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Research Article

Cytotoxicity and antiproliferative activity of essential oils from lemon, wild orange and petitgrain against MCF-7, HepG2 and HeLa cancer cells

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Abstract

The purpose of this study was to determine the chemical composition and biological properties of the citrus essential oils (EOs) derived from orange rinds (peels) of lemon (*Citrus limon*), wild orange (*Citrus sinensis*) from Brazil extracted by the cold pressed/expressed method, and leaves and twigs of petitgrain (*Citrus aurantium*) from Paraguay extracted by steam distillation. These food grade EOs were evaluated for their cytotoxic activity in breast, liver, and cervical cancer cells (MCF-7, HepG2 and HeLa) via MTT assay, antiproliferative activity via colony formation assay, and antimigratory activity via wound healing assay, and apoptosis via DNA fragmentation and morphology assessment. The major compounds found in lemon EO were D-limonene (66.75%), beta-pinene (12.82%), and gamma-terpinene (11.57%), totaling over 90% of the identified compounds. For wild orange, the only predominant compound was limonene (96.60%), and the rest, found in minor amounts, included alpha-pinene, bicyclohexane, beta-pinene, beta-myrcene, 3-carene, and o-cymene. For petitgrain EO, linalyl isobutyrate (51.76%) and linalool (26.86%) were mainly detected. Based on the MTT assay, petitgrain EO was the most effective against MCF-7, HepG2 and HeLa. However, wild orange EO was the most antiproliferative and antimigratory against all three cells using the anticolony formation assay and wound healing assay, respectively. The results showed that cell death is associated with the apoptotic process, with morphological hallmarks of apoptosis including membrane blebbing and DNA fragmentation. These findings imply that the three citrus EOs might be used as active components in functional food products for chemopreventive benefits.

Keywords: antimigration; apoptosis; *Citrus aurantium*; *Citrus limon*; *Citrus sinensis*; MTT assay

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Introduction

In Thailand, there were 190,636 new cancer cases in 2020. According to the Global Cancer Observatory (2020), there will be 290,000 new cancer cases by 2040. The most prevalent kinds of cancer and their recognized risk factors among males in Thailand include the liver and bile duct. These types of cancer are generated by damage from inherited hemochromatosis diseases, cirrhosis, alcoholism, or infections like hepatitis. However, the most prevalent malignancies in women in Thailand differ (Virani *et al*., 2017). Breast cancer may be caused by a family history of breast cancer, and cervical cancers may be triggered by HPV infection, smoking, early sexual contact, having multiple sexual partners, using birth control pills, and smoking as risk factors. Currently, surgical treatments, chemotherapy, hormonal, biological, and radiation therapies are among the clinical treatment options for cancer in Thailand (Kulthanachairojana *et al*., 2021). These available treatments are invasive, have severe side effects, and/or are prohibitively expensive for Thai cancer patients with limited financial resources. As a result, there is a need for less expensive and more pleasant alternative therapies or chemopreventive programs using natural products.

Citrus fruits such as lemons, limes, oranges, wild oranges, tangerines, petitgrains, and grapefruits are readily accessible and reasonably priced throughout the world. These fruits include bioactive chemicals such as phenols, flavonoids, and terpenes, which have pharmacological activities in breast cancer (Nipin *et al*., 2017). Citrus fruit processing generates a substantial quantity of waste (peels, seeds, and pulps). The most important by-product of citrus processing is essential oil (EO). Limonene, the most basic monocyclic monoterpene in citrus oil, has significant chemotherapeutic potential (Chebet *et al*., 2021). Citrus EOs are widely utilized as food additives in a variety of foods and drinks (Dosoky and Setzer, 2018), owing to their classification as generally regarded as safe (GRAS).

Citrus limon (Lemon) EO was found to be cytotoxic to human lung, breast, and prostate cancer cells (Zu *et al*., 2010). The chemicals, citral, decanal, and octanal, contributed to apoptosis in human leukaemia (HL-60) cells (Hata *et al*., 2003). *Citrus sinensis* (Wild orange) EO demonstrated antitumor effects in HL-60 cells and human colon cancer cells, by induction of apoptosis and preventing invasion and metastasis (Chidambara Murthy *et al*., 2012). Furthermore, high D-limonene content in the EO has been linked to strong radical-scavenging action (Asjad *et al*., 2013; Yu *et al*., 2017).

Citrus aurantium (Petitgrain) is a small tree with its fruit juice rarely utilized due to the presence of the bitter-tasting flavanone naringin as well as high quantities of citric acid, which is responsible for the intense sour flavour (Sherif *et al*., 2015). Petitgrain is the most affordable EO derived from bitter orange. Paraguay is the top producer of petitgrain oil, followed by Egypt, Spain, France, and Italy (Do *et al*., 2015). The high D-limonene level may account for the robust antioxidant activity (De Pasquale *et al*., 2006).

The worldwide EOs market was worth USD 18.6 billion in 2020. It is predicted to increase at a 7.4 percent compound annual growth rate (CAGR) from 2021 to 2028 (Grandview Research Essential Oils Market Size, 2022). The market is expected to grow due to rising trend from key end-use sectors such as food and beverages, personal care and cosmetics, and aromatherapy. EOs have the major advantages over conventional medications and chemicals in that there are no significant adverse effects and can be easily incorporated into foods and drinks for daily ingestion. This is expected to be the primary motivator for market expansion.

