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Research Article
Phytochemical Analysis, Antioxidant and Cytotoxic Potentials of *Pelargonium graveolens* Extract in Human Breast Adenocarcinoma (MCF-7) Cell Line

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Abstract

Background: *Pelargonium graveolens* is an important medicinal plant traditionally used to heal wounds, ulcers, skin disorders and diarrhea. Despite its well-known antioxidant activity, the cytotoxicity of *P. graveolens* crude extract has not been well documented. **Methodology:** Methanolic fraction was extracted from *P. graveolens* leaves and was analyzed by GC/MS. Two modes of experiments were performed: First, assessing antioxidant activity using DPPH free radicals, while the second, detecting the effect of the extract on MCF-7 cell morphology, viability, stress responses, mitochondrial potential and Reactive Oxygen Species (ROS) generation were examined with MTT and High Content Screening (HCS) scanning. **Results:** Twenty four compounds have been identified which constitute 90.02% of the extract. A dose-dependent reduction in DPPH was recorded with IC_{50} of 484 μg mL^{-1}. The MTT results showed a dose-dependent anti-tumor activity and reduction in cell viability against MCF-7 cells with IC_{50} of 288 μg mL^{-1}. Further examination of HCS results indicated a dose-dependently growth inhibition of MCF-7 cells (53.92% at 200 μg mL^{-1}). Membrane permeability also increased in a dose-dependent manner, while only high concentrations caused alterations in nucleus morphology. A 40.3% reduction in the mitochondrial potential was observed. Such disruptions resulted in the release of cytochrome C, for which a 0.7 fold increase was detected in the treated MCF-7 cells compared with untreated cells. Treatment also resulted in a 0.53 and 1.32 folds increases in generation of ROS at 100 and 200 μg mL^{-1}, respectively. **Conclusion:** Methanolic extract of *P. graveolens* exhibited a significant antioxidant activity and has profound cytotoxic activity against MCF-7 cell line.

Key words: High content screening, antioxidant, *Pelargonium graveolens*, cytotoxicity, MCF-cell line

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

For centuries plants were considered to be one of the most abundant sources of medicines and drugs. The vast diversity of plant species all around the world as well as their constituents and active compounds, made their applications range from simple traditional rituals into disease specific treatments, after extensive research on chemical composition, mode of action and toxicity.

One of these plants is *Pelargonium graveolens*. It is an aromatic plant, belonging to the Geraniaceae family that grows in temperate areas of the world[1]. Essential oils are the major components of *P. graveolens.* The oils mainly consist of β-citronellol, geraniol, δ-selinene and l-menthone[2]. In addition to its benefits in the food and beverage industries[3], current research has focused on the importance of *P. graveolens* essential oils and their wide array of biological activities. Examples of this include: Antimicrobial and antimalarial activity[4] and antiasthmatic, antiallergic, antidiarrhoeic and antihepatotoxic activities[5]. Extracts of *P. graveolens* have been shown to possess antioxidant properties and the ability to scavenge free radicals and reduce reactive oxygen species[6,7]. In addition, some reports suggest that extracts of *P. graveolens* exhibit a cytotoxic and antitumor activity[8,9]. Haag *et al.*[10] proposed monoterpenes as the principle component of geranium essential oils, which have been shown to prevent mammary, lung, skin, liver and stomach cancers in rat models. However, few studies revealed the exact cytotoxic effects of *P. graveolens* extract on tumor cells and the cell morphological changes that occur after exposure to the extract.

Identifying the toxicity of new drugs is an important step before beginning marketing and general consumption of new drugs. Many procedures are currently used to detect the level of cytotoxicity of new compounds or drugs. Multi-parametric analysis is one of the most common approaches and uses flow cytometry and cellular imaging–based techniques, including high-content screening (HCS) to detect the level of compound toxicity and classification of the compound based on observed patterns of reversible and irreversible cellular injury[11,12]. It provides an easy and rapid method for identifying substances with apoptosis inducing characteristics using high throughput analysis. The standard measurements that multi-parametric analysis provides are (a) Nuclear morphology and intensity, (b) Mitochondrial trans-membrane potential, (c) Plasma membrane permeability, (d) Cell proliferation and (e) Cytochrome C release[13]. Accordingly this study aims to evaluate the antitumor and antioxidant properties of *P. graveolens* crude extracts *in vitro* using the human breast adenocarcinoma MCF-7 cell line via detection of the potential antioxidant activities through the DPPH-scavenging assay and investigate the anti-tumor properties by employing MTT assays and HCS for detecting the following cellular parameters: Cell viability, total nuclear intensity, membrane permeability, mitochondrial membrane potential changes, cytochrome C release and ROS generation.

