

# Anticancer, Antimicrobial and Antioxidant Activities of the Essential Oils of Some Aromatic Medicinal Plants (*Pulicaria inuloides*-Asteraceae)

Nabil Qaid M. Al-Hajj<sup>1,2</sup>, Algabr MN<sup>3</sup>, Khamis Ali Omar<sup>1</sup>, Hongxin Wang<sup>1,\*</sup>

<sup>1</sup>School of Food Science and Technology, Jiangnan University, Wuxi 214122, P.R. China

<sup>2</sup>Marine Science and Biological Research Authority, Aden, P.O. Box-1231, Aden, Yemen

<sup>3</sup>Hajjah University, Department of chemistry, Faculty of Applied Science, P.O. Box 80004, Yemen

\*Corresponding author: [alwossabi2007@yahoo.com](mailto:alwossabi2007@yahoo.com)

**Abstract Purpose:** To determine the anticancer, antimicrobial and antioxidant activities of *Pulicaria inuloides* essential oil (PiEO). **Methods:** 3-(4, 5-dimethylthiazol- 2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was used to assess cytotoxic activity. Steam distillation of the aerial parts of *P. inuloides* was performed using a Clevenger apparatus. Antioxidant activities were determined by using 2, 2-diphenyl-1,1-picrylhydrazyl (DPPH). Disc diffusion and microtiter broth microdilution assays were employed to determine antimicrobial activity. **Results:** PiEO inhibited six tested strains of *Listeria* with minimum inhibitory concentrations of 5.49 µg/mL against *L. monocytogenes*, *L. innocua*, and *L. ivanovii*; 6.24 µg/mL against *L. welshimeri*, and *L. grayi*; and 11.55 µg/mL against *L. seeligeri*. PiEO also possessed more efficient free radical scavenging activities than butylated hydroxytoluene (BHT). PiEO possessed significant anticancer properties, indicated by growth inhibition and changes in the morphological structure of MCF-7 cancer cells treated with PiEO.

**Keywords:** *Pulicaria inuloides*, essential oil, MCF-7, antimicrobial, antioxidant activity

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## 1. Introduction

Cancer is the second leading cause of death worldwide. Breast cancer accounts for 30% of all cancers, and is the primary cause of cancer-related mortality, in women [1]. A major problem with current cancer treatment is the severe lack of effective anti-tumor drugs [2]. Due to multiple side effects and problems related to common cancer treatments such as chemotherapy, surgery, and radiotherapy, alternative treatments are frequently investigated [3].

Natural products, especially plants, have played a significant role in the treatment and prevention of human diseases, worldwide and for thousands of years [4]. Plants are rich sources of chemically diverse compounds, and have properties that are beneficial to human health. Some medicinal plants have shown promising activity against various types of cancer. About 50% of known anticancer therapeutic agents are derived from plants [7]. For example, compounds such as taxol and vinca alkaloids act to destabilize the microtubules of cancer cells and prevent the rapid proliferation of tumors [8].

The application of medicinal plants often involves the use of their essential oils. Essential oils are complex mixtures of many different aromatic compounds and the composition of the oils is dependent on the genetic

make-up of each cultivar, although it can also be influenced by the distillation process [11]. In the last two decades, there has been increasing interest in the pharmacological activities of essential oils [5]. Several studies on essential oils have revealed anti-cancer properties due to the presence of a variety of biologically active components [6].

*Pulicaria inuloides* (family Asteraceae, tribe Inuleae) is a well-known Yemeni medicinal plant [11]. There have been many studies on the applications of *P. inuloides* essential oil (PiEO) and on its anticancer, antispasmodic, anti-inflammatory, anti-diabetic, antibacterial and antioxidant activities [11,14]. However, there are no reports on the inhibition of cancer cell proliferation by PiEO. Therefore, in this study, we investigated the cytotoxic effects of PiEO on MCF-7 cells, a breast cancer cell line isolated from a 69-year-old Caucasian woman in 1970 that is widely used in studies of tumor biology and hormone mechanisms of action [1].

## 2. Materials and Methods

### 2.1. Plant Material

*P. inuloides* leaves were collected in 2016 during the flowering season (August–September) from Bany Matar, a mountainous region located northwest of Sana'a city,

Yemen, at 15° 21.1217' N, 44° 12.4474' E, 2,200 m above sea level. Botanical identification was made by Prof. Dr. Abdallah Amine (College of Agriculture, Sana'a University). The leaves were dried for 10 days at room temperature (23–25°C) in a shaded place and milled using an electrical mill. The powdered samples were stored in a dry, cool, dark place.