Thus, this work aspired to determine the cytotoxicity and antiproliferative activity of commercial food grade EOs from lemon, wild orange, and petitgrain against MCF-7, HepG2, and HeLa cancer cells. Thus far, no previous research on the cytotoxic effects of the less common petitgrain EO against MCF-7, HepG2, and HeLa has been conducted. It was expected that the findings would make consumers better aware of the chemopreventive benefits of food grade EOs for daily ingestion, and that could be used as a low-cost alternative therapy for cancer patients or as a chemopreventive program for consumers.

Materials and Methods

Sample preparation

EOs of orange rinds (peels) of *Citrus sinensis* (Wild Orange; WO) and *Citrus limon* (Lemon; L) from Brazil were extracted by the cold pressed/expressed method. EO from leaves, and twigs of *Citrus aurantium* (Petitgrain; P) from Paraguay was extracted by steam distillation. These EOs (food grade) filled in 15 mL amber bottles with screw lids were purchased from dōTERRA Intl. (Pleasant Grove, UT, USA). Each EO was then diluted in dimethyl sulfoxide (DMSO) (final concentration in culture media was less than 0.1% (v/v)) and filtered through a 0.22 μm. The EO dilutions were stored at -20 °C.

GC/MS analyses of EOs

The chemical analysis of the EOs was performed as previously reported (Monajemi *et al*., 2010) in Shimadzu QP2010 equipment, equipped with a capillary column of fused silica HP5-MS (5% phenylmethylsiloxane, 30 m × 0.25 mm i.d.; film thickness, 0.25 μm) and a mass spectrometer as a detector. Operating conditions were as previously conducted (Monajemi *et al*., 2010). The data was gathered and processed using ChemStation software. Compound identification was accomplished by comparing MS spectra to standard spectra from the NIST 2014 library. The total peak areas of all the compounds present in each sample was used to calculate the relative percentage content of the analyzed compounds.

Cancer cell culture

Human breast adenocarcinoma (MCF-7 ATCC® HTB-22™), human hepatocellular carcinoma (HepG2 ATCC® HB-8065™), and human cervical adenocarcinoma HeLa cell (ATCC® CCL-2™) were obtained from the American Type Culture Collection (ATCC, Manassasa, VA, USA). These cells were maintained in DMEM medium for MCF-7, HepG2 and RPMI-1640 for HeLa cells supplemented with 10% FBS and 100 U/mL penicillin G, and 100 μg/mL streptomycin incubated at 37°C with 5% CO_2 atmosphere. The new medium culture was replaced every 3 days and trypsinized with 0.25% trypsin-EDTA.

Determination of cytotoxicity of EOs

Viability of cancer cells was detected using the MTT (3, 4, 5-dimethylthiazol-2-yl-2-5-diphenyltetrazolium bromide assay. Five thousand of the MCF-7, HepG2 and HeLa cells were added to 96-well plates for 24 h at 37°C in an incubator. Each EO solution in the range of 0.0-2.0% (v/v) (25 μL) was added to the cell culture to obtain a final volume of 200 μL; DMSO (0.1%) served as the vehicle control. After incubation in an incubator at 37°C for 24, 48, and 72 h. MTT reagent was added to replace the media and incubated for 4 h. Next, 200 μL of DMSO was added to dissolve the formazan crystals, and the absorbance at 590 nm was recorded by a microplate reader (Microplate Reader model M965+). Cell viability was evaluated compared to the control (not treated with EO). Maximal cytotoxic effect (*E*max) and half-maximal inhibitory concentration (IC_{50}) values were calculated.

Colony formation assay

The determination of the formation of colonies after treatment with EOs was performed in triplicate. The MCF-7, HepG2 and HeLa cells were cultured in a 6-well plate at 500 cells/well for 24 h. After that, cells received EO treatment (0.0-1.0% (v/v)) for 24 h, and then the medium was subsequently removed from the culture plates, the cells were washed with PBS. Next, cells were cultivated in fresh media at 37°C with 5% CO_2 for 14 days, and fresh media were added and changed every 2 days. The following cells were fixed with 4% formaldehyde for 30 min and were stained with 0.5% Coomassie brilliant blue g-250 for 30 min. Excess color was removed with tap water several times. Colonies in the given area were counted. Colony formation was expressed as a percentage relative to untreated cells.

Cell morphology

The MCF-7, HepG2 and HeLa cells (5×10^3 cells/well) were seeded into a 24- well plate overnight. Cancer cells were treated with EO (0.25% (v/v)) for 24 h. Cell morphology was observed by an inverted light microscope.

DNA fragmentation analysis

The MCF-7, HepG2 and HeLa cells (2×10^5 cells/well) were seeded into 6-well plates overnight. Treatments with EOs (0.25% (v/v)) were carried out for 24 h. Afterwards, cells were washed with PBS. Genomic DNA was then extracted using a DNA extraction kit (Vivantis, Malaysia) according to the kit's manual. The genomic DNA (1 μg/ml) was mixed with 6X loading dye, and 100 ng of 1 kB DNA was used as a marker. The DNA was analyzed on gel electrophoresis in 1% agarose gel with 1x SYBR Safe dye (Vivantis, Malaysia) at 100 V for 40 min. The band of DNA was viewed using a Gel documentation (Syngene Gene Flash, UK).