MATERIALS AND METHODS

Materials: All chemicals were thankfully provided from Al-Nahrain University, Department of Biotechnology. The MTT kit was purchased from Intron Biotech (Korea), while Cellomics® Multiparameter Cytotoxicity 3 kit and ROS Cellomics kit were purchased from ThermoScientific (USA).

Plant materials: Leaves of *P. graveolens* were collected from local plant nurseries in Baghdad, Iraq. The collected leaves were washed thoroughly under tap water to remove trace chemicals and dust. After thorough washing, the *P. graveolens* leaves were dried by incubation overnight at 37°C. After incubation the fully dried leaves were blended in order to obtain a fine powder, which was used for extraction purpose.

Methanolic extraction: Dried powder (100 g) of *P. graveolens* leaves was treated with 500 mL methanol. The mixture was homogenized with a magnetic stirrer for 4 h at room temperature and then filtrated using Whatman filter paper No. 1. The filtrate solvent was cooled and centrifuged with 1500 rpm at 15°C for 30 min. The crude fraction was evaporated at 45°C using a rotary evaporator (IKA, Germany) under vacuum to obtain the final dried *P. graveolens* crude extract. The resulting pellet was subjected to further extraction by repeating the same procedure. The methanol fraction yielded 1.4 g.

GC/MS analysis: The *P. graveolens* extract was analyzed by GC-2010 Plus (Shimadzu, Japan) equipped with DB-5MS column (30 m long 0.25 mm i.d. and 0.25 μm thick, Agilent Technologies, J and W Scientific Products, USA). The injector and detector temperature were set at 250 and 230°C, respectively. The oven temperature started at 100°C and raised at a rate of 5°C min^{-1} then held at 260°C for 1 min. Aliquot of 1 μL of sample was injected and helium was used as the carrier gas. The mass range was scanned from 50-550 amu. Identification for possible compounds and essential oils was carried out in Ministry of Science and Technology, Department of Water and Environmental Research (using NIST Library).

Evaluation of antioxidant activity using DPPH: Antioxidant activity was detected by using the DPPH radical scavenging assay according to the previously described procedure[14]. Aliquots (0.5 mL) of 2 folds serial dilutions of the extract and ascorbic acid (75, 125, 250, 500 and 1000 μg mL^{-1}) were added in reaction test tubes. Simultaneously, 3 mL of methanol-DMSO (9:1 v/v) and 0.3 mL of DPPH solution (0.1 mg mL^{-1}) were added to each concentration. Samples were shaken for a few seconds and allowed to stand in the dark at room temperature for 60 min. After 60 min the absorbance detected against bank at 517 nm. The percent inhibition of radical formation in the samples was calculated according to the following formula:

$$\text{Percentage of decoloration} = \left(\frac{\text{Control absorbance-Sample absorbance}}{\text{Control absorbance}}\right)\times 100$$

Tests were carried out in triplicate and the IC_{50} values of the samples were calculated using a log dose inhibition curve.

Cell line culture: Human breast adenocarcinoma MCF-7 cells[15] were obtained from American Type Culture Collection (ATCC) and cultured in DMEM medium supplemented with 10% fetal bovine serum, 1% sodium bicarbonate, 10^3 IU penicillin G and 100 μg mL^{-1} streptomycin. The MCF-7 cells were maintained in a CO_2 incubator (5%) at 37°C.

MTT cytotoxicity assay: Protocol was performed following the manufacturer instructions. Cells (1×10^4 to 1×10^6 cells mL^{-1}) were grown in a 96 flat-well plate with a final volume of 200 mL $well^{-1}$. The plate was covered by sterilized parafilm, agitated gently and incubated at 37°C, 5% CO_2 for 24 h. After incubation, the medium was removed and 200 mL of the 2 folds serial dilutions of *P. graveolens* crude extract (25, 50, 100, 200, 400 mg mL^{-1}) were added to the wells. Triplicates were performed at each concentration as well as controls. Plates were incubated at 37°C, 5% CO_2 for 24 h. After exposure to extract, 10 mL of the MTT solution was added to each well. Plates were further incubated at 37°C, 5% CO_2 for 4 h. The medium was then carefully removed and 100 mL of solubilization solution was added per well and incubated for 5 min. The absorbance was determined with an ELISA reader (Bio-rad, Germany) at a wavelength of 575 nm. The optical density readings were subjected to statistical analysis in order to calculate[16] the IC_{50}.