## 2.2. Isolation of the Essential Oil

Essential oil was obtained from *P. inuloides* leaves (500 g) by subjecting them to hydro-distillation for 6 h in a Clevenger apparatus according to the methods outlined by the British Pharmacopeia [15]. The oil was dried over anhydrous sodium sulfate and then stored at 4°C prior to cell analysis. The extraction yield was calculated as the ratio of the weight of the oil to the weight of the leaves, using the following equation:

$$\% \text{ yield of oil} = \frac{\text{Weight of oil}}{\text{Weight of dried leaves}} \times 100. \quad (1)$$

## 2.3. Chemicals

All chemicals and solvents used were of analytical grade. 2, 2-diphenyl-1,1-picrylhydrazyl (DPPH), 2,6-bis(1,1-dimethylethyl)-4-methylphenol (butylated hydroxytoluene; BHT), and 2-(1,1-dimethylethyl) were obtained from TCI Europe N.V. (Zwijndrecht, Belgium). Nutrient Broth and Nutrient Agar were procured from Sinopharm Chemical Reagent Co. Ltd. (SCR; Shanghai, China). Penicillin was from AMRESCO Life Science Research Products through VWR Co., Ltd. (Shanghai, China).

## 2.4. Anti-pathogenic Bacterial Activity

### Pathogenic bacterial strains

Anti-pathogenic bacterial activity was tested against six *Listeria* species: *L. monocytogenes* ATCC 7644, *L. innocua* ATCC 33090, *L. ivanovii* ATCC 19119, *L. seeligeri* SLCC 3954, *L. welshimeri* ATCC 35897, and *L. grayi* ATCC 25923, purchased from the China General Microbiological Culture Collection Center (Beijing, China). All strains were first identified and then cultured in sterile test tubes containing nutrient broth and incubated for 24 h at 37°C. Suspensions were prepared in sterile deionized distilled water with a density of McFarland standards (0.5) to obtain a bacterial concentration of about  $1.5 \times 10^6$  colony-forming units (CFU)/mL. The anti-pathogenic bacterial activity of PiEO was assessed at a concentration of 20 µL using the agar disk diffusion method described by [11], taking into consideration the proposed amendments by [11].

### Agar disk diffusion

Sterile nutrient agar was prepared according to the manufacturer's specifications and autoclaved at 121°C under 15 lbs of pressure for 20 min. After cooling, the sterile media was poured into sterile petri dishes (90 mm; 20 mL/dish). Bacterial suspensions ( $100 \mu\text{L}$ ;  $1.5 \times 10^8$  CFU/mL) were spread onto the plates. PiEO was mixed with 1 mL of dimethyl sulphoxide (DMSO) at different concentrations (5 mg/mL); then, 50 µL was loaded onto

6-mm diameter sterile filter paper discs and individually impregnated on the surface of the poured media in the culture plates. The inoculated plates were incubated for 24 h at 37°C. Penicillin (20 µg/mL) was used as a positive control. All experiments were performed in triplicate and the anti-pathogenic bacterial activity of PiEO was measured as the diameter of the inhibition zones (DIZ) in mm.

### Determination of minimal inhibitory concentration (MIC)

The MIC of the essential oil was determined using the microtiter broth microdilution assay described by [11]. The essential oils were diluted to 50 µL/mL and subjected to serial dilution in a microtiter plate containing tryptic soy agar. The bacterial and yeast strains were suspended in liquid culture medium at a final concentration of  $10^4$  CFU/mL. After incubation at 37°C for 24 h, optical density was measured at 520 nm using a spectrophotometer. MIC was defined as the lowest concentration of the essential oil at which the microorganisms did not exhibit visible growth.