Wound healing assay

The MCF-7, HepG2, and HeLa cells (2×10^5 cells/well) were seeded into a 24-well plate overnight. Next, a 200 µL sterile pipette tip was used to scratch a wound and cell scrap was cleared with PBS. After that, cell treatment with EO (0.125% (v/v)) in the new media culture at 37 °C for 24 h was conducted. A scratch of the wounds was captured and a percentage relative closure of the scratch, compared with untreated control cells, was calculated.

Results

Chemical composition of the EOs

The quantitative and qualitative analysis of the chemical composition of the EOs are shown in Table 1. Fourteen, seven, and sixteen compounds were identified in lemon, wild orange, and petitgrain EOs, respectively (Figure 1, Table 1). The four common components found in all three EOs are alpha-pinene, beta-pinene, beta-myrcene and bicyclohexane. The major compounds found in lemon EO were D-limonene (66.75%), beta-pinene (12.82%), and gamma-terpinene (11.57%), totaling over 90% of the identified compounds. The six compounds that were uniquely detected in lemon EO were camphene (inducing intrinsic apoptosis in melanoma cells) (Girola *et al*., 2015), gamma-terpinene (no significant effect) (Fitsiou *et al*., 2016), trans-alpha-bergamotene (no report on anticancer activity yet), 1,3-cyclohexadiene, bicycloheptane and tricycloheptane (no activity).

For wild orange EO in this work, the only predominant compound was D-limonene (96.60%), and the rest, found in minor amounts, included alpha-pinene, bicyclohexane, beta-pinene, beta-myrcene, 3-carene and o-cymene. The only compound found in wild orange but not found in lemon, was 3-carene. Interestingly, 3-carene was found to be cytotoxic to A549 lung cancer cells (Yang *et al*., 2017).

For petitgrain EO in this work, linalyl isobutyrate (51.76%) and linalool (26.86%) were mainly detected. The former has yet to be reported with anticancer ability; however, the latter is known for apoptosis induction (Iwasaki *et al*., 2017).

In summary, most chemical components of these three EOs present in Table 1 play a role in chemoprevention and may contribute to the cytotoxic, antiproliferative, and antimigratory effects of EOs in the following tests.

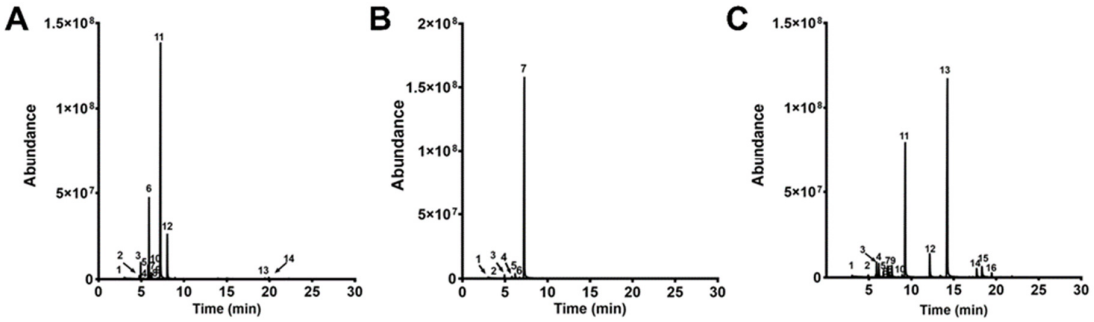

Figure 1. GC-MS chromatograms of EOs (**A**) Lemon (**B**) Wild orange (**C**) Petitgrain

Table 1. Chemical composition of EOs

Peak no.	Retention time (min)	% Abundance	Constituents	Chemo preventive activities	Ref.
Lemon					
1	4.708	0.01	Tricycloheptane	No report	No report
2	4.775	0.43	Bicyclohex-2-ene	No report	No report
3	4.935	2.16	alpha-Pinene	Inducing natural killer cells via ERK/AKT pathway	Jo *et al*. (2021)
4	5.269	0.06	Camphene	Induces intrinsic apoptosis in melanoma cells	Girola *et al*. (2015)
5	5.807	2.15	Bicyclohexane	No report	No report
6	5.91	12.82	beta-Pinene	Apoptosis stimulation and cell cycle arrest	Salehi *et al*. (2019)
7	6.185	1.55	beta-Myrcene	Inducing oxidative stress and apoptosis	Bai and Tang (2020)
8	6.573	0.04	Bicyclohex-2-ene	No report	No report
9	6.885	0.17	1,3-Cyclohexadiene	No report	No report
10	7.114	0.20	o-Cymene	No report	No report
11	7.212	66.75	D-Limonene	Suppressing angiogenesis and metastasis	Chidambara Murthy *et al*. (2012).
12	8.044	11.57	gamma-Terpinene	No significant effect	Fitsiou *et al*. (2016)
13	19.476	1.67	Beta-Caryophyllene	Modulating STAT3 signaling, oxidative stress, DNA damage response Cytotoxic against MCF-7, A549, Hela	Di Sotto *et al*. (2020); Su *et al*. (2013)
14	19.935	0.43	trans-alpha-Bergamotene	No report	No report