Multi-parameter cytotoxic assay: The multi-parameter cytotoxicity assay was performed to measure the five orthogonal MCF-7 cell health parameters after exposure to *P. graveolens* crude extract *in vitro*. The parameters were: Viability cell count, total nuclear intensity, cell membrane permeability, mitochondrial membrane permeability and cytochrome c release. Briefly, after 24 h of exposure with different concentrations of *P. graveolens* crude extract, the treated MCF-7 cells were stained with cell staining solution (MMP dye+permeability dye) for 30 min at 37°C. Cells were fixed, permeabilized and blocked before probing with primary cytochrome C antibody and secondary DyLight 649 conjugated goat anti-mouseIgG for 60 min each. Plates were analyzed using the ArrayScan HCS analyzer (ThermoScientific, USA).

ROS detection: MCF-7 cells (1×10^4) were seeded in 96-well plates for 24 h prior to exposure with different concentrations of *P. graveolens* crude extract. At the indicated time, 50 μL of staining solution (DMEM), containing 500 nM Hoechst 33342 and 2.5 μg mL^{-1} dihydroethidium (DHE) were added to each well. Plates were incubated at 37°C for 30 min. The cells were fixed with PBS containing 3.5% formaldehyde for 15 min at room temperature. Finally, cells were washed with PBS and plate was evaluated using an ArraySacn HCS analyzer (ThermoScientific, USA).

Statistical analysis: A one-way analysis of variance (ANOVA) was performed to assess whether group variance was significant. Data were expressed as Mean±Standard Deviation (SD) and statistical significances were carried out using a GraphPad Prism version 6.

RESULTS

GC/MS analysis: The GC/MS analysis of *P. graveolens* methanol extract was listed in Table 1 and it was found that the extract contained 24 identified constituents which represent 90.02%.

The major component was citronellol 22.61% followed by palmitic acid 10.59, decanediol 10.39%, geraniol 9.04%, l-menthone 5.29%, pyroglutamic acid 4.83, linalool 4.82%, stearic acid 2.96%, germacrene-d 2.67%, agarospirol 2.43%, geranyl tiglate 2.38%, linalool oxide 2.23%, germacrene D 1.68% and γ-cadinene 1.38%. In addition 10 components were present in amount less than 1%. On the other hand, 9.98% out of the total was considered as non-identified constituent.

Antioxidant activity of *Pelargonium graveolens* crude extract: The scavenging activity of *P. graveolens* methanol extract was evaluated using stable DPPH free radical scavenging assays. Results, in Fig. 1, revealed that with

Table 1: Chemical composition of *P. graveolens* methanolic extract

Compounds	Peak area (%)
Germacrene D	1.68
α-pinene	0.50
Limonene	0.95
Linalool oxide	2.23
Linalool	4.82
Trans-rose oxide	0.47
Citronellol	22.61
Geraniol	9.04
β-myrcene	0.42
l-menthone	5.29
Geranyl acetate	0.90
Phenyl ethyl tiglate	0.67
γ-cadinene	1.38
Agarospirol	2.43
Decanediol	10.39
Pyroglutamic acid	4.83
Stearic acid	2.96
Geranyl tiglate	2.38
Propanoate	0.91
1-bromooctane	0.73
Palmitic acid	10.59
Germacrene-d	2.67
Epizonaren	0.49
α-humulene	0.68
Non-defined	9.98

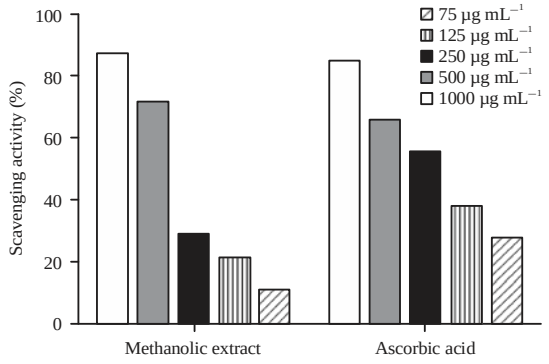

Fig. 1: DPPH free radical scavenging activity of methanolic extract of *P. graveolens* depending on concentrations compared with ascorbic acid

increasing concentrations of extract, the higher the percentage of scavenged free radicals. The concentrations that were used ranged from 75-1000 μg mL^{-1} and the extract showed significantly high antioxidant activity (87.7% at 1000 μg mL^{-1}).

The above result was compared with the free radical scavenging activity of ascorbic acid as a positive control, in which, according to the Fig. 1, the extract showed almost the same dose-dependent pattern as ascorbic acid free radical scavenging activity, especially at concentrations of 500 and 1000 μg mL^{-1}.

The was calculated and demonstrated that the inhibitory effects of methanol and ascorbic acid for DPPH were 484 and 292 μg mL^{-1}, respectively.

