RESEARCH ARTICLE

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Schleichera oleosa Seed Extract Reduced the Proliferation of Breast Cancer by Regulating the *BRCA1* and *p16* Genes

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Abstract

Background: Breast cancer is the most commonly diagnosed cancer and the leading cause of cancer death in females worldwide. *Schleichera oleosa* (kusum tree) belongs to the Sapindaceae family commonly found in many states of India. This plant is traditionally being used in various pathological conditions. **Methods:** *In vitro* studies were performed using seed extract of Schleichera oleosa. Different concentrations of seed extracts were treated on MCF-7 breast cancer cell line and its effect on migration and colony formation were observed. *BRCA1* and *p16* gene expression was analyzed by real-time PCR and Western blotting. **Results:** We have analyzed anticancer and anti-metastatic effects of seed extract in breast cancer and IC_{50} was 140µg/ml concentration. Further, its inhibitory role in cell migration and colony formation was at 140µg/ml ($P<0.0001$) concentration and reduced significantly growth of sphere at 140 µg ($P<0.0031$) and 150µg ($P<0.0010$) concentration after 5 days of treatment. The apoptosis study was shown a significant increase at 140 µg ($P<0.0001$) in apoptotic cells. Expression of *BRCA1* and *p16* were found to be over-expressed as 1.4 and 1.7 fold, respectively, at 140µg/ml concentration after 24 h of treatment at the transcription level. BRCA1 protein was up-regulated but p16 expression down-regulated at 140 to 150µg/ml (One-Way ANOVA, $P<0.0001$) concentration. **Conclusion:** In this study, we found a significant role of *S. Oleosa* seed extract has an anti-cancer as well as anti-metastatic via up-regulation of *BRCA1* and *p16* genes in breast cancer cells.

Keywords: Schleichera oleosa- kusum tree- Breast cancer- BRCA1- p16- Anticancer

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Introduction

Breast cancer is the most commonly diagnosed cancer and the leading cause of cancer death in females worldwide (Ferlay et al., 2019). With the expansion of urban population and changes in lifestyle, there is increasing incidence of breast cancer. According Globocon 2020 there will be an estimated 19.3 million new cancer cases and 10.0 million cancer related deaths in 2020. And 2.3 million (11.7%) new cases and 6,85,000 of death in females worldwide due to breast cancer (Globocon, 2020; Sung et. al., 2021). Urban population of India has most common cases of breast cancer; however, it is the second most common after cervix cancer in the rural population (Global Cancer Observatory, 2020).

Schleichera oleosa (kusum tree) belongs to the Sapindaceae family occurs at the foothills of the Himalayas and commonly found in many states of India such as South India, Chhattisgarh, Bihar, West Bengal, Madhya Pradesh and Uttar Pradesh. It is also found in China and Sri Lanka as well (Kundu et al., 2011). Bark and seed of this plant is traditionally being used in various pathological conditions (such as rheumatic pain, abnormal hair conditions, acne, tropical itching, burn and other skin problem) in India. Parts of *S. Oleosa* plants such as seed, bark leaves contain some phenolic compounds as shown in Table-1 and seeds contain 40.3% oil with yellowish brown color. Which have antioxidant properties and reported as their protective role for the vital molecules and help generate metabolic energy (Meshramet al., 2015). The fatty acid profile of Seeds showed 16 components. Linolelaidic acid, the transform of linoleic acid, was found as dominant fatty acid (49.7%) and others were eicosenoic acid or gondoic acid (29.5%), palmitic acid (7.6%), linoleic acid (5.6%) and oleic acid (2.8%) (Table 2) analyzed in GCMS by Palanuvej (2008). Linolelaidic acid induces apoptosis, cell cycle arrest and inflammation in human umbilical vein endothelial cells (Li et al., 2017). Schleicherastatin is a phytochemical present in *S. Oleosa* has significant inhibitory activity against P-388 in lymphocytic leukemia

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cell line. However, other phytochemical Schleicheols exhibited marginal activity against colon, lung, ovary, pancreas and prostate cancer cell lines (Pettit et al., 2000). The new series of sterol hydroxylation at C-22 appears to be potential cancer cell growth inhibitor. Third et al. 2010 was studied the cytotoxic effect of bark extract of S. Oleosa in different cancer cell lines such as 502713 (colon), SW-620 (colon), HCT-15 (colon), A-549 (lung), HEP-2 (liver) and SK-NS-H (central nervous system). The bark extracted in water has shown cytotoxic effects in all three colon cancer cell lines, whereas bark extract in methanol and water was shown cytotoxic effects in A-549 (lung cancer) and Hep-2 (Liver cancer) cell lines, respectively. However, in chloroform and hexane, plant extract did not show any cytotoxic effect on the cell lines (Thind et al., 2010).

The breast carcinoma susceptibility *(BRCA1*) genes encode various multifunctional proteins have major role in DNA repair mechanisms. However, mutations in *BRCA1* gene may lead to altered cell growth patterns in breast as well as ovarian tissue (Bianconi et al., 2013; Antoniou et al., 2003). A mutation in the *BRCA1* gene unable to repair DNA damage causes mutations in other genes as well. These mutations can accumulate over time that might transform a normal cell into malignant cells (Deng, 2006).

p16 gene has been well studied as a tumor suppressor in various cancers. It discovered as a cyclin-dependent kinase inhibitor (CDKI) have shown frequent deletion mutation suggested that it plays a crucial role in carcinogenesis (Li et al., 2011). p16 as a CDKI, binds and inhibit the activity of CDK4/6 for further activation of Rb (retinoblastoma) molecule that leads to cell cycle arrest in G1 phase. Another role of p16 was reported in cell senescence, and its physiologic involvement is still unclear (Wang et al., 2000; Liggett et al., 1998).

However, so far in our knowledge no study has reported the effects of Schleichera oleosa on *BRCA1* and *p16* genes in breast cancer. In this study, we have focused on the seed extract of *S. Oleosa* on the basis of IC_{50} in MCF-7 cells and phytochemical constituents identified by GC-MS, which induces apoptosis in endothelial cells reported earlier (Palanuvejet al., 2008; Li et al., 2017).We have identified its anti-cancer as well as anti-metastatic role in breast cancer by up regulation of *BRCA1* and *p16* genes.

Materials and Methods

Sample Collection

Plant materials (leaves, seed, pulp, fruit coat and bark) of the S. Oleosa tree (Fig-1A) were collected from ICMR-NICPR campus, Noida, Gautam Budha Nagar, Uttar Pradesh.