Wild orange					
1	4.939	0.66	alpha-Pinene	Same as above	Same as above
2	5.813	0.45	Bicyclohexane	No report	No report
3	5.092	0.06	beta-Pinene	Same as above	Same as above
4	6.191	2.00	beta-Myrcene	Same as above	Same as above
5	6.717	0.20	3-Carene	Cytotoxic to A549 lung cancer cells	Yang *et al*. (2017)
6	7.126	0.03	o-Cymene	No report	No report
7	7.212	96.60	D-Limonene	Same as above	Same as above
Petitgrain					
1	4.942	0.15	alpha-Pinene	Same as above	Same as above
2	5.814	0.26	Bicyclohexane	No report	No report
3	5.908	1.59	beta-Pinene	Same as above	Same as above
4	6.192	1.32	beta-Myrcene	Same as above	Same as above
5	6.719	0.75	3-Carene	Same as above	Same as above
6	7.131	0.80	1,3,8-p-Menthatriene	No report	No report
7	7.212	1.02	D-Limonene	Inducing apoptosis in lung cancer	Yu *et al*. (2018)
8	7.433	0.85	trans-beta-Ocimene	No significant effect	Su *et al*. (2013)
9	7.726	2.52	1,3,6-Octatriene	No report	No report
10	8.941	0.49	2-Carene	Cytotoxic to HeLa	Wang *et al*. (2021)
11	9.269	26.86	Linalool	Induced apoptosis	Iwasaki *et al*. (2016)
12	12.177	5.25	L-alpha-Terpineol	Cytotoxic to murine Sarcoma 180 cell line	Negreiros *et al*. (2021)
13	14.233	51.76	Linalyl isobutyrate	No report	No report
14	17.722	2.13	(R)-lavandulyl acetate	Inducing apoptosis and necrosis	Gezici *et al*. (2018)
15	18.351	3.56	2,6-Octadien-1-ol	Inducing cell cycle arrest in MCF-7	Cho *et al*. (2016)
16	19.482	0.67	Beta-Caryophyllene	Same as above	Same as above

Cytotoxicity of EOs

All three EOs exhibited dose-dependent cytotoxicity on MCF-7, HepG2, and HeLa cells as assessed by the MTT assay (Figure 2). After EO treatment 2% (v/v), the highest cytotoxicity (% *E*max) was 98.71% from lemon in MCF-7 and 96.00% from petitgrain in HeLa after 72 h (Table 2). HepG2 was the least susceptible, with an *E*max of 81.08% from petitgrain at 2% (v/v) (Table 2), thus underscoring a selective cytotoxic property of the EOs towards the tested cancer cells. Petitgrain had the lowest IC_{50} values of 0.14% (v/v) for MCF-7 and 0.25% (v/v) for HeLa. Overall, according to the MTT assay, petitgrain EO was the most cytotoxic to all three cancer cells. Three EOs exhibited considerably higher cytotoxicity towards MCF-7 and HeLa than HepG2.

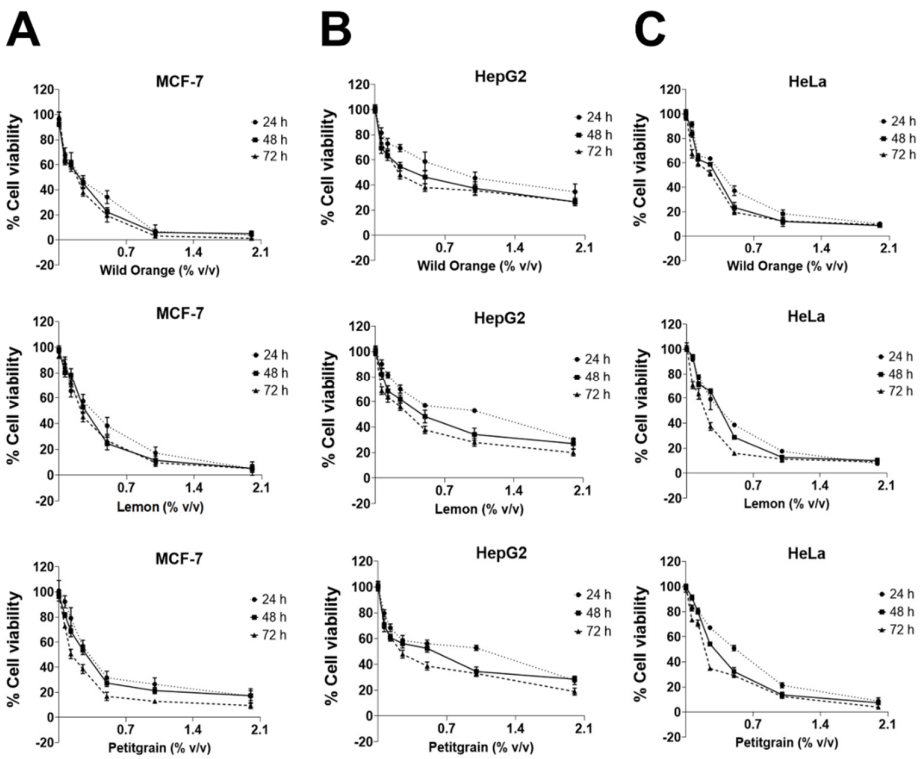

Figure 2. Effect of EOs on the cell viability (**A**) MCF-7 (**B**) HepG2 (**C**) HeLa. Cells were treated with EOs (0.0–2.0% (v/v)) for 24, 48 and 72 h. Data represent means ± SEM of three independent experiments