Table 2: Count of viable cell after exposure to different concentrations of *P. graveolens* methanolic extract on MCF-7 cells

Treatment (μg mL^{-1})	Viable cell count (Mean±SD)	Inhibition (%)
Untreated cells	3975±35.35	0.00
Doxorubicin 20 μM	1050±98.99	72.27
200	1908±63.36	53.92
100	2839±172.45	29.50
50	3377±94.04	15.12
25	3567±55.15	10.20

Cytotoxic effects of *P. graveolens* extracts on MCF-7 cells using the MTT assay: The assay of 3-(dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was used to determine the cytotoxic effects of *P. graveolens* extract on MCF-7 cells. This assay was performed to measure the cell viability and inhibition rate through applying different concentrations of *P. graveolens* extract to the tumor cell lines.

Data analysis carried out in μg mL^{-1} and log values of μg mL^{-1} was plotted on a Graphpad Prism 6 using log (Inhibitor) versus response curve. The most effective concentrations were chosen based on the most significant IC_{50} values. Cell viability at each time-point was determined by MTT colorimetric assays.

Results indicated that exposure of MCF-7 cells to methanol extract at concentrations from 25-400 μg mL^{-1} for 24 h showed a reduction in cell viability in a dose-dependent manner, in which the cell viability decreased with increasing concentrations of methanolic extract. The lowest MCF-7 cell viability (38%) was recorded at 400 μg mL^{-1}. On the other hand, the extract significantly showed the most potent cytotoxic activity with an IC_{50} value of 288 μg mL^{-1}.

Multi-parameter cytotoxic activity of *P. graveolens* extract: A multi-parameter cytotoxic activity with the *P. graveolens* methanolic extract was implemented in HCS using MCF-7 cells. Five different measurements (cell count viability, nuclear intensity, cell membrane permeability, mitochondrial membrane potential and cytochrome C release) were detected in this assay and images of treated (extract and doxorubicin) and untreated MCF-7 cells were also captured.

Results showing in Fig. 2, demonstrate that the viable count of MCF-7 cells decreased with increasing concentrations of *P. graveolens* extract when compared with untreated cells. The reduction in cell count (percentage inhibition rate) was observed at rates of 10.20, 15.12, 29.5 and 53.92% for cells treated with 25, 50, 100 and 200 μg mL^{-1} of *P. graveolens* methanolic extract, respectively (Table 2).

This result suggests highly that the methanolic extract is cytotoxic against MCF-7 cells as observed by the MTT assay, in which the reduction of the cell count was dose dependent and the most significant reduction appeared ($p<0.0001$) after applying a high dose (200 μg mL^{-1}) of extract.

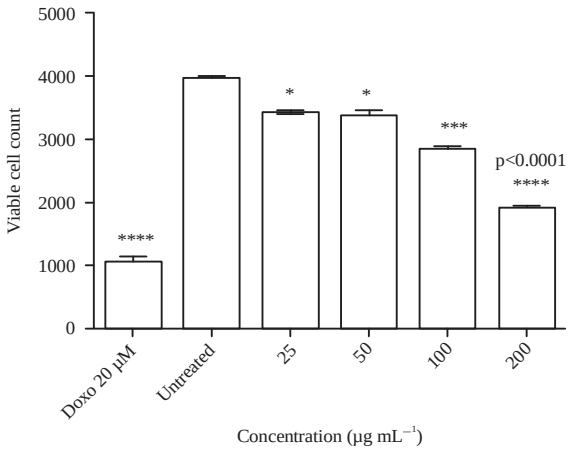

Fig. 2: Reduction in MCF-7 cell count after 24 h exposure to different concentrations of *P. graveolens* methanolic extract at 37°C and evaluated on the ArrayScan HCS reader, mean differences for significance were made between the untreated cells and the whole group

Table 3: Effect of different concentrations of *P. graveolens* methanolic extract on nuclear intensity, CMP, MMP and cytochrome C release

Treatment (µg mL^{-1})	NI	CMP	MMP	Cytochrome C
	(Mean±SD)			
Untreated cells	360±15.50	88±10.60	855±21.21	318±12.63
Doxorubicin 20 µM	1109±14.10	347±31.81	240±20.50	802±60.39
200	815±28.76	292±17.67	510±28.46	535±13.17
100	609±36.99	208±10.60	712±11.32	362±15.55
50	430±28.20	182±90.89	748±36.55	322±14.84
25	383±40.90	176±15.55	383±40.90	176±15.55

NI: Nuclear intensity, CMP: Cytoplasmic membrane permeability, MMP: Mitochondrial membrane potential

This result suggests highly that the methanolic extract is cytotoxic against MCF-7 cells as observed by the MTT assay, in which the reduction of the cell count was dose dependent and the most significant reduction appeared ($p<0.0001$) after applying a high dose (200 µg mL^{-1}) of extract.