Extraction of phytochemicals

The leaves, seeds, pulp, fruit coat, bark of the *S. oleosa* tree were collected and washed with tap water followed by distilled water and dried in a hot air oven at 65°C. Plant samples were crushed/powdered using a mortar-pestle taken 5 gram of each plant sample in separate flask and 10-ml methanol was added to it. The mixture was heated at 55°C for around 15 h in shaker water-bath. After evaporating methanol, concentrated extract was collected. The extracted plant material was dissolved in 1ml DMSO (Larson et al., 2016).

Cell Culture

The breast cancer cell line MCF-7 was maintained in DMEM medium with 10% FBS and (1%) antibiotics (penicillin and streptomycin) and kept in 37°C incubator with 5% CO2. When the cells became 80%-90% confluent, cells were washed twice with phosphate-buffered saline, then 0.25% trypsin- 0.53mM EDTA solution is added to the flask and incubated for 2–3 min so that the cells could detach from the flask. After the cells have been successfully detached, equal number of media is added to neutralize the effect of trypsin and the cells are carefully dispersed by pipetting repeatedly to make a single cell suspension. The cells were seeded in a 6-well plate for experiments (Altenburg et al., 2011).

MTT Assay

MTT assay was performed in MCF-7 breast cancer cell lines using plant extract. Plant extracts were dissolved in DMSO to make 100 µg stocks. Further stock was diluted and made 10-200 µg working concentration. MTT assay was performed in 96-well plates, cells were counted using a hemocytometer and 5,000 cells seeded in each well of 96-well plates and kept in an incubator for 24 hr. Next, day-treated cells with different concentrations of plant extract and incubated for 24 hr after MTT dye (5mg/ml) were added to each well and incubated for 4hr. The lysis solution was added and incubated for 1hr again and read at 570 nm wavelength. The percentage of cell viability was calculated using the following formula: Percentage cell viability = OD of experiment sample x 100/OD of the control (Yang et al., 2017).

Real-time quantitative PCR

RNA was extracted using TRI reagent, MCF-7 cells collected and washed with 1x PBS, 500 µl TRIzol reagent added to the cell pellet. RNA was isolated by chloroform and precipitated by chilled isopropanol, precipitated RNA was washed with 75% ethanol, the RNA pellet was air-dried and suspended in nuclease-free water. The quality of isolated RNA was checked using 1% agarose gel. cDNA was prepared using the First Strand cDNA synthesis kit (Thermo Scientific), 1µl of RNA samples used for cDNA preparation and quantified using nanodrop at 260/280 nm wave length. Reverse transcriptase PCR was performed to check the cDNA quality by using GAPDH primer (Forward-5'-GACCACTTTGTCAAGCTCATTTC-3',Reverse-5'CTCTCTTCCTCTTGTGCTCTT-3' 147 bp) and cycle condition was 95°C in 5min, 95°C for 30 sec, 60°C for 30sec, 72°C for 1min and repeated 35 cycles. Real time quantitative PCR of *BRCA1* and *p16* gene was performed by Syber green method using RT primers of BRCA1 (Forward5'-ACAGCTGTGTGGTGCTTCTGTG-3'and Reverse-5'-CATTGTCCTCTGTCCAGGCATC-3'107 bp) and p16 (Forward-5'GCTGCCCAACGCACCGAATA-3' and Reverse-5'-ACCACCAGCGTGTCCAGGAA-3'180

bp) and GAPDH (Forward-5'-GACCACTTTGTCAAGCTCATTTC-3', Reverse-5'-CTCTCTTCCTCTTGTGCTCTT-3' 147 bp) at Tm 630C and 600C respectively for 40 cycles. CT values of each sample were normalized with house-keeping gene and fold change of gene expression was calculated using the relative quantification method using GAPDH as housekeeping gene (Wang et al., 2008)

SDS-PAGE and Western blotting

The whole cell protein is isolated from the cultured cells, treated with different concentrations of freshly prepared seed extract. Cells were harvested, washed with 1x PBS and re-suspended in ice cold whole cell lysate along with inhibitors PMSF, DTT, NaV, PIC. After an hour of incubation on ice, the lysate was centrifuged at 14,000 rpm for 20 min at 4°C. The supernatant was transferred to a fresh tube and stored at −800C until further analysis. The isolated protein is quantified by taking OD at 230 nm, 260 nm and 280nm and protein concentration is calculated using the following formula: Protein concentration = (1.55 x OD at 280 nm) – (0.76 x OD at 260 nm). SDS-PAGE electrophoresis was done for protein expression of *BRCA1* and *p16* genes by adding 50µg of denatured protein of each sample was loaded onto the wells of 10% gel with a protein marker and run on 90 V at 4°C.Transfer of proteins on a PVDF membrane was done by semi-dry method for 1hr. After the successful transfer of the proteins on the PVDF membrane, the membrane was blocked with 5% nonfat skim milk for an hour, primary antibody is added and kept on shaker-incubator at 4°C overnight. The next day, the membrane was washed thrice by 1x TBST for 15 min each time, to remove any unspecific bound antibody. Secondary antibody was added and incubated again for an hour followed by washing three times with 1x PBS.The membrane was developed using a chemiluminescence in the dark room with the help of ECL. The X-ray film was exposed to the membrane and protein bands was developed (Mirza et al., 2013).

Clonogenic assay

MCF-7 cells were cultured and counted 1,000 cells seeded in each well of six-well plates and were incubated for 24 h to adhere to the plate surface. After 24 h, cells were treated with different concentrations of seed extract for the next 24 h. After which, the media were removed from the wells and cells washed with 1x PBS twice then fresh media was added. Cells were allowed to grow until control well cells colonies are formed but not joined. The media were removed and colony fixed by fixation solution (Methanol, acetic acid 7:1), plates are left for 30 min then staining was done using crystal violet for 2 h. Washed the plates in tap water twice and air-dried. Colonies are then counted. Plating efficacy and survival fraction was calculated by following formula: PE= No. of colony counted x 100/No. of colony seeded, SF= No. of colony counted x PE/No. of colony seeded (Rafehi et al., 2011).

Cell migration assay (Wound healing assay)

MCF-7 cells are seeded in a six-well plate and incubated for 24 h or until they reach 100% confluence, Monolayer of cells scratched with a 10µl pipette tip across the center. After scratching, washed the cells were gently with 1x PBS to wash away the detached cells. Cells were treated with different concentrations of seed extract and image of the scratch was captured every 6 h by inverted phase contrast microscopy. The distance of migration was calculated using Image-J software and graph was plotted to see migration inhibition of treated cells of different concentration seed extract (Jonkman et al., 2014).