Table 2. *E*max and IC_{50} of EOs on cancer cells

Treatment	Time (h)	MCF-7		HepG2		HeLa	
		*E*max (%)	IC_{50} (%)	*E*max (%)	IC_{50} (%)	*E*max (%)	IC_{50} (%)
Wild Orange	24	96.17±2.74^{a}	0.19±0.01^{b}	65.37±6.19^{b}	0.80±0.10^{c}	89.72±1.26^{b}	0.32±0.01^{b}
	48	94.93±2.09^{a}	0.15±0.01^{a}	73.31±3.11^{b}	0.35±0.04^{a}	91.22±0.57^{b}	0.24±0.01^{b}
	72	98.71±0.62^{a}	0.15±0.01^{a}	73.23±2.01^{b}	0.30±0.04^{a}	90.50±1.42^{b}	0.18±0.01^{a}
Lemon	24	94.92±3.11^{a}	0.28±0.03^{b}	69.63±1.16^{b}	0.83±0.03^{c}	92.27±1.22^{b}	0.33±0.03^{b}
	48	94.76±2.31^{a}	0.25±0.02^{b}	72.91±4.16^{b}	0.45±0.08^{b}	89.91±1.92^{b}	0.31±0.01^{b}
	72	94.86±5.20^{a}	0.23±0.01^{b}	80.00±2.56^{a}	0.27±0.03^{a}	90.94±2.14^{b}	0.16±0.02^{a}
Petitgrain	24	83.00±5.91^{b}	0.34±0.04^{c}	72.41±3.26^{b}	0.64±0.09^{b}	91.14±2.54^{b}	0.42±0.03^{c}
	48	82.67±3.86^{b}	0.26±0.02^{b}	71.45±2.04^{b}	0.37±0.06^{a}	92.52±1.07^{b}	0.30±0.02^{b}
	72	90.54±2.34^{b}	0.14±0.01^{a}	81.08±2.74^{a}	0.25±0.02^{a}	96.00±1.35^{a}	0.19±0.01^{a}

Data were represented as mean ± SEM of three independent experiments. Different lowercase letters in the columns indicate significant differences ($p < 0.05$).

On the basis of the chemical composition, D-limonene, the most abundant compound of lemon and wild orange EO, and linalyl isobutyrate and linalool of petitgrain EO are believed to strongly contribute to antiproliferative effects. However, the participation of additional minor elements should not be excluded; in fact, the terpenes limonene, and beta-caryophyllene, have also demonstrated strong anticancer effects, indicating possible synergy among EO components.

Anticolony formation effect

One of the most important factors for carcinogenesis is cancer cell colonization. An anti-colony formation test was conducted to examine the antiproliferative capacity of the three EOs on the long-term viability of cancer cells. It was indicated that all three EOs reduced the colony forming capability of all three cancer cells in a dose-dependent manner (Figure 3). At 1.0% (v/v) of all three EOs, complete inhibition (100%) of colony formation was detected in MCF-7 and HeLa. Similar to the cytotoxic results, HepG2 was the least susceptible, as indicated by the highest IC_{50} values of all three EOs (Table 3). For MCF-7, the most pronounced antiproliferative effect was found in wild orange EO, with the lowest IC_{50} of 0.14% (v/v), followed by petitgrain EO and lemon EO. Likewise, wild orange EO was the most effective against HepG2 (IC_{50} of 0.54% (v/v)) and HeLa (IC_{50} of 0.14% (v/v)), followed by lemon EO and petitgrain EO. Interestingly, wild orange EO exerted the strongest antiproliferative effect over a longer period (14 days) in a colony formation assay when compared to petitgrain EO, with the strongest cytotoxic effect over a shorter period (24 h, 48 h, and 72 h). All three EO decreased the colony formation capability of MCF-7, HepG2 and HeLa cells in addition to cell viability, suggesting that certain EO elements may impact single cancer cell survival in order to suppress cancer cell colonization.