### Free radical-scavenging effect of PiEO

The free radical-scavenging effects of PiEO were measured according to DPPH free radical-scavenging activity (DPPH\*) as described previously [11], with slight modification. Briefly, 20 µL of each PiEO treatment was mixed with 3.5 mL of DPPH\* methanolic solution ( $6 \times 10^5$  Mol/L). The mixture was allowed to react in the dark at room temperature. Then, the absorbance was measured after 30 min at 517 nm, and converted to DPPH\* % by the following equation (Eq. 2):

$$\text{DPPH} = \left[ \frac{A_0 - A_1}{A_0} \right] \times 100. \quad (2)$$

where  $A_0$  is the absorbance of the control and  $A_1$  is the absorbance of the targeted treatment. BHT was used as a positive control at 120 mg/L. All treatments were carried out in triplicate.

### Cancer Cell Lines

The MCF-7 human breast cancer cell line frozen in liquid nitrogen (-80°C) was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). 9.4.6.

### Cell culture media RPMI 1640 proportions

Roswell Park Memorial Institute culture medium 1640 (RPMI 1640) powder was added into water and stirred until completely dissolved; then, 2 g/L sodium bicarbonate was added and dissolved completely to the desired pH (pH 7.0–7.2). The media was filtered immediately under sterile conditions.

### Cell culture

MCF-7 cells were used for the cytotoxicity assay. All cancerous cell lines were grown in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA) and antibiotics: a mixture of streptomycin (50 µg/ml) and penicillin (50 UI/mL). The cancer cell line was maintained at 37°C in an atmosphere of 5% CO<sub>2</sub> and 95% air (100% humidity). At 70–80% confluence, cells were detached from culture flasks by treatment with 0.05% trypsin-EDTA. The cells were counted, and their viability determined, using trypan blue exclusion dye (Sigma-Aldrich) on a hemocytometer (Boeckel & Co., GmbH, Hamburg, Germany). After

counting, cultures were diluted in 96-well tissue culture plates to obtain the required cell density.

#### Cell subculture

The cells were placed immediately in a 37°C water bath for rapid thawing, and then transferred to 15 mL tubes with culture media followed by centrifugation at 1,000 rpm for 5 min. Fresh RPMI 1640 media was placed into the flasks. Supernatant was aspirated off the centrifuged cells, and an appropriate volume of fresh culture medium was added to each well of cells by pipetting. An aliquot of cells was transferred to new culture containers according to the recommended ratio of the cell line. The cells were observed under a microscope before incubation at 37°C.

#### Cytotoxicity assay

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was used to assess cytotoxic activity [16]. MCF-7 cell suspensions (100 µL;  $1.0 \times 10^4$  cells/mL) were added to 96-well plates and incubated for 24 h at 37°C under a 5% CO<sub>2</sub> /95% air (100% humidity) atmosphere in an incubator, to allow stabilization before adding extracts. The cells were then treated with PiEO dissolved in DMSO to a final concentration of 0.5% in the culture media and incubated for 48 h, then fixed by adding 50 µL/well cold 50% (w/v) trichloroacetic acid (TCA) to a final concentration of 10% in the culture media and incubated for 1 h at 4°C. After 48 h, the media was removed and the cells were washed with distilled water.

#### Morphological examination

To observe morphological changes, about  $1 \times 10^4$  cells were cultured in 96-well plates, allowed to adhere overnight, and then treated with various concentrations of PiEO for 24, 48 and 72 h. After the treatment period, the cells were washed twice with phosphate-buffered saline (PBS) and then observed and photographed under an inverted microscope.

### 2.5. Statistical Analysis

Data were subjected to analysis of variance (ANOVA) following a completely random design to determine the

least significant difference (LSD) at  $P < 0.05$  using SPSS software (ver. 11.5; SPSS, Inc., Chicago, IL, USA). Cytotoxicity analyses were performed in triplicate.

## 3. Results

### 3.1. Antimicrobial Activity of PiEO

The bacteriostatic activity of PiEO showed pronounced activity against all of the *Listeria* spp. tested in this study, as assessed by the disc diffusion assay. In addition, MIC was assessed by the microtiter broth microdilution assay (Table 1).