Sphere formation assay

MCF-7 cells are cultured, counted and 2,000 cells were seeded in ultra-low adherent 96-well plate. Cells are seeded in serum-free media containing 1% antibiotics and different concentrations of seed extract and are allowed to grow and form spheres. The image of the spheres were taken and the diameter of the spheres were calculated from imaging software (Radical scientific) and graph was plotted with control verses different concentrations of seed extract treated cells for sphere size reduction (Liu et al., 2015; Bartosh et al., 2014; Johnson et al., 2013).

Apoptosis assay

The cells were cultured, counted and approximately 5,000 cells were seeded in a 96-well plate and incubated for 24 h, so that the cells could adhere to the wells. Along with this separately sphere of MCF-7 cells were generated in ultralow adhere plate using serum free media. Drug treatment was given the next dayand incubated overnight at 37°C at 5%CO_2. The cells were fixed using 4% PFA for 30 min after fixing, the cells were washed with 1x PBS followed by staining with 1mg/ml AO/EtBr for 10 min and again washed with 1x PBS. Images of the cells were captured by an inverted fluorescence microscope at 485 nm wavelength (Ribble et al., 2005).

Statistical analysis

Statistical analysis was done by Prism 5 software.

Results

Phytochemical extraction of Schleichera oleosa

S. Oleosa's tree parts (leaf, bark, seed, fruit coat and fruit pulp) were collected (Figure 1A) and phytochemical extraction was done by a methanolic extraction method and dried the extracts. The dry extract was weight and dissolved in DMSO (Figure1B,C) to make working solution of 50, 100, 120, 140, and 150 µg/ml.

Inhibitory concentration IC_{50} calculation of plant extract

The MTT assay was performed in MCF-7 cells using plant extracts. After 24hr treatment absorbance of MTT was recorded at 570 nm wavelength and percentage cell viability was calculated. Here Seed extract has shown their cytotoxic effects as IC_{50} values on MCF-7 cell lines at 140 µg/ml concentration after 24 h of treatment (Figure 2). Whereas the extract from leaf, bark, fruit coat and fruit pulp did not show any cytotoxic effects on MCF-7 cell lines. Now for further experiment we have used the IC_{50} value concentration (140 µg/ml) of seed extract.

Table 1. Phytochemicals Present on the Different Part of the *S. Oleosa* Plant. (Dan and Dan, 1986; Ghosh et al., 2011)

Sr. No.	Plant Materials (S. oleosa)	Phytochemicals
1	Bark	Lupeol, lupeol acetate, beta-sitosterol, scopoletin, taraxerone and tricadenic acid A, tannin, betulin and betulinic acid, schleicherastatins 1−7 and two related sterols, schleicheols 1 and 2.
2	Fruit	Luteolin, rutin, quercitin and kaempferol, phenolic acids, protocatechuic acid, vanillic acid, caffeic acid and syringic acid.
3	Seed oil	Oleic acid, Stearic acid, Gadoleic acid and arachidic acid as well as cyanogenic compounds, the oil also contains Linoleic acid, Palmitic acid, and hydrocyanic acid.

Table 2. Fatty Acid Profile of *S. oleosa* Seed Oil and Their Percent by HPLC- GC/MS (Basu, 1974; Palanuvej et al., 2008)

Sr. No.	Fatty acid	%
1	Myristic acid	0.01
2	Palmitic acid	7.59
3	Palmitoleic acid	1.8
4	Cis Oleic acid	2.83
5	Trans Linolelaidic acid	49.69
6	Cis Linoleic acid	5.56
7	alpha-Linolenic acid	0.26
8	Eicosenoic acid	29.54
9	Eicosadienoic Acid	0.24
10	Heneicosanoic Acid	0.04
11	Behenic Acid	1.14
12	Erucic acid	1.22
13	Lignoceric Acid	0.03
14	Docosahexaenoic Acid	0.02

S. Oleosa seed extract Inhibit colony formation on breast cancer

Clonogenic assay was performed in 6-well plates and treated with different concentrations of seed extract of *S. Oleosa* for 24hr and allowed to grow the cells until the colony of untreated cells reached nearby. Cell images were captured and the number of colonies counted with the help of J software, result (Figure 3) indicates that after drug treatment colony formation reduced at 120µg to 150µg are statically significant. It clearly indicates that S. Oleosa seed extract reduces colony formation after treatment and were significant (t-test P<0.0001, One-way ANOVA P<0.004, R2 = 0.8194).

High conc. of seed extract of S. Oleosa reduced the migration of breast cancer cells

The migration assay was performed by the scratch method in 6-well plates. Cells were treated with different concentrations of seed extract for 24 hrs and wound healing image was captured in every 6hrs from 0 to 24hrs using a microscope. The gap distance of the scratch was measured by Image-J software. Cell migration was gradually reduced from 120 to 150 µg/ml on seed extract treated cells, and however at 140µg/ml (P<0.0008) the cell migration was drastically reduced (Figure 4).

S. Oleosa seed extract reduced the Sphere formation of breast cancer cells

Sphere formation of MCF-7 cells was developed in ultralow attachment 96-well plate containing serum-free