Figure 3 shows the representative images acquired from the multi-parametric (nuclear intensity, cell membrane permeability, mitochondrial membrane potential and cytochrome C release) cytotoxic effects on a MCF-7 cell nucleus and mitochondria. The exposure of MCF-7 cells to *P. graveolens* extract at higher concentrations causes increased nuclear size, due to nuclear swelling and increased cell membrane permeability. The morphology of the nucleus (Hoechst blue) showed a nuclear condensation, which often appeared at high concentrations of *P. graveolens* extract (100 and 200 µg mL^{-1}). Both these effects were significantly different ($p<0.1$ and $p<0.0001$, respectively) from that of untreated cells (Fig. 4a) and such events were not induced at lower concentrations of *P. graveolens*.

On the other hand, Fig. 4b shows that the intensity of MCF-7 cell membrane permeability (green) gradually increased in a dose dependent pattern and that significant increase were observed after exposure to 100 and 200 µg mL^{-1} of *P. graveolens*. Data analysis (Table 3) for the resulting fluorescent intensity of the extract and doxorubicin treated cells, compared with that of untreated cells, revealed that the nuclear intensity significantly increased approximately 1.3 fold after exposure to 200 µg mL^{-1} of the extract, compared with 20 µM doxorubicin, a strong antitumor chemotherapy intercalating DNA molecule that causes massive DNA damage[17], which showed a 2 folds increase in intensity.

However, the average fluoresce data for the cell membrane permeability showed a dose dependent pattern, starting from 1 fold at 25 µg mL^{-1} and increasing to 2.3 folds at 200 µg mL^{-1}, with significant differences (ranging from $p = 0.01$ to $p<0.0001$) as compared with untreated MCF-7 cells.

Another two parameters, mitochondrial membrane potential and cytochrome C release were also measured. In comparison as shown in Fig. 5a, only higher concentration of *P. graveolens* extract (200 µg mL^{-1}) induced a significant (40.3%, $p<0.0001$) reduction in mitochondrial membrane potential, while the positive doxorubicin control cause a reduction up to 71.9%. The measurement of mitochondrial membrane potential depended on the average intensity

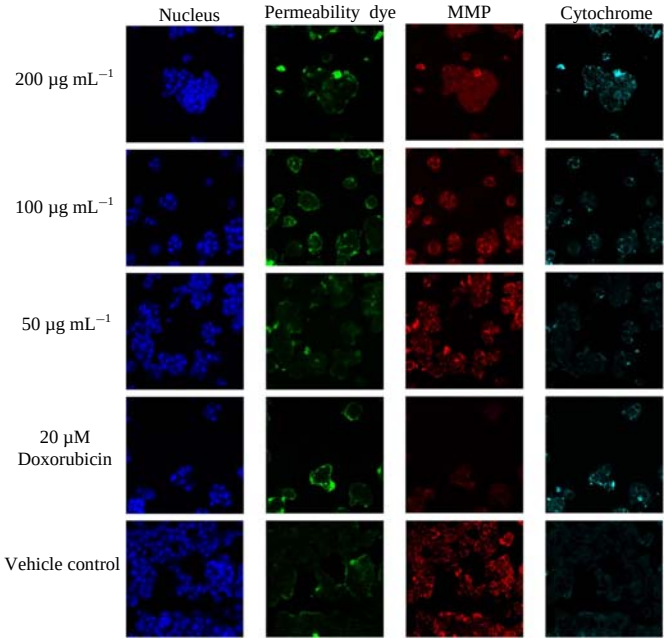

Fig. 3: Multiparameter cytotoxicity (HCS) analysis of *P. graveolens* extract-treated MCF-7cell line after 24 h of incubation at 37°C. Cells were stained with Hoechst 33342 (Blue) (Ex 330 nm/Em 420 nm) dye which enables monitoring of cell loss, nuclear morphology changes and DNA content, permeability dye (Green) (Ex 491 nm/Em 509 nm) for membrane permeability monitoring, MMP dye (Red) (Ex 552 nm/Em 576 nm) for mitochondrial membrane potential changes and with goat anti-mouse secondary antibody conjugated with DyLightTM for cytochrome C releasing

