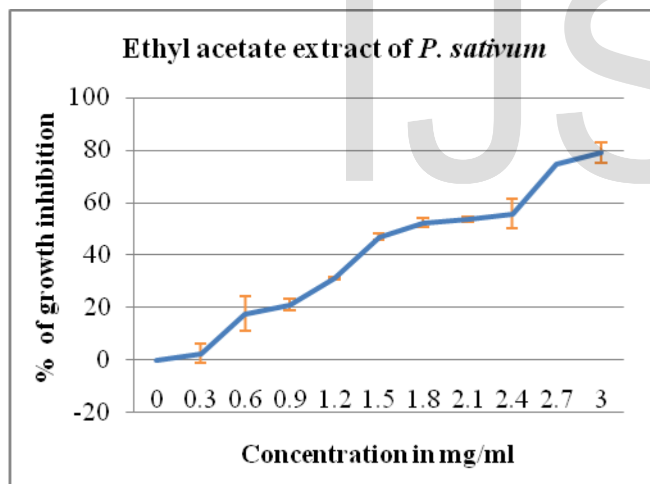

Fig. 2: Cytotoxic activity of ethyl acetate extract of *P. sativum* seed against MCF-7 breast cancer cell line by MTT assay.

3.4 Morphology of normal and extract treated MCF-7 cells has been observed under a fluorescent microscope.

MCF-7 cells treated with ethyl acetate extract of *P. sativum* exhibited notable morphological changes with related to apoptosis like blebbing formation, nuclear formation and apoptotic bodies. The treatment with ethyl acetate extract apoptotic cells showed early apoptotic cells (perinuclear chromatin condensation is shown as bright green patches.) The mean percentage of apoptosis was found to be high (63.0%) and necrosis was found to be 14.0% (Table 4) in the MCF-7 cells treated with ethylacetate extract of *P. sativum*. Ethylacetate extract of *P. sativum* induced more necrosis in the MCF -7 cells than other extracts (Fig. 3).

Table 4: Percentage of normal, apoptotic and necrotic cells on human breast cancer cell line (MCF-7) after the treatment of *P. sativum* bark powder extract.

S. No	Type of Extract	Normal Cells (%)	Mode of Cell Death	
			Apoptosis (%)	Necrotic (%)
1	Control	90.3 ± 2.08 (88.0 - 92.0)	5.3 ± 2.52 (3.0 - 8.0)	4.3 ± 1.53 (3.0 - 6.0)
2	Ethylacetate extract of *P. sativum*	23.0 ± 2.65 (20.0 - 25.0)	63.0 ± 1.00 (62.0 -64.0)	14.0 ± 3.61 (11.0 -18.0)

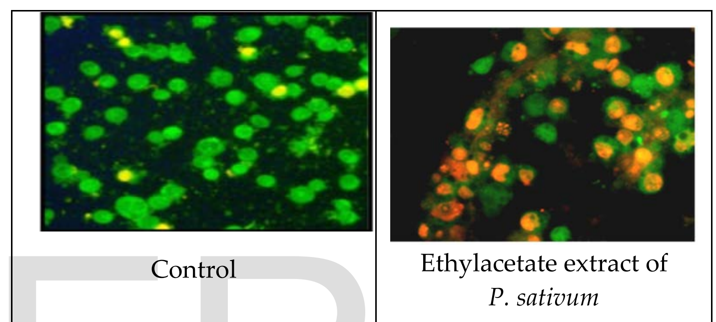

Fig 3: Morphological changes observed for control ethyl acetate extract of *P. sativum* treated (24 hrs) MCF-7 cells stained with acridine orange and ethidium bromide and 1) Viable cells have uniform bright green nuclei with organized structure (2) Early apoptotic cells have green nuclei, but perinuclear chromatin condensation is visible as bright green patches or fragments (3) Late apoptotic cells have orange to red nuclei with condensed or fragmented chromatin.

3.5 Assessment of mitochondrial membrane potential by JC-1 staining technique

The loss of mitochondrial membrane potential (Δψm) is an early event in apoptosis. To detect the changes in mitochondrial function, JC-1 staining assay was employed. The JC-1 stain is fluorescent cation which emit red fluorescence when sequested into the mitochondria of healthy cells with high mitochondrial membrane potential (Δψm). When the apoptotic cells are stained with JC-1, the red fluorescence turned into green fluorescence due to mitochondrial membrane potential (Δψm). Because, the cells undergoing apoptosis are no longer able to retain the JC-1 cation into the mitochondria and so apoptotic cells are in fluorescent green. Red fluorescence was observed in the

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control (untreated healthy MCF-7 cells) and they are presented in (Fig. 4).

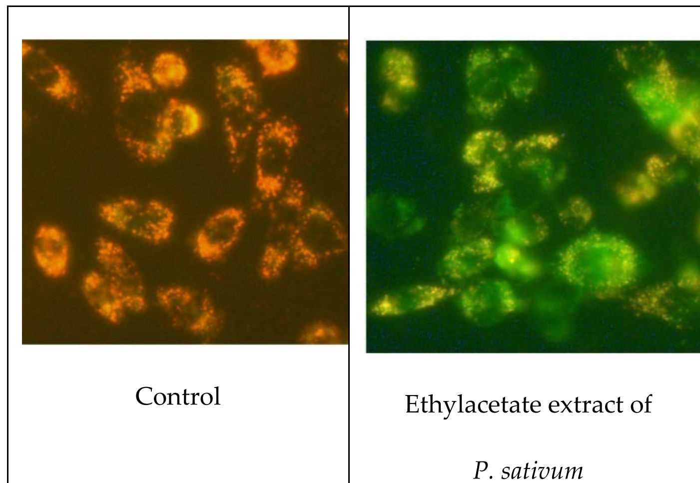

Fig 4: Photomicrographs of MCF-7 breast cancer cells, JC-1 dye accumulated in the mitochondria of healthy cells as aggregates (red-orange fluorescing); in cells treated with the various extracts of different plants for 24 hrs, due to collapse of mitochondrial membrane potential, the JC-1 dye remained in the cytoplasm its monomeric form, which fluoresced green.

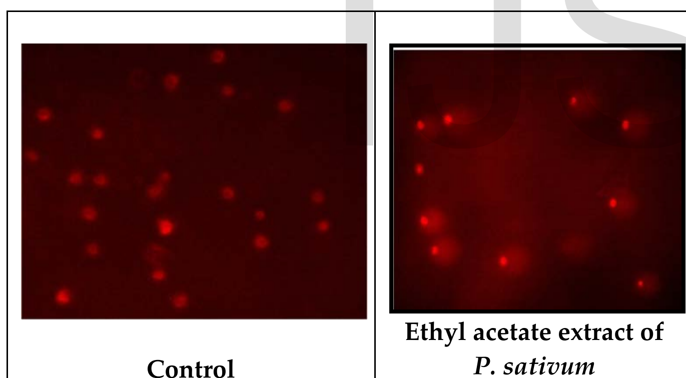

Fig 5: DNA damage in ethyl acetate exrtact of *P. sativum* treated MCF-7 cells is revealed in the Comet assay. Comet images of DNA strand breaks at 12 hrs treatment of the ethylacetate exrtact of *P. sativum.*

4. CONCLUSION

The results obtained from the *invitro* studies performed using the MCF-7 cell lines reveals that the ethyl acetate extract of *Pisum sativum* seed has a anticancer activity. The anticancer activity of *Pisum sativum* will provide a useful information in the possible application in the prevention and treatment of cancer.

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