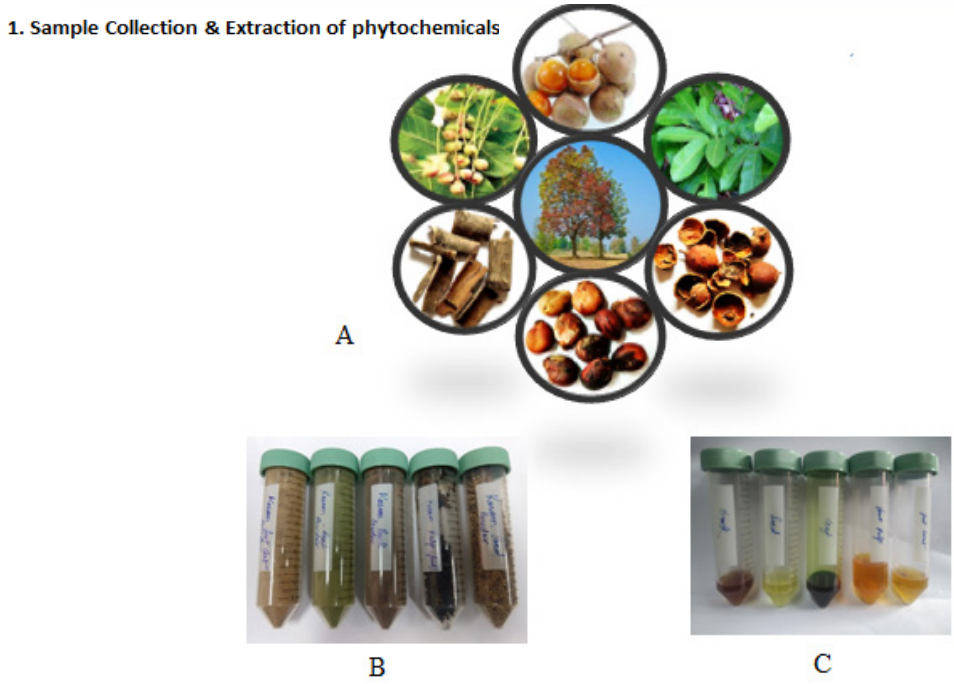

Figure 1. A, Kusum tree (*S. Olieosa*) and its different parts taken for the study Fruit, leaves, fruit coat, seed, bark, pulp; B, Fruit, leaves, fruit coat, seed, bark, pulp were washed dried and powdered in motor pestle; C, Methanolic extraction of Fruit, leaves, fruit coat, seed, bark, pulp were done and evaporated then weigh and dissolved in DMSO.

2. MTT Assay

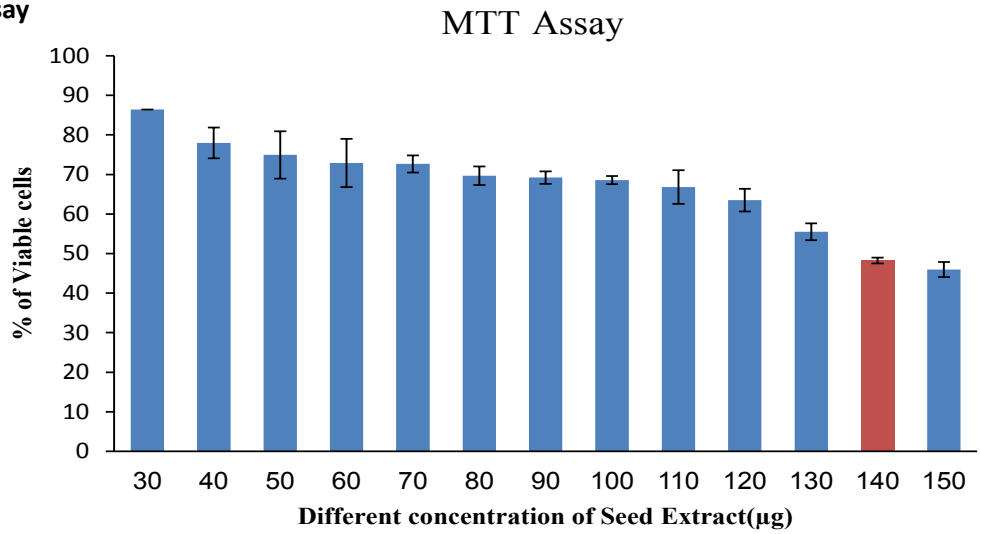

Figure 2. MTT Assay on MCF-7 Cells Treated with Different Concentration of Seed Extract of *S. Oleosa*

DMEM medium along with different concentrations of *S. Oleosa* seed extract, Cells were allowed to grow and formed sphere, the images of 1st, 3rd, and 5th days were captured. The diameter of spheres was measured by imaging software (ProCam, Radical scientific) of an inverted microscope (Figure 5). Sphere size was found significantly reduced after seed extract treatment at 140µg/ml ($P<0.0031$) and 150µg/ml ($P<0.0010$) (One-way ANOVA $P<0.0001$ $R^2=0.9412$) concentrations on 5th day.