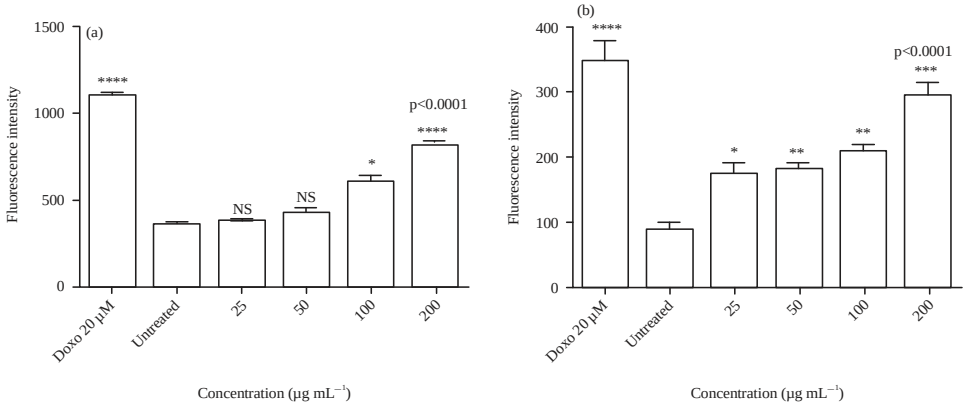

Fig. 4(a-b): Effects of *P. graveolens* methanolic extract exposure on MCF-7 cell line for 24 h, (a) Neuclear intensity effect and (b) Cell membrane permeability effect. The effect was evaluated on the ArrayScan HCS reader, mean differences for significance were made between the untreated cells and the whole group

of MMP dye that punctuate the mitochondria within the per-nuclear cytoplasmic region[18] and the lower the fluorescent intensity, the higher the effect against the mitochondria. On the other hand, the release of cytochrome C was only significantly ($p = 0.0005$) induced after MCF-7 cell were exposed to 200 µg mL^{-1} extract (Fig. 5b). The release of cytochrome C was detected by a 0.7 fold increase in the average fluorescent intensity of the treated MCF-7 cells, compared to untreated cells, whereas doxorubicin recorded a 1.5 fold increase in intensity (Table 3).

Detection of ROS: The ability of *P. graveolens* to affect the intracellular redox parameters related to oxidative stress was detected by measuring ROS generation, using

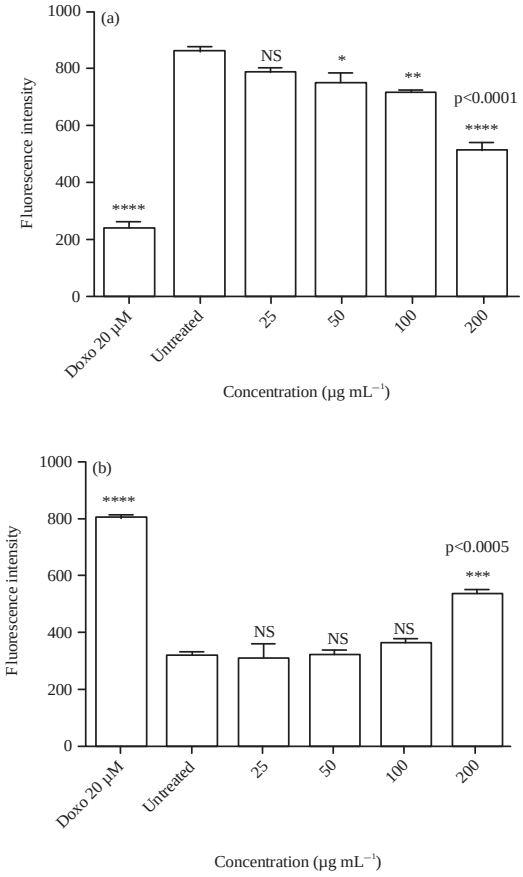

Fig. 5(a-b): Effects of *P. graveolens* methanolic extract exposure on MCF-7 cell line for 24 h, (a) Mitochondrial membrane potential and (b) Cytochrome C release. The effect was evaluated on the ArrayScan HCS reader; mean differences for significance were made between the untreated cells and the whole group

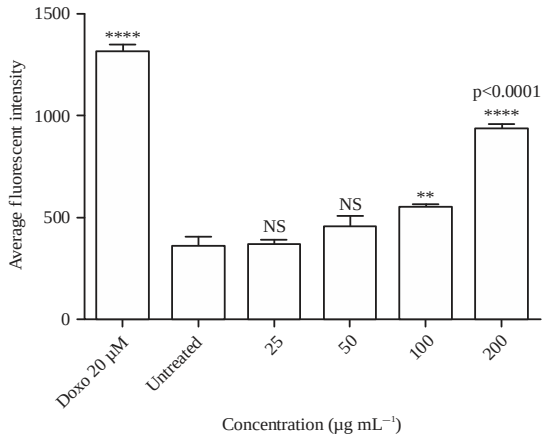

Fig. 6: Effect of different concentrations of *P. graveolens* methanolic extract on ROS generation in MCF-7 cells

Table 4: Mean intensity of different *P. graveolens* methanolic extract concentrations on ROS induction in MCF-7 cells

Treatment (µg mL^{-1})	Intensity (Mean±SD)
Untreated cells	359±14.67
Doxorubicin 20 µM	1312±38.18
200	934±22.62
100	552±13.34
50	453±20.50
25	367±11.58

MCF-7 tumor cells, for 24 h. The level of ROS was determined using Cellomics ROS Kit (ThermoScientific).