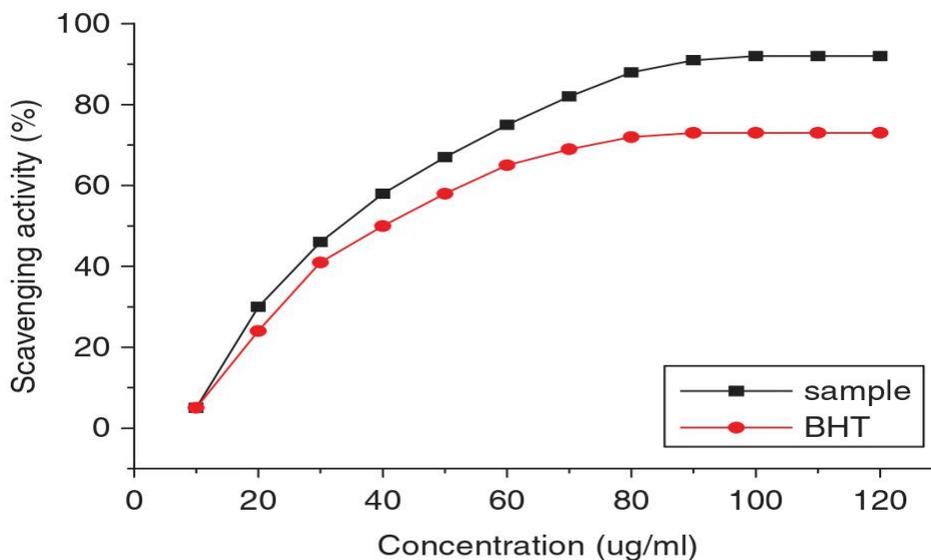
**Table 1.** Diameter of the inhibition zone of *P. inuloides* essential oil (PiEO) compared to antibiotics

| Pathogen bacterial strains | Inhibition zone diameter <sup>a</sup> (mm) |                          |                |
|----------------------------|--|--------------------------|----------------|
|                            | PiEO<br>(20 mg/mL)                         | Penicillin<br>(20 µg/mL) | MIC<br>(µg/ml) |
| <i>L. monocytogenes</i>    | 15 ± 0.8                                   | 20 ± 0.2                 | 5.49           |
| <i>L. innocua</i>          | 15 ± 0.2                                   | 20 ± 0.7                 | 5.49           |
| <i>L. ivanovii</i>         | 13 ± 0.8                                   | 18 ± 0.8                 | 5.49           |
| <i>L. seeligeri</i>        | 13 ± 0.4                                   | 14 ± 0.5                 | 11.55          |
| <i>L. welshimeri</i>       | 15 ± 0.3                                   | 16 ± 0.2                 | 6.24           |
| <i>L. grayi</i>            | 15 ± 0.5                                   | 19 ± 0.3                 | 6.24           |

<sup>a</sup>DIZ, diameter of inhibition zone (mm) including disk diameter (6 mm).

### 3.2. Free Radical Scavenging Effect

The antioxidant activity of PiEO was determined by the DPPH free radical scavenging assay. The DPPH scavenger effect (DPPH-Pi) assay depends on the existence of natural phenols and their ability to donate a hydrogen ion to convert stable free radicals (DPPH) to non-radicals (DPPH-H). The results are presented in Figure 1.



**Figure 1.** DPPH radical scavenging activity of *Pulicaria inuloides* essential oil (PiEO) versus butylated hydroxytoluene (BHT) (Each value represents mean ± standard deviation (SD) (n = 3).)

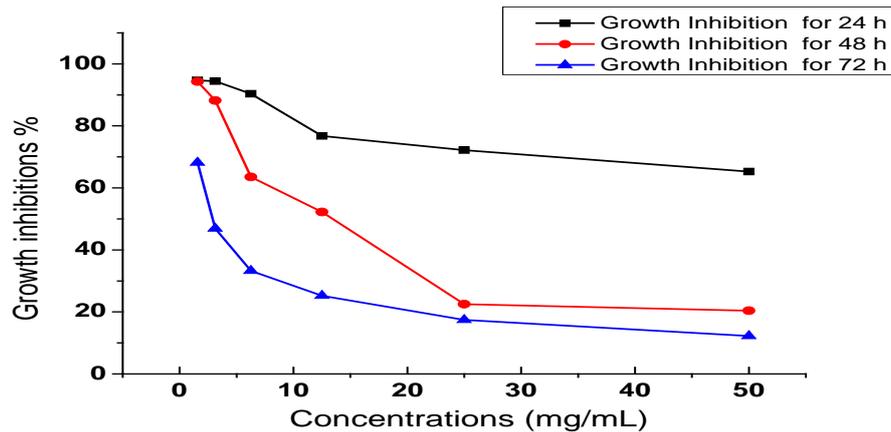


Figure 2. Percentage growth inhibition of MCF-7 cells by PiEO.

### 3.3. Cytotoxic Activity of PiEO on MCF-7 Cells

PiEO showed significant cytotoxic activity against the MCF-7 cells used in the study. The results are given in Table 2 and Figure 2.

Table 2. The cytotoxic activity of *P. inuloides* essential oil (PiEO) on MCF-7 cells

| Concentration (mg/mL) | MTT1 (24h)              | MTT2 (48h)              | MTT3 (72)               |
|-----------------------|-------------------------|-------------------------|-------------------------|
| Control               | 100                     | 100                     | 100                     |
| 1.56                  | 65.25±5.07 <sup>e</sup> | 20.40±1.11 <sup>d</sup> | 12.21±1.11 <sup>e</sup> |
| 3.125                 | 72.15±2.53 <sup>d</sup> | 22.48±0.60 <sup>d</sup> | 17.41±2.35 <sup>e</sup> |
| 6.25                  | 76.74±0.76 <sup>c</sup> | 2.19±1.18 <sup>c</sup>  | 25.22±1.43 <sup>d</sup> |
| 12.5                  | 90.33±0.91 <sup>b</sup> | 63.54±1.11 <sup>b</sup> | 33.22±1.47 <sup>c</sup> |
| 25                    | 94.40±0.31 <sup>a</sup> | 88.18±2.99 <sup>a</sup> | 46.92±2.25 <sup>b</sup> |
| 50                    | 94.67±0.76 <sup>a</sup> | 94.28±1.3 <sup>a</sup>  | 68.91±6.29 <sup>a</sup> |
| IC <sub>50</sub>      | --                      | IC <sub>50</sub> =3.0   | IC <sub>50</sub> =12.9  |

Different letters indicate significant differences between PiEO and MTT ( $p < 0.05$ ). Results are expressed as means  $\pm$  SD.

### 3.4. Morphological Analysis

Morphological analysis of MCF-7 cells was carried out to gain insight into morphological alterations caused by treatment with PiEO. Untreated MCF-7 cells were spherical in shape. The nuclei and nucleoli and were larger than those of the treated cells (Figure 3). Our study showed that cells treated by 6.25, 12.5, 25.0 and 50.0  $\mu$ l/ml oil had become vacuolated and shrunken and some cells were death in 24h and 48 h while cells treated in the same concentrations at 72h was begin to growth and increased.

## 4. Discussion

### 4.1. Antimicrobial Activity of PiEO

The *in vitro* anti-pathogenic bacterial effect of PiEO was assessed and the results are presented in Table 1. There was a positive relationship between PiEO concentration and DIZ. All DIZ values less than 10 mm were ignored. The results also demonstrated that PiEO had noticeable activity against all species of *Listeria*. There were no

significant differences in the DIZ induced by treatment with 20 mg/mL PiEO for *L. monocytogenes*, *L. grayi*, *L. welshimeri*, and *L. innocua*. The DIZs induced by 20 mg/mL PiEO were comparable to those induced by 20  $\mu$ g/mL penicillin. While the antimicrobial activity of PiEO has been reported previously, with DIZs against *S. aureus*, *S. pneumoniae* and *B. subtilis* of 16.6, 18.2 and 18.0 mm, respectively [11], our current study is the first to report the antibacterial activity of PiEO against *Listeria*. The MIC values indicate that PiEO has a greater inhibitory action against all *Listeria* strains (Table 1). The Gram-positive strains were less sensitive to inhibition than the Gram-negative strain. This variation in inhibition may be attributed to the bacterial cell membrane constituents. The cell membranes of Gram-positive bacteria contain an outer peptidoglycan layer, which is an ineffective permeability barrier, while the cell membranes of Gram-negative bacteria contain a more effective permeability barrier in the outer membrane consisting of lipopolysaccharides. [11]