3. Clonogenic assay

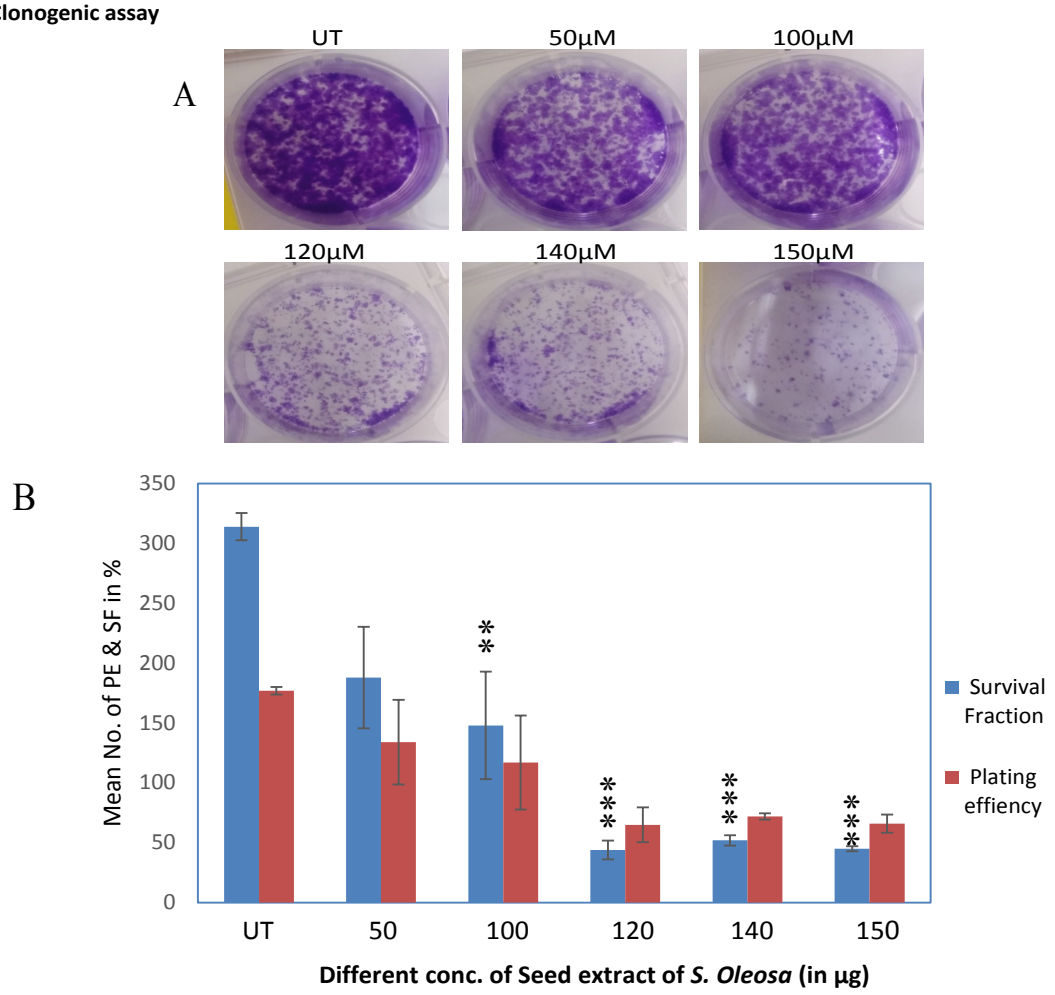

Figure 3. A, Clonogenic assay of MCF-7 cells treated with different concentration of seed extract; B, Survival and plating efficacy in different concentration.

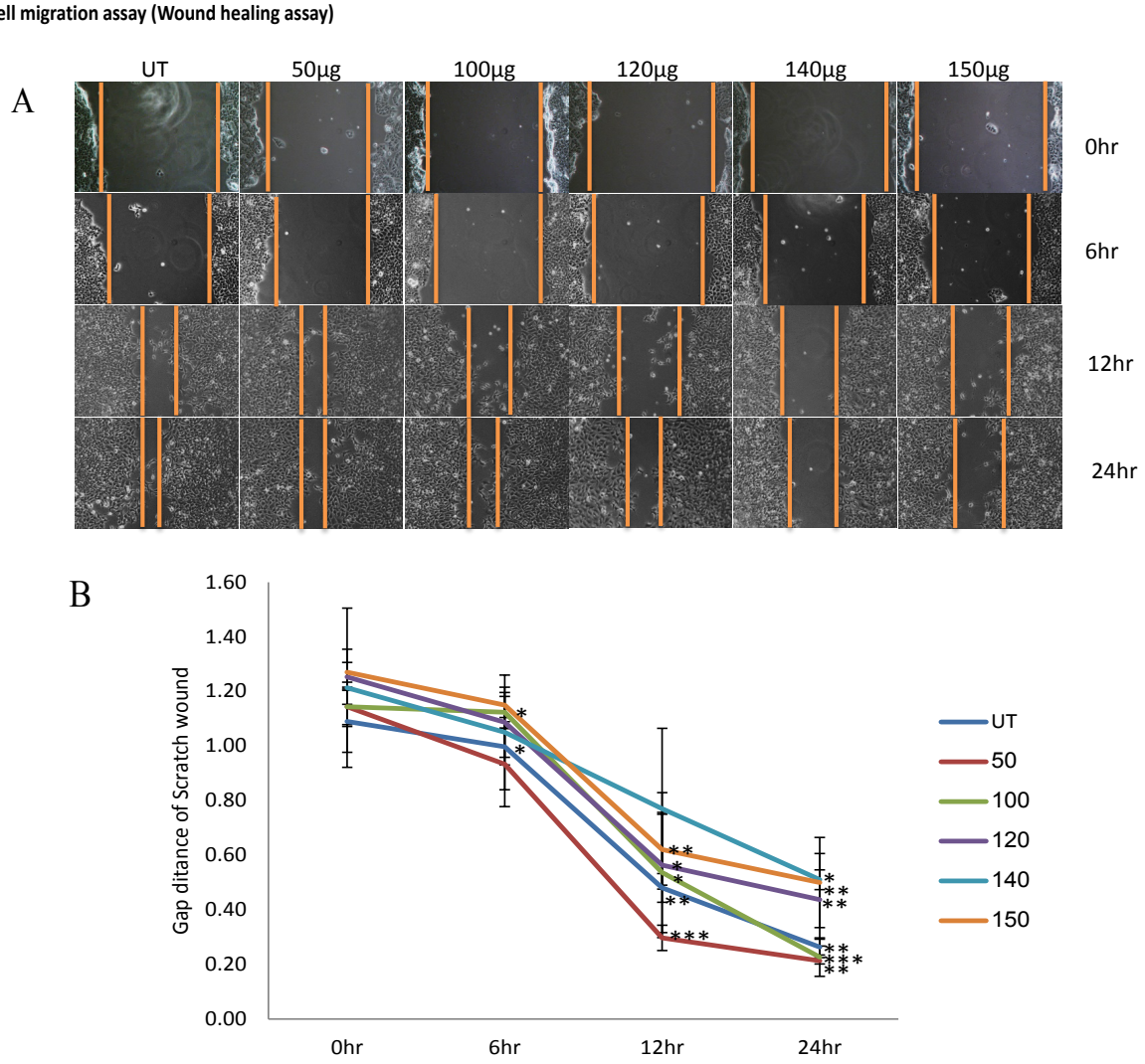

Figure 4. A, Migration assay of MCF-7 breast cancer cells treated with different concentration of seed extract; B, Graphical presentation of migration of cells in different time intervals treated with different concentration of seed extract of *S. Oleosa*

S. Oleosa seed extract has cytotoxicity and increases apoptosis in breast cancer cells

Apoptosis analysis on MCF-7 cells sphere and monolayer was done in 96-well plates; cells were treated for 24 h and stained with acridine orange/ethidium bromide stain. Image was captured at 485 nm wavelength by inverted fluorescence microscope and it showed that the number of stained cells was significantly increased in the seed extract treated MCF-7 cells Sphere at 120 to 150 µg where as it was highly significant at 140µg ($P<0.0061$) and 150µg ($P<0.0012$) of treated cells. Figure 6 (A-B) However in cells monolayer it was significant at 120µg ($P<0.0015$), 140µg ($P<0.0001$) and 150µg ($P<0.0007$) seed extract treated cells as shown in Figure 6 (C-D). Whereas it was drastically increased at 140µg/ml concentration of seed extract treated cells.

S. Oleosa seed extract up regulate BRCA1 and p16 gene expression in breast cancer

MCF-7 breast cancer cell line was treated with different concentration 50 µg to 150 µg of seed extract from Kusum tree (*S. Oleosa*). Genes expression of BRCA1 and p16 were performed by real-time quantitative PCR and observed that *BRCA1* expression was increased 1.4 fold as compare to control at 140µg/ml and it was statistically significant ($P<0.0299$).Similarly, *p16* gene expression was increased 1.7 fold as compare to control at 140 µg of seed extract and was significant ($P<0.0093$) (Figure 7A).