Figure 6 shows that only higher concentrations of *P. graveolens* extract exhibited significant increases in the ROS levels, specifically 0.53 fold ROS generation at 100 µg mL^{-1} and 1.32 folds ROS generation at 200 µg mL^{-1} as compared with untreated MCF-7 cells. Meanwhile, lower concentrations of *P. graveolens* extract showed no significant alteration in redox balance, also as compared with non-treated cells (Table 4).

DISCUSSION

The DPPH is characterized as a stable radical, owing to the delocalization of the spare electron, with a maximum absorption at approximately 517 nm and it has been frequently used to determine the antioxidant activity of different substances, including plant extracts[19].

The antioxidant activity of the *P. graveolens* methanolic extract can be attributed, at least partially to the presence of essential oil compounds, including β-citronellol and geraniol and its ability to reduce free radicals by quenching reactive oxygen species and trapping radicals before they reach their cellular targets[5]. Many studies have exposed the radical-scavenging activity of extracts and essential oils of *P. graveolens* using the DPPH assay with values ranged from 63.70 mg mL^{-1} (leaves) to 64.88 mg mL^{-1} (stems) for essential oils[20]. In addition, Marangoni and de Moura[21], mentioned that the use of essential oil extracted from *Coriandrum sativum* presented stronger synthetic antioxidant effects than butyl hydroxyl toluene on the delay of lipid oxidation.

Free radical scavenging activity is highly dependent on the type of solvent used in the extraction process, due to the differences in polarities and extraction abilities of the potentially antioxidant phytochemicals[22]. Since methanol is an amphiphilic compound, it's capable of extracting most of the various chemical groups from the plant material. Therefore, the methanol extract of *P. graveolens* had the highest activity among different extracts[23]. This observation is

in agreement with a previous study of Chahmi *et al.*[24], in which they compared the antioxidant power against DPPH of *Inula viscosa* extracts using different organic and alcoholic solvents. They also reported that ethanol was more effective organic solvent than ethyl acetate.

Free radicals play a key role in the development of a number of adverse health conditions, including cancer, cardiovascular disease and cataracts and have also been responsible in both initiation and acceleration of the aging process[25]. It is well known that free radicals cause oxidation, which can be controlled or prevented through a range of antioxidant substances[26].

Natural antioxidants present in medical plants may be useful as a treatment for the prevention of oxidative damage during aging[27]. Many medicinal plants have great antioxidant potential, which can reduce the oxidative stress in cells and is therefore useful in the treatment of many human diseases, including cancer, cardiovascular diseases and inflammatory diseases[28].

The *P. graveolens* is often used as a medicinal herb in different areas of the world due to its biological activities, which include bactericidal, antifungal and antiviral activities as well as antioxidant activities[29]. However, very little research has been reported on the antitumor activity of *P. graveolens* extracts. The cytotoxic effect observed with MCF-7 cells exposed to methanolic extract may be attributed to the presence of essential oils, which are considered to be the major constituent in *P. graveolens* that causes a wide variety of biological activity. Such indications are in agreement with Fayed[8] which reported that the essential oils from the aerial parts of *P. graveolens* showed a potent cytotoxic effect in HL-60 and NB4 human leukemic cell lines with an LC_{50} value up to 86.5 μg mL^{-1}. In addition, significant inhibition (60-90%) of MIAPaCa2 pancreatic tumor cells was achieved with geraniol, one of the major essential oil constituents of *P. graveolens*[30].

Some studies correlated the anti-inflammatory activity and cytotoxic activity (anti-tumor activity) of *P. graveolens* essential oils in such a way that the essential oils can provoke immunity in patients suffering from cancer. Zhuang *et al.*[31] reported an approved trial where 66 breast cancer patients (stage I-IV) were treated with surgery followed chemotherapy or radiotherapy and were enrolled in administration of herb complex, which included essential oils from *P. graveolens*. Their results suggested that using herb complex had the capacity to delay the reduction in levels of leucocytes and neutrophils experienced by patients during cancer treatment.