### 4.2. Free Radical-scavenging Effects

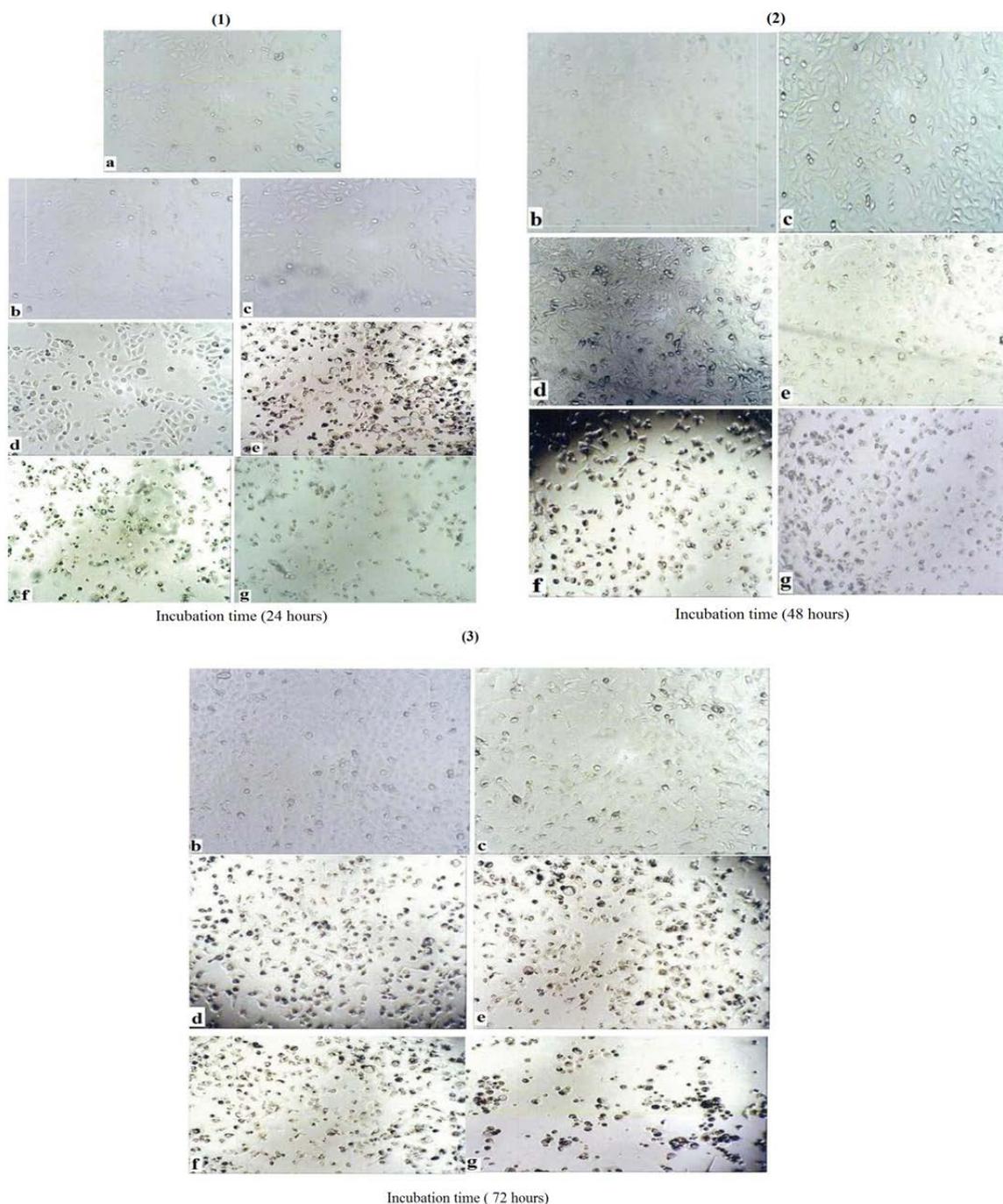
Antioxidant ability is most commonly determined by the DPPH\* scavenging activity assay [18] because of its simplicity and accuracy. The DPPH\* scavenging assay is based on the presence of natural phenolic compounds and their ability to donate a hydrogen ion to convert stable free radical DPPH\* to a non-radical state, DPPH-H. The stability of DPPH\* is primarily due to steric crowding around the divalent nitrogen atom [19]. This change depends on the number of hydrogen atoms absorbed, which can be monitored spectrophotometrically by measuring the bleaching of DPPH\* color from purple to yellow [20]. Figure 1 shows that PiEO was more active than BHT in converting DPPH\*, with PiEO converting 55–98% while BHT converted 50–80%. The ability of PiEO to quench hydroxyl radicals, which are implicated in some diseases, revealed the presence of antioxidant compounds in this plant [16].

### 4.3. Cytotoxic Activity of PiEO on MCF-7 Cells

The results for growth inhibition of MCF-7 cells by various concentrations of PiEO are shown in Table 2. There significant differences between all the Times (24h,

48h and 72 hours. The values in 24h and 48h were the better than 72 hours. In concentrations 50  $\mu\text{l/ml}$  oil, the values were 94.67, 94.28 and 68.91  $\mu\text{l/ml}$  in all times tested respectively **Table 2**. In the present study, the growth of MCF-7 was inhibited by PiEO treatment in a dose-dependent manner (**Figure 2**). The 50% cytotoxic effect ( $\text{IC}_{50}$ ) of PiEO was found to be 12.9 and 3.0  $\mu\text{l/ml}$ . In our study, PiEO showed broad spectrum cytotoxicity, with a prominent effect on MCF-7 breast carcinoma cells at high concentrations. PiEO treatment significantly decreased the growth rate of MCF-7 cells compared with controls. This may be attributable to isothiocyanates (ITCs), which have been reported to be important and promising chemopreventive compounds [17]. The essential oil of *P. jaubertii* from southern Saudi Arabia had a higher proportion of carvotanacetone (98.6%), and showed

excellent in vitro cytotoxic activity against MCF-7 and Hep-G2 cells ( $\text{IC}_{50} = 3.8$  and  $5.1 \mu\text{l/ml}$ , respectively) [17]. In recent years, numerous investigations have shown that sulfur-containing compounds induce apoptosis in multiple cell lines (including breast and colorectal cell lines) and animal models [18]. In this study, PiEO significantly inhibited growth in an MCF-7 cell line at high  $\text{IC}_{50}$  values (**Table 2**). An  $\text{IC}_{50}$  of an oil extract less than 100  $\mu\text{g/ml}$  is categorized as a potentially cytotoxic substance [18]. In previous investigations of PiEO on MCF-7 cells, the  $\text{IC}_{50}$  was  $5.36 \mu\text{g/ml}$  [19]. In the present study, PiEO was found to be cytotoxic towards human MCF-7 cells in an MTT assay, and the concentrations required for 50% cell death were 12.9 and 3.0  $\text{mg/ml}$ . Hence, our study shows the efficacy of PiEO against MCF-7 cells, suggesting a protective effect against breast cancer.



**Figure 3.** Morphological changes to the MCF-7 cells treated with PiEO at **a** (control-untreated), **b** (1.65), **c** (6.25), **d** (12.5), **f** (25) and **g** (50) after 24, 48 and 72 h incubation. (1) =24 hours, (2) =48 hours and (3) = 27 hours incubation

### 4.3. Morphological Analysis

Cells incubated with 1.56 and 3.125 mg/mL PiEO for 24 h, and 1.56 mg/mL for 48 h, showed the characteristic morphological changes of apoptosis, including membrane blebbing, chromatin condensation, and the formation of apoptotic bodies. The cell shrinkage increased progressively in a dose- and time-dependent manner. This shrinkage may have been due to the growth-inhibiting effects of some bioactive compounds of PiEO (Figure 3). Interestingly, in this study, treating cancer cells with PiEO produced a significant dose-dependent decrease in cell viability as measured by the MTT assay. A significant decrease was seen in cell viability at multiple concentrations of PiEO. Cells on the control plate did not show any morphological changes, and were irregular, confluent aggregates with rounded and polygonal cell morphology (intact).

A previous toxicology study focused on the effects of PiEO on breast cancer [20]. However, various bioactive compositions of *P. inuloides* may be attributed to synergy between a number of secondary metabolites, including citrinin, monacolin K, dimeric acid, flavonoids, total phenols, and other compounds. On the other hand, the percentage of viable cells in cell lines increased with increasing treatment duration. These results were similar to studies on the cytotoxic effects of goniothalamin on human breast cancer cells [20].

## 5. Conclusion

PiEO exerted a strong antimicrobial action against all tested strains of *Listeria*. This study also showed that PiEO possessed strong antioxidant activity and effective cytotoxic activity against MCF-7 cells. These properties are likely to be related to the oil constituents. Our findings suggest that PiEO has potential as an inexpensive and easily accessible natural anti-cancer agent, as a novel broad-spectrum bioactive compound.

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