S. Oleosa seed extract up regulate BRCA1 protein expression in breast cancer

Expression of BRCA1 protein in western blotting experiments (Figure 7B,C) was up regulated as compare to control, however with increasing drug concentration from 100 to 140 µg/ml, ($P<0.0005$; $P<0.0009$; $P<0.0113$) expression of BRCA1 protein was significantly increased (One-Way ANOVA, $P<0.0001$, $R^2 = 0.8729$). Expression of p16 protein was down regulated at 140 and 150µg/ml (t-test, $P<0.0404$; $P<0.0001$) of seed extract treatment and (One-way ANOVA, $P<0.0001$, $R^2 = 0.8735$).

5. Sphere formation assay

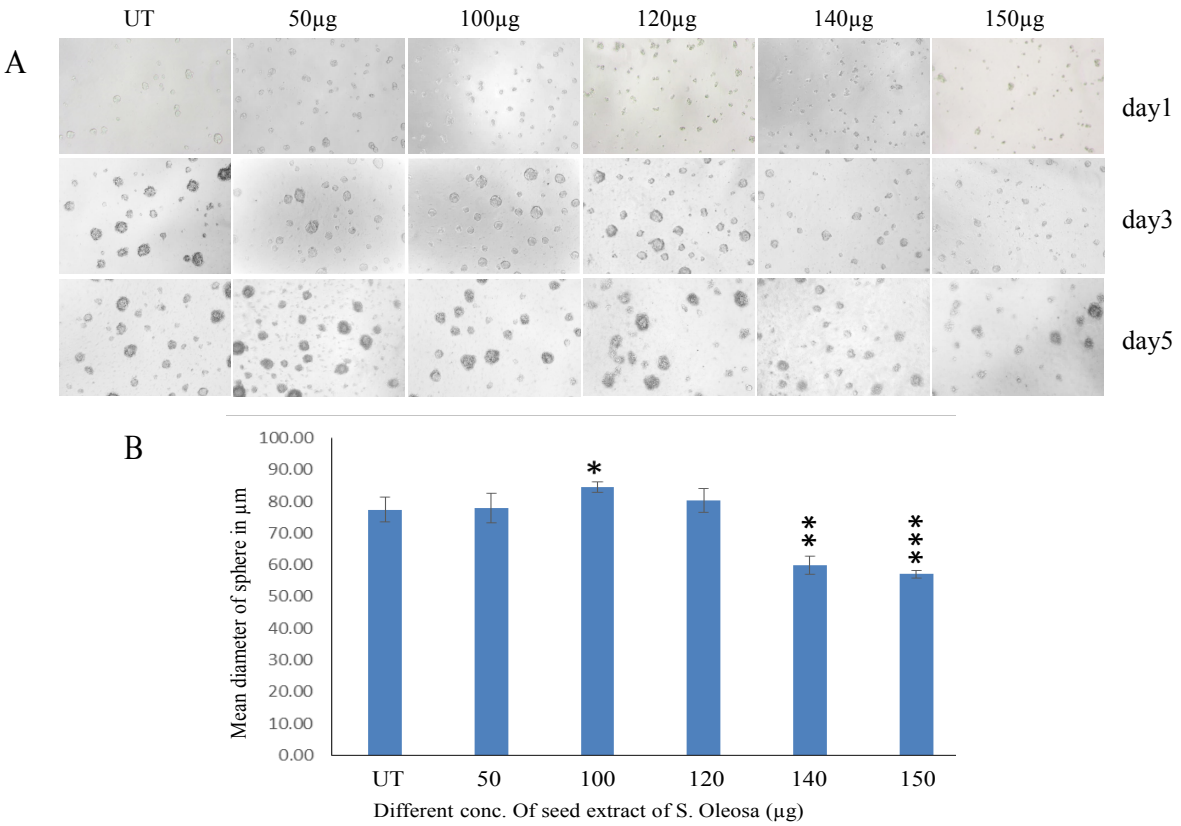

Figure 5. A, Sphere formation of MCF-7 breast cancer cells treated with different concentration of seed extract; B, Graphical presentation of sphere size treated with different concentration of seed extract at different time intervals.