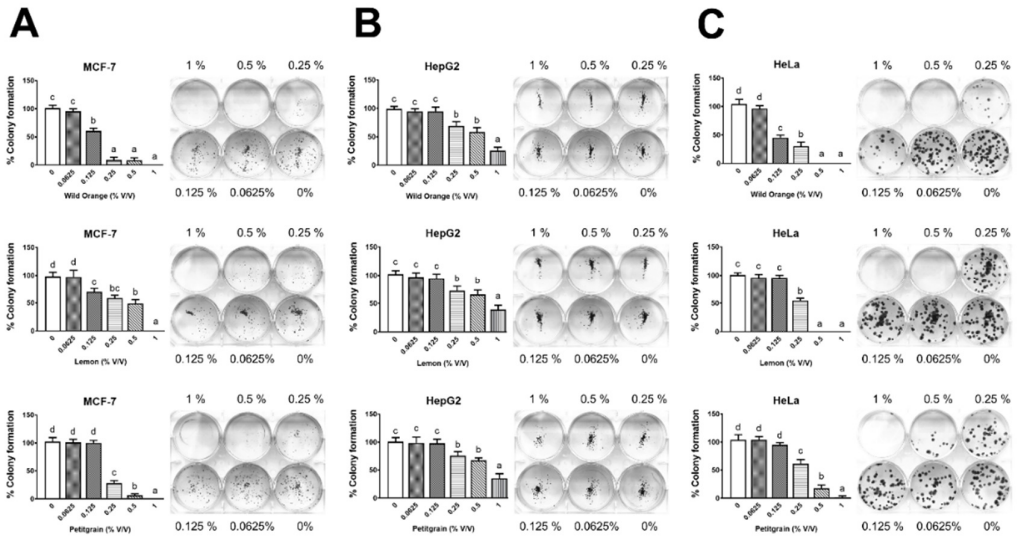

Figure 3. Effects of EOs on colony formation. (**A**) MCF-7 (**B**) HepG2 (**C**) HeLa.
Data were represented as mean ± SEM of three independent experiments. Different letters indicate significant differences ($p < 0.05$).

Table 3. IC_{50} (%) of EOs on colony formation

Treatment	IC_{50} (%)		
	MCF-7	HepG2	HeLa
Wild Orange	$0.14\pm0.01^{a, A}$	$0.54\pm0.07^{a, B}$	$0.14\pm0.02^{a, A}$
Lemon	$0.32\pm0.04^{c, A}$	$0.64\pm0.06^{ab, B}$	$0.26\pm0.01^{b, A}$
Petitgrain	$0.23\pm0.01^{b, A}$	$0.72\pm0.08^{b, B}$	$0.30\pm0.03^{c, A}$

Different lowercase letters in the columns and uppercase letters in the rows indicate significant differences ($p < 0.05$) using One-way ANOVA and Duncan's Multiple Range Test. Data represent means ± SEM of three independent experiments.

Changes in cancer cell morphology and DNA fragmentation

According to light microscopy observations, the number of viable cells was reduced in EO-treated cancer cells (Figure 4). Furthermore, treated cancer cells showed morphological changes that included decreased cell size and roundness, both of which are markers of apoptotic cells, as well as an abundance of detached and floating dead cells (Figure 4). According to the MTT experiment results, the decreased cell viability of all three cancer cells was caused by the apoptotic process.

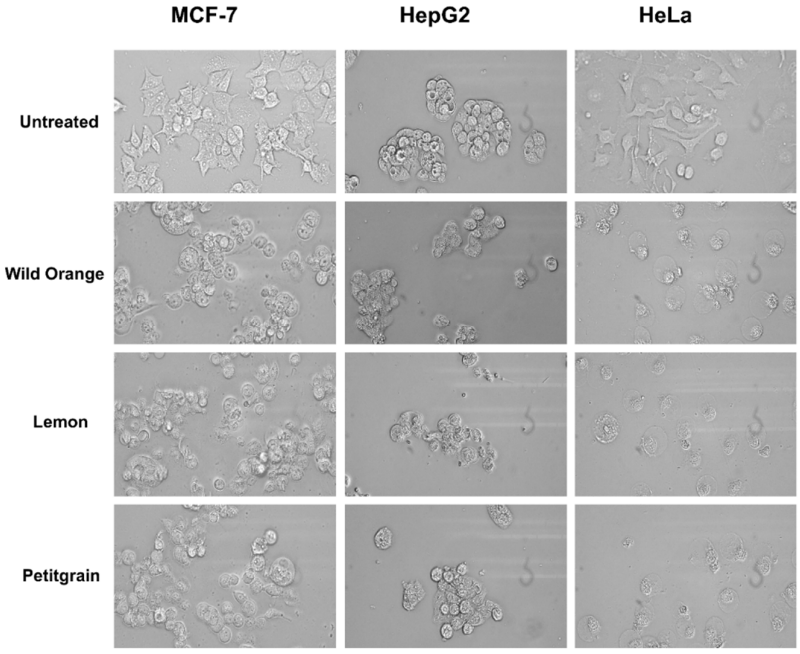

Figure 4. Effects of EOs on cell morphology (**A**) MCF-7 (**B**) HepG2 (**C**) HeLa. Cells were treated with EOs at 0.25% for 24 h

The genomic DNAs from MCF-7, HepG2, and HeLa cells were electrophoresed for integrity check after being treated for 24 h with 0.25 % (v/v) citrus EOs. In comparison with the control (untreated cells), this distinctive laddering of apoptotic activity was found in all three cancer cells treated with each citrus EO (Figure 5). This impact was more noticeable with wild orange EO than with the other two.

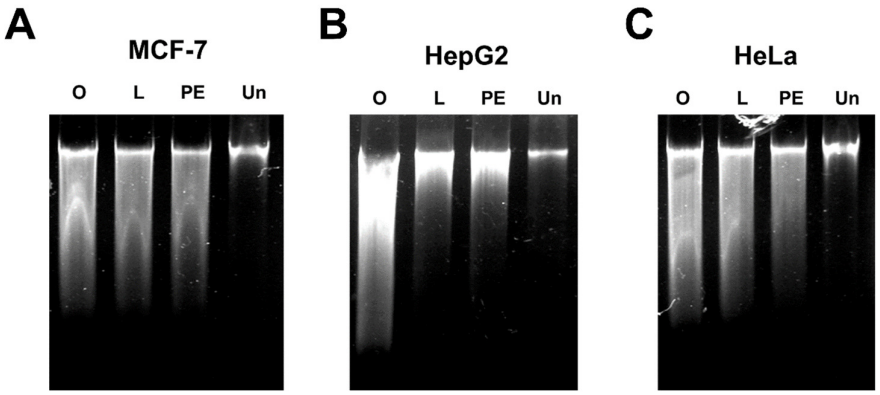

Figure 5. Effects of EOs on DNA fragmentation in cancer cells (**A**) MCF-7 (**B**) HepG2 (**C**) HeLa. Cells were treated with the EOs at 0.25% for 24 h. O = Wild orange; L = Lemon; PE = Petigrain; Un = Untreated cells (Control)

This is in accordance with the findings of an anticolony formation test, which revealed that wild orange EO had the best antiproliferative action against all three cancer cells. This is the first study to indicate that wild orange EO and petitgrain EO had an effect on DNA fragmentation in MCF-7, HepG2 and HeLa cells.

Apoptosis is characterized by the cleavage of chromosomal DNA into oligonucleosomal-sized pieces. Our findings demonstrated that when MCF-7, HepG2, and HeLa cells were treated with wild orange, lemon, and petitgrain EOs, DNA fragmentation occurred, confirming apoptosis induction by the EOs.

Antimigratory effect

Migration and invasion are often regarded as critical aspects of cancer metastasis. To detect the effect of citrus EOs on the migratory capabilities of MCF-7, HepG2, and HeLa cells, a wound scratch assay was conducted. As a result, the wound closure was observed and imaged under an inverted microscope. Migration of all three cancer cell lines treated with wild orange EO was significantly inhibited (Figure 6) with the percentage of wound closure reduced to below 40% at both 24 and 48 h whilst lemon EO led to the percentage of wound closure reduced to below 50%. Petitgrain EO was the least effective antimigratory activity. The most common cause of death in cancer patients is metastasis. One of the critical steps is cancer invasion and cell migration. The present data showed that wild orange EO most significantly decreased the migratory ability of all three cancer cells.

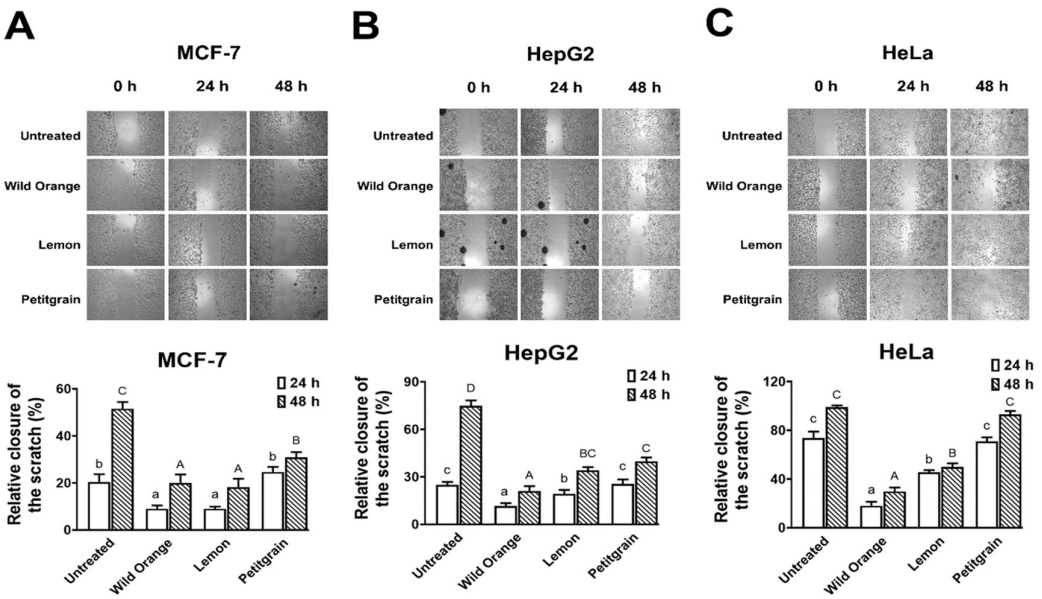

Figure 6. Effects of EOs on cancer cell migration using wound healing assay (**A**) MCF-7 (**B**) HepG2 (**C**) HeLa. Cells were treated with EOs (0.125% v/v) for 24 h and 48 h. Different small letters and capital letters indicate significant differences ($p < 0.05$) at 24 h and 48 h, respectively

Discussion

The wild orange and lemon EOs from Brazil and petitgrain EO from Paraguay were shown to display chemical compositions similar to the previous reports, yet certain components were distinct from the other reported citrus EOs of different origins. The three compounds, alpha-pinene, beta-pinene, and beta-myrcene in lemon EO exhibited chemo preventive activities (Table 1), for examples, inducing natural killer cells via ERK/AKT pathway (Jo *et al*., 2021), apoptosis stimulation and cell cycle arrest (Salehi *et al*., 2019), inducing

oxidative stress, respectively (Bai and Tang, 2020). Interestingly, beta-caryophyllene, a sesquiterpene found in both lemon and petitgrain, was found to modulate STAT3 signalling, oxidative stress, DNA damage response (Di Sotto *et al*., 2020) and was cytotoxic against MCF-7, A549, and Hela (Su *et al*., 2013).

In comparison to the earlier publication (Ben Hsouna *et al*., 2017), their investigations indicated a complex blend of hydrocarbon monoterpene, oxygenated monoterpene, and nitrogen compounds in the lemon EO. The nine significant identified components included D-limonene, beta-pinene, alpha-terpineol, nerolidol, farnesol, acetate geranyl, linalyl acetate, and acetate neryl. Alpha-pinene, sabinene, myrcene, 1,8-cineol, cis-linalool oxide, and geranial were found as minor components (<1%). Similar to the previous report, sweet orange EO from Maharashtra, India consisted of 15 compounds, of which D-limonene was a dominant contributor, followed by 5,5,10,10-tetrachlorotricyclodecane, butane, 1-(2,2-dichloro-3,3-dimethylcyclopropyl)-pentane, 3-chloro-2-nitrobenzyl alcohol, and others (Cholke *et al*., 2017).

Likewise, sweet orange EO from Vietnam contained D-limonene (98.238%), beta-myrcene (1.169%) and alpha-pinene (0.548% and 0.413%) (Ngân *et al*., 2020). However, sweet orange EO from Uganda and Rwanda showed a total of 51 and 55 volatile chemical components, respectively. The major constituents found were D-limonene (87.9 and 92.5%), myrcene (2.4 and 2.0%), alpha-pinene (0.5 and 2.4%), linalool (1.2 and 0.9%), octanal (1.3 and 0.6%) and decanal (both 0.2%) (Simon Muhoho Njoroge *et al*., 2009).

Similar to the Poland petitgrain EO, 20 compounds were identified. The major components of petitgrain EO were linalyl acetate (48.06%) and linalool (26.88%) (Gniewosz *et al*., 2017). Linalool was the most abundant of the primary components of Tunisian oil (22.35-62.57%), with linalyl acetate and alpha-terpineol being less abundant (Ellouze *et al*., 2014). The Greek petitgrain oil contained 88.09% oxygenated monoterpenes, the vast majority of which were linalool (58.21%). Also, Greek petitgrain oil contains neryl acetate and trans-ocimene, both of which are absent from commercial petitgrain oil. Sicilian petitgrain oil, on the other hand, was found to have greater levels of linalyl acetate (0.3-73.1%) and less of linalool (8.7-16.7% (De Pasquale *et al*., 2006).

According to the earlier publication, the IC_{50} of lemon EO from China on MCF-7 was 0.143% (v/v) (Zu *et al*., 2010), which was similar to our findings. However, EOs from other plants were more cytotoxic than our EOs to HepG2 cells. At 72h, the IC_{50} value of *Origanum dictamnus* EO was 0.00699% (v/v), and the impact was mostly due to carvacrol (Mitropoulou *et al*., 2015). EOs of the plants, *Satureja thymbra* and *Satureja parnassica,* were examined for chemopreventive effects against MCF-7 cells and displayed stronger cytotoxicity [IC_{50} = 0.002% (v/v) and 0.08% (v/v), respectively] than our results (Fitsiou *et al*., 2016). For petitgrain EO, the IC_{50} values against MCF-7, HepG2 and HeLa cells in the range of 0.14-0.25% (v/v) at 72 h were reported for the first time.

Previously, beta-caryophyllene was able to inhibit colony formation of HCT 116 cells (Dahham *et al*., 2015) and beta-myrcene showed an antiinvasive effect on metastatic MDA-MB-231 human breast cancer cells (Lee *et al*., 2015). Limonene, a main component of citrus EOs, plays a role in apoptosis in tumor cells (Hata *et al*., 2003). Similarly, DNA nucleosomal fragmentation was seen in tumoral HL60 cells treated with the median to maximum concentrations of lemon juice (0.8-2.0% v/v) and the three highest concentrations of limonene (0.6, 1.2, and 2.35 mM) (Fernández-Bedmar *et al*., 2011). These findings might point to the start of the apoptotic process. Limonene appears to be an apoptotic-inducing agent with anticancer effects and also suppressing angiogenesis and metastasis (Chidambara Murthy *et al*., 2012). The presence of chemical components in all three citrus EOs (Table 1) contributed to cytotoxic activity, antiproliferative ability, and antimigratory effect against MCF-7, HepG2 and HeLa cells.

Conclusions

This is the first study to show the chemical composition and cytotoxic evaluation of the lesser-known petitgrain EO in comparison to well-known lemon and wild orange EOs, contributing to the increase of recent studies regarding the therapeutic attributes of petitgrain, which is currently scarce. All three EOs exhibited cytotoxic activity, antiproliferative ability, and antimigratory effects against MCF-7, HepG2, and HeLa cells in a dose and time-dependent manner and induction of apoptosis. These three citrus EOs are crucial sources of novel bioactive compounds that can replace synthetic chemicals since they are less hazardous and favorable to the environment, and they can be utilized as an alternative to anticancer therapies that are more inexpensive, sustainable, and ecological.

Authors' Contributions

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by them. W.S. and T.K. conducted the experiments. B.B. and N.L.M. designed the experiments. T.K. curated cancer cells. V.L. designed the experiments, analysed data and wrote the manuscript. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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