Cell-based-high content screening is considered to be a more predictive assay when compared with conventional MTT cytotoxicity. It covers a broad range of effects and monitors multiple and independent toxicities in the same cell with a quantitative measurement of many parameters related to toxicity[12,32]. Therefore, this assay was adopted to monitor the different parametric effects of *P. graveolens* methanolic extract on MCF-7 cells. Depending on the results, the significant reduction of MCF-7 cells at higher concentrations of extract may be attributed to the induction of cellular apoptosis. The condensed and bright intensity of the Hoechst blue stain was attributed to nuclear condensation and the following observations are typical features of apoptotic cell morphology: Nuclear condensation, nuclear fragmentation, cell shrinkage and formation and aggregation of apoptotic bodies[33,34]. In addition, the use of membrane permeability dye and the increasing intensity of this dye, especially at the highest exposure concentration, supporting the fact that the extract can induce apoptosis in MCF-7 cells, since this dye can only stain the cells when the plasma membrane permeability increases due to the loss of plasma membrane integrity[18,35].

This is little information available that is related to the cytotoxic effect of *P. graveolens* extract on tumor cells. However, since essential oils are the major constituents of *P. graveolens*, many studies and reviews indicate that essential oils extracted from different plants species show antitumor and cytotoxic activity through multiple pathways and mechanisms. These proposed pathways include: Apoptosis, cell cycle arrest, anti-metastatic and anti-angiogenic[36,37]. Many studies have investigated the cytotoxic and suppressing activity of plant natural products on tumor cells and revealed that these cells show an increase in expression of the *p53* protein by triggering the up-regulation of the *p53* gene and down-regulation of the *Bcl-2* anti-apoptotic gene[13,38].

One of the most distinctive features of cell death or when a cell undergoes apoptosis is the disrupting of active mitochondria[39]. The MMP dye was used to detect the functionality of active mitochondria; it has the ability to accumulate in mitochondria that maintain their inner membrane potential[40]. Compared with the doxorubicin exposure, results indicated that only 200 μg mL^{-1} of *P. graveolens* methanolic extract triggered a significant reduction in mitochondrial brightness, which may be due to changes in the mitochondrial transmembrane potential caused by the triggering of apoptosis in MCF-7 cells. It has been suggested that these changes in the membrane

potential are due to the opening of the mitochondrial permeability transition pores, allowing the transition of ions and small molecules, like calcium ions and consequently leading to the decoupling of the respiratory chain and release of cytochrome c into the cytosol[41]. Finally, the release of cytochrome c activates a series of caspases, specifically cysteine proteases, which are mainly responsible for the degradation and digestion of the cell from inside[41,42].

The ROS are constantly generated and eliminated in a biological system and normal cells control the levels of ROS by balancing their generation and elimination using scavenging systems[43]. However, for cells under chemical or environmental stress, ROS are overproduced and this leads to modification of cell morphology, structure and cell apoptosis[13].

Increased levels of ROS generation are associated with the control of multiple interacting molecules, including antioxidant enzymes. Cellular injury or exposure to chemicals will dramatically affect the intracellular balance between antioxidant enzymes and lead to overproduction of ROS[44].

Some natural compounds as well as doxorubicin, can induce the generation of ROS and trigger cells to undergo apoptosis[13]. The excessive production of ROS after exposure to the higher concentrations of *P. graveolens* extract may contribute to the destruction of the mitochondrial membrane potential and eventually result in releasing of apoptotic cytochrome C. Furthermore, Gibellini *et al.*[45] described how higher levels of ROS can destroy cellular DNA and plasma membrane, resulting in higher permeability of the plasma membrane and DNA fragmentation. Similar findings were observed throughout this study.

The *P. graveolens* is a well-known plant with uses in traditional medicine and pharmacy, in addition to its activity as an antioxidant[46]. However, little information is available regarding the ability or the cellular and molecular mechanism of *P. graveolens* in reducing ROS and scavenging oxidants, especially cellular redox that is not influenced by only one factor or molecule. The observed reduction in oxidative stress may be attributed to the important role of essential oils, including geraniol and β-citronellol as antioxidants. In addition, geraniol has been reported to exhibit significant antioxidant properties by stabilizing and protecting the cell membrane against oxidative stress[47].

CONCLUSION

In conclusion this study report that the methanolic extract of *P. graveolens* exhibited a significant antioxidant activity and has profound cytotoxic activity against MCF-7 cell line. The extract induces ROS cell generation and in turn causes DNA damage, increasing in cytoplasmic membrane permeability and affecting the mitochondrial membrane potential and the releasing of cytochrome C release.

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