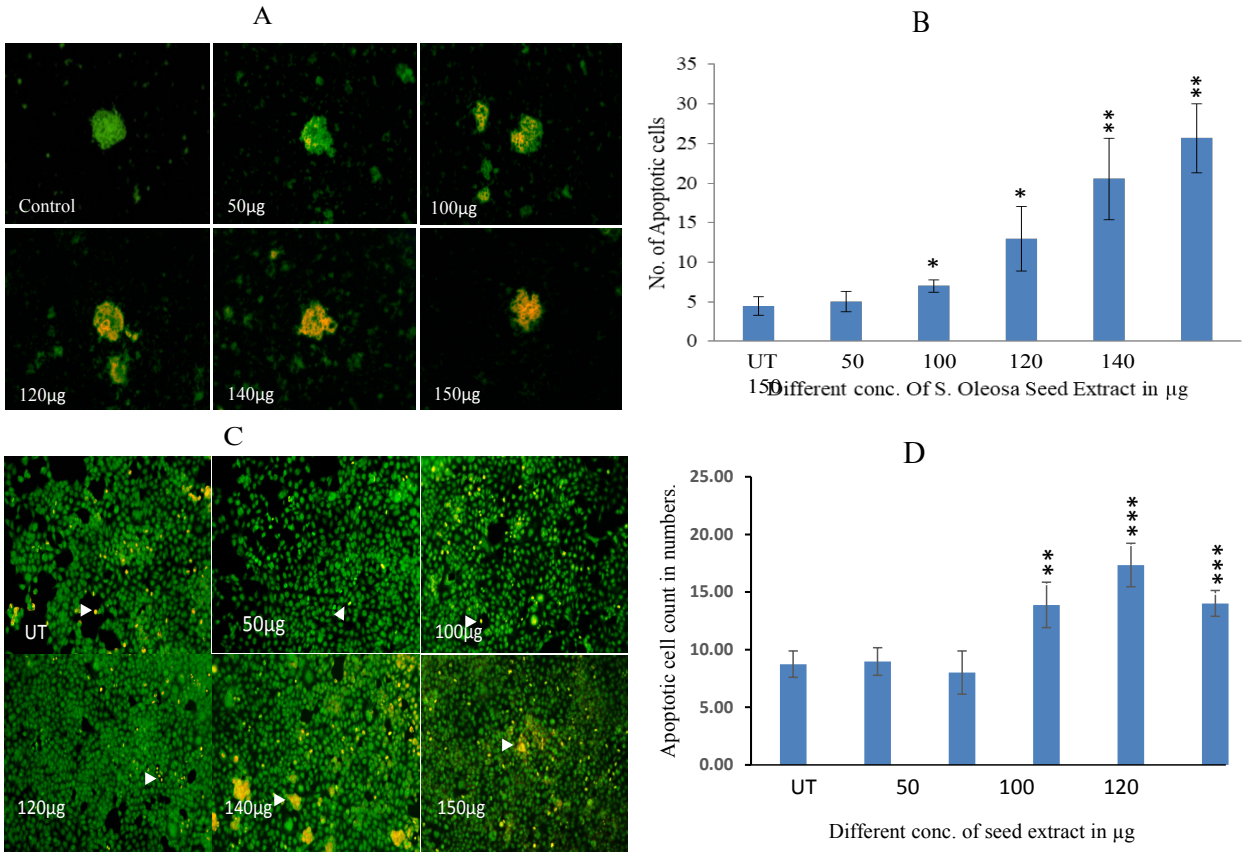

Figure 6. A-B, Cytotoxity of *S. Oleosa* seed extract on sphere cells of MCF-7 breast cancer cells. After 24 hr of treatment stained with acridin orange/ Etbr staining dye for apoptosis cells observation in florescence microsope; C, Apoptosis analysis of MCF-7 cells monolayer by acridin orange/etbr staining method treated with different concentration of seed extract; D, Its Graphical presentation of apoptotic cells in treated cells.

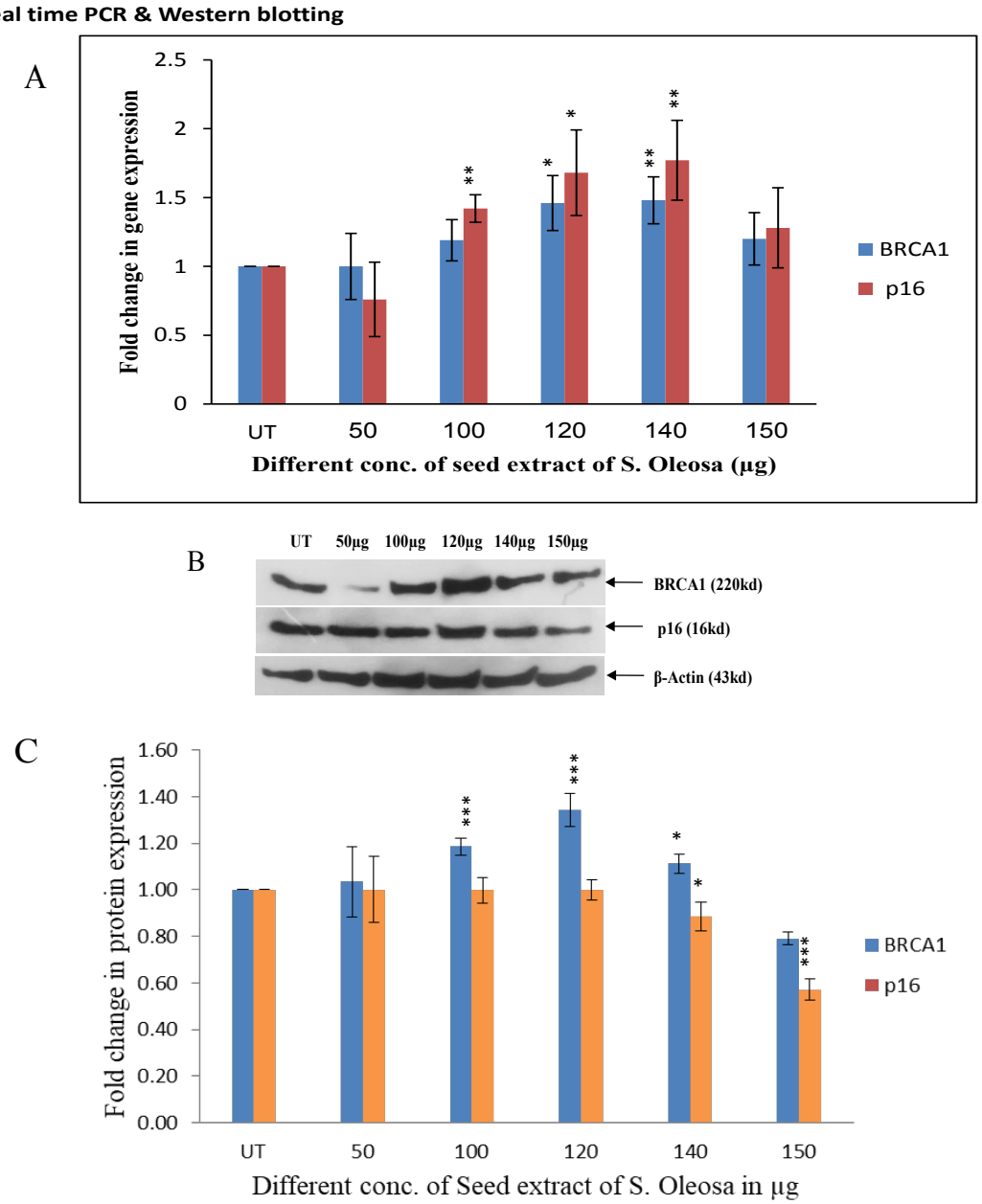

Figure 7. A, Real time gene expression analysis of *BRCA1* and *p16* genes in seed extract treated MCF-7 cell line by real time quantitative PCR normalized with *GAPDH* housekeeping gene; B, Protein expression analysis of *BRCA1, p16* and *β-Actin* gene by western blotting; C, graphical representation of protein expression normalized with housekeeping gene.

