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Antioxidant and Anticancer Activities of *Citrus reticulate* (Petitgrain Mandarin) and *Pelargonium graveolens* (Geranium) Essential Oils

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Abstract: The essential oils isolated by hydro-distillation from *Pelargonium graveolens* (geranium) and *Citrus reticulate* (petitgrain mandarin) were analyzed by GC/MS and assessed for antioxidant and anticancer activities. Twenty five components of petitgrain mandarin essential oil were identified and the major components were γ-terpinene (47.89%), methyl N-methyl anthranilate (13.17%), α-terpinene (7.40%), β-phellandrene (6.26%) and trans-isolimonene (5.87%). On the other hand, Thirty two compounds constituting 99.23% of geranium essential oil have been identified. The major components were citronellol (29.90%), trans-geraniol (18.03%), 10-epi-γ-eudesmol (8.27%), isomenthone (5.44%) and linalool (5.13%). The DPPH free radical scavenging activities of petitgrain mandarin and geranium essential oils at various concentrations ranging from 25 to 200 µg/ml were determined. The essential oils of both plants reduced the concentration of DPPH free radical with an efficacy near to that of standard antioxidant (ascorbic acid). 50% effective concentration (EC_{50}) of petitgrain mandarin essential oil (79.84 µg/ml) was higher than geranium essential oil (66.45 µg/ml). It was indicated that the antioxidant activity of the geranium essential oil was higher than that of the petitgrain mandarin essential oil. In addition to the antioxidant activity, the anticancer activity of the two essential oils on two human promyelocytic leukemia cell lines (HL-60 and NB4) using trypan blue assay were investigated *in vitro*. HL-60 and NB4 cell lines were treated with the essential oils samples at different concentrations ranging from 25 to 200 µg/ml. Geranium essential oil showed the highest anticancer activity with the LC_{50} values of 62.50 µg/ml in NB4 cell line and 86.5 µg/ml in HL-60 cell line, whereas petitgrain mandarin essential oil gave the LC_{50} values of 85.05 µg/ml in NB4 cell line and 105.73 µg/ml in HL-60 cell line. The results demonstrated the potential of the two essential oils for cancer treatments.

Key words: *Citrus reticulate*, *Pelargonium graveolens*, Essential oils, antioxidant, anticancer.

INTRODUCTION

In recent years the general public has shown an increased interest in the use of herbal medicines in preference to synthetic drugs. This is based on the belief that natural products are intrinsically less dangerous and can be obtained at a lower cost [1]. Many diseases, such as cancer, atherosclerosis and inflammation are caused by free radicals and lipid peroxidation inside human bodies. This kind of risk can be reduced by an appropriate dietary pattern including a great portion of fruit and vegetables [2,3] because of the great amount of natural antioxidants in these plant foods [4].

Volatile oils, also known as essential oils are lipophilic compounds containing volatile aroma compounds. The constituents of the oils are mainly monoterpenes and sesquiterpines which are hydrocarbons with the general formula $(C_5H_8)_n$. Oxygenated compounds derived from these hydrocarbons include alcohols, aldehydes, esters, ethers, ketones, phenols and oxides. It is estimated that there are more than 1000 monoterpene and 3000 sesquiterpene structures. Other compounds include phenylpropenes and specific compounds containing sulphur or nitrogen. Hundreds of new natural substances are being isolated and identified every year, but data concerning their biological activities are known for only some [5]. The volatile compounds of oils are aromatic or odoriferous that occurs in plants as such or less frequently may result from the degradation of glycerides by enzyme action [6]. They are used in perfumes, cosmetics, flavoring food and drinks and for scenting. Medicinal uses proposed by sellers of essential oils vary from skin treatments to remedies for cancer [7]. Essential oils exhibit various and variable antimicrobial activities, including antifungal, antiviral, antibacterial, insecticidal, and antioxidant properties [8].

Geranium (*Pelargonium* species) is an essential oil plant that belongs to the Geraniaceae family [9]. Out of 25 *Pelargonium* species, only four species are important in the production of essential oil. The four

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essential oil species are *P. graveolense, P. odoratissium, P. capitatum* and *P. radens* [10]. The geranium essential oil is largely utilized in the perfumery, cosmetic and aromatherapy industries all over the world. It has since become indispensable aromatherapy oil. It is one of the best skincare oils because it is good in opening skin pores and cleaning oily complexions [9-11]. Other uses of geranium essential oil that are becoming more and more popular include the treatment of dysentery, hemorrhoids, inflammation, heavy menstrual flows, and even cancer. The French community is currently treating diabetes, diarrhea, gallbladder problems, gastric ulcers, jaundice, liver problems, sterility and urinary stones with this oil. The leaves are used as a form of herbal tea to de-stress, fight anxiety, ease tension, improve circulation and cure tonsillitis [11]. On the other hand, petitgrain mandarin essential oil is extracted from *Citrus reticulata* of the Rutaceae family. This essential oil has some great properties to help relieve stress and digestive problems, but is mostly used to increase circulation to the skin, reducing fluid retention and to help prevent stretch marks. Mandarin oil is soothing to the nervous system and has a tonic effect on the digestive system, while helping flatulence, diarrhea and constipation [12,13]. However, few studies on antioxidant and anticancer activities of geranium and petitgrain mandarin essential oils have been performed until now. The objective of current study was to evaluate antioxidant activity using DPPH radical scavenging assay and anticancer activity against two human promyelocytic leukemia cell lines (HL-60 and NB4) of *Pelargonium graveolens* (geranium) and *Citrus* reticulate leaves (petitgrain mandarin).

MATERIALS AND METHODS

Plant Material: The fresh aerial parts (stems, stalks and leaves) of *Pelargonium graveolens* (geranium) purchased from experimental station of medicinal plants, Faculty of Pharmacy, Cairo University, Egypt. Fresh *Citrus reticulate* leaves (petitgrain mandarin) were collected from local farm in Badr Center, El-Behira, Egypt.

Essential Oil Isolation: Geranium and petitgrain mandarin samples (100 g) were hydro-distilled in Clevenger-type apparatus [14]. The essential oils were collected and dried over anhydrous sodium sulphate. The essential oil samples were stored in the dark at 4ºC. The amount of oil obtained from plant material was calculated as:

Oil (% v/w) = observed volume of oil (ml)/weight of sample (g) × 100

GC/MS Analysis of Essential Oil: The essential oils were analyzed by GC-MS according to Adams[15]. GC/MS analysis was performed on a Thermoquest-Finnigan Trace GC-MS equipped with a DB-5 (5% phenyl) methylpolysiloxane column (60 m \ 0.25 mm i.d., film thickness 0.25 μm). The injection temperature was 220 ºC and the oven temperature was raised from 40 ºC (3 min hold) to 250 ºC at a rate of 5 ºC/min, then held at 250 ºC for 2 min; transfer line temperature was 250 ºC. 1 μl of sample was injected and helium was used as the carrier gas at a flow rate of 1.0 ml/min. The mass spectrometer was scanned over the 40 to 500 m/z with an ionizing voltage of 70 eV and identification was based on standard mass library that National Institute of Standards and Technology (NIST Version 2.0) to detect the possibilities of essential oil components.

Antioxidants Activity Using DPPH Radical Scavenging Activity: The hydrogen atom-or-electron donating ability of the corresponding essential oils was measured from the bleaching of the purple colored methanol solution of DPPH. This spectrophotometric assay uses the stable radical, 2,2′-diphenylpicrylhydrazyl (DPPH˙), as a reagent [16]. Fifty microliters of various concentrations (25, 50, 75, 100 and 200 μg/ml) of the essential oils in dimethyl sulphoxide (DMSO) were add to 5 ml of a 0.004% methanolic solution of DPPH˙. The reaction mixture was covered and left in the dark at room temperature. After one hour, the absorbance was read against blank at 517 nm. Ascorbic acid was used as standard control. The antiradical scavenging activity of the two essential oils were evaluated in comparison with the reference ascorbic acid as described above for extracts. The antioxidant capacity to scavenge the DPPH radical for the oils was calculated by the following equation:

$$\text{Scavenging effect (\%)} = ((A_{blank} - A_{sample}) / A_{blank}) \times 100$$

where A_{blank} is the absorbance of control reaction (containing each reagents except the sample), and A_{sample} is the absorbance of sample.

The percentage of Scavenging activity was plotted against the essential oil concentration to obtain the effective concentration (EC_{50}), defined as the essential oil concentration necessary to cause 50% scavenging. Tests were carried out in triplicate.

Cell Growth and Viability Assay: Human promyelocytic leukemia cell lines (HL-60 and NB4), obtained from the American Type Culture Collection (ATCC), were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mmol/L glutamine, penicillin (100 U/ml), and streptomycin (100 mg/ml) in humidified atmosphere containing 5% CO_2 at 37 ºC for 24 h. Cell counts were determined. HL-60 and NB4 cells were seeded at a density of 2 x 10^5/ml. After that the cells were treated with different volumes of geranium and petitgrain mandarin essential oils to give final concentrations (25, 50, 75, 100 and 200 μg/ml) and incubated under the same condition for another 24h. The final volume in each experiment was made up to 100 μl with the media containing 1% DMSO. Control cells were treated with the equivalent amount of vehicle dimethyl sulphoxide. After this period the cell viability was evaluated using trypan blue assay. The viability percentages were calculated according to Bennett *et al.*[17]. This method depended up on the ability of trypan blue dye to stain the dead cells with blue color then easier to count using hemocytometer slide (under microscope). Each experiment was carried out in triplicate.

The percentage of dead cells was plotted against the essential oil concentration to obtain the LC_{50}, defined as the essential oil concentration necessary to cause 50% death.

Statistical Analyses: Statistical analyses (standard deviation ''SD'' and standard error ''SE'') was carried out according to Fisher[18]. LSD (Least significant difference) test was used to compare the significant differences between means of treatment [19]. The statistical package for social science S.P.S.S.[20] program version was used for all analysis.

RESULTS AND DISCUSSION

Essential Oils Composition: The hydro-distillation of *Citrus reticulate* leaves (petitgrain mandarin) and the aerial parts of *Pelargonium graveolens* (geranium) yield 0.46% (v/w) and 0.26 % (v/w) respectively. The GC-MS analysis of petitgrain mandarin (leaves) and geranium (aerial parts) essential oils are presented in Table (1) and Table (2), respectively. It seems that there were no similarities among chemical compositions of the two essential oils.

A total of 25 components constituting 99.38% of petitgrain mandarin essential oil were identified and the major components were γ-terpinene (47.89%), methyl N-methyl anthranilate (13.17%), α-terpinene (7.40%), β-phellandrene (6.26%), trans-isolimonene (5.87%), α-terpinolene (4.63%), myrcene (3.18%), α-Pinene (2.77%), 4-Terpinol (1.66%), Linalyl propionate (1.39%) and Cuminic aldehyde (1.15%). All other components were present in amount lower than 1%. In case of petitgrain mandarin essential oil components, a hydrocarbons and anthranilate are the two main groups, with oxygenated compounds as a minor fraction.

Table 1: Chemical composition of petitgrain mandarin essential oil

Compound	Peak area (%)
α-Terpinene	7.40
δ-3-Carene	0.26
Myrcene	3.18
α-Pinene	2.77
p-Cymene	0.01
β-Phellandrene	6.26
Trans-Isolimonene	5.87
γ-Terpinene	47.89
α-Terpinolene	4.63
Z-Citral	0.02
β-Ocimene-x	0.25
Alloocimene	0.65
Limonene oxide	0.66
4-Terpinol	1.66
Linalyl propionate	1.39
Cuminic aldehyde	1.15
Linalyl acetate