Discussion

About 60 percent of traditional medicines are used by the world's population. These are used for primary health care in rural areas not only in developing countries, but also in developed countries, where modern medicines are predominantly used. The traditional medicines are derived from medicinal plants, minerals, and organic matter, while the herbal drugs are prepared from medicinal plants only. Use of plants as a source of medicine has been inherited and is an important component of the healthcare system in India. It has been reported that Schleichera oleosa plant possesses antimicrobial, antioxidant, anticancer activity; this species contains important phytochemicals such as terpenoids, betulin, betulinic acid etc in the bark (Table 1) (Dan et al., 1986; Ghoshet al., 2011) and Seeds contains 16 fatty acid constituents (Table 2) Oleic acid (2.83%), Palmitic acid (7.59%), Trans-Linolelaidic acid (49.69%), CisLinolelaidic acid(5.56%), Eicosenoic acid (29.54%), etcanalyzed in GC-MS by Basu (1974) and Palanuvej (2008) out of these, Trans-Linolelaidic present 49.69% in the fatty acid constituents extracted by methanol in S. Oleosa seed oil. The studies also reveal that this medicinal plant can be used as an alternative to synthetic compounds for use in preventing and treating several diseases. Considering the medicinal uses of this plant further we have studied its anticancer properties in breast cancer cells.

We have collected Plant materials from ICMR-NICPR campus, Noida, and extracted the it's phytochemical by

methanolic extraction method followed by the published article as its methanolic extraction shown the anticancer property in lung and liver cancer (Thind et al., 2006; Larson et al., 2016). After dissolving in DMSO further, we have investigated its IC_{50} concentration and found that only seed extract at 140 µg/ml gives IC_{50}. However, we have also done MTT assay of other samples bark, leaves, pulp, and fruit coat but didn't get its cytotoxic effect on breast cancer cells.

Colony formation is the process where cells migrate from one site to other site and proliferate to form tumor. In this study, we have performed the colony formation assay as per procedure followed by Rafehi (2011) using seed extract of *S. Oleosa*. Interestingly, we have found significantly reduction in the colony formation as well as survival frequency at 120 to150 µg/ml seed extract. Cell proliferation is associated with many signaling pathways one of them is MEK/ERK. Inhibition of this pathway leads to cell cycle arrest and delayed cell growth (Wang et al., 2000). However, up-regulation of p16 and other tumor suppressor genes might be reduce the tumor cell growth as reported by Denchi (2005) in his previous work.

To check *S. Oleosa*'s anti-metastatic role in breast cancer cells, we have performed migration assay. It was done by the scratch method and gap filling was reduced from 120 to150 µg/ml seed extract treated cells. It might occur due to inhibitory effects of seed extract on histone deacetylase, resulting in down-regulation of cancer metastasis promoter genes such as MMP-2 and MMP-9 as well as up-regulation of cancer metastasis suppressor, e.g. E-cadherin in cancer cells (Yang et al., 2017). Due to down regulation of these genes metastasis of cancer cells inhibited or reduced in seed extract treated breast cancer cell.

We have also studied the tumor forming capacity of breast cancer cells in the presence of seed extract of S. oleosa and mimicked the tumor in vitro as mamosphere model. Mamospheres were developed using ultra-low adherence 96 plate having serum free media containing *S. Oleosa*'s Seed extract and we found that a significant reduction in sphere formation after treatment with seed extract might happened due to low expression of *E-cadherin* gene which is responsible for short life and lower size of mamosphere formation as reported previously by Lombardo, (2015).

The cytotoxic effect of S. Oleosa's seed extract on MCF-7 cells were analyzed using acridin orange and Etbr staining method and found that it was gradually increased the number of apoptotic cells from 120 to 150 µg/ml concentration. It was significantly increased at 140 µg/ml concentrations which are seen by dual staining of acridin orange and Etbr stain. Early to late stages of apoptotic cells were seen as yellow and orange color, respectively, however necrotic cells were in red color as reported by Liu (2015) in previously published article.

BRCA1 has multiple cellular functions along with the DNA-binding activity. Its nuclear-localizing region interacts with *TP53, RB, ATM, BRCA2*, and other genes involved in DNA repair and transcriptional regulation (Haber, 2000; Hofmann et al., 2000). In response to DNA damage, the BRCA1 protein is phosphorylated by ATM protein and then moves to the nucleus, where it becomes part of a DNA repair multiprotein complex to mediate homologous DNA repair (Yang et al., 2017).In this study, we have found up-regulation in *BRCA1* gene expression that might be involved in DNA repair mechanisms which could have enhance the other tumor suppressor genes expression and may reduce the oncogene level.

The p16-Rb pathway controls G1 to S transition (Yang et al., 2017). Irreversible arrest of cell growth is due to cellular senescence and its molecular mechanism involves p16 and p53 tumor suppressor genes and telomere shortening (Rayess et al., 2012) p16 protein, through p16-Rb pathway mediates senescence mechanisms by inhibiting the CDK4/6 leads to cell cycle arrest in G1 phase. Retinoblastoma (Rb) is maintained in hypo phosphorylated state resulting in the inhibition of transcription factor E2F1 localization to the nucleus and thus preventing its target genes expression which are crucial for the G1/S transition (Yang et al., 2017). It has also been shown that p16-Rb pathway linked to the mitogenic signaling cascade for inducting reactive oxygen species which in turn activates the protein kinase C delta that leads to irreversible cell cycle arrest (Takahashi et al., 2006). In our study we have found up-regulation of *p16* gene. Thus, p16 seems to play an important role in the cell cycle and cellular senescence.

Based on the above results, we have concluded that *BRCA1* and *p16* genes pathway could have induce the expression of tumor suppressor genes and cell cycle arrest respectively. Further, it would have reduced the migration and sphere formation of the cells which leads to increase apoptotic condition of breast cancer cells treated with *S.Oleosa* seed extract. Based on this preliminary study, *S. Oleosa* plant seed extract might have an anti-cancer potential in breast cancer therapy.

In conclusion, based on above mentioned results, we concluded that *S. Oleosa* seed extract has anti metastatic, anticancer property in breast cancer by up regulated expression of *BRCA1* and *p16* genes. Further details phytochemical analysis study should be done to identification of compounds responsible to induces apoptosis in breast cancer cells. *S. Oleosa* seed extract (Kusum oil) may be used to understand the molecular mechanism in breast carcinogenesis.

Author Contribution Statement

Ram Krishna Sahu:Conceptualization, Investigation, Methodology and Writing, Dr. Binayak kumar, Dr. Ragini Singh and Dr. Soni kumari: Methodology and Review the paper. Prof. Simran Tandon, Prof. Bhudev Chandra Das Review and Editing. and Dr. Suresh T Hedau: Critical review the manuscript and Editing.

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Data availability

This article and its supplementary file include all data generated andanalyzed during the study.

Conflict of Interest

The authors declare no conflict of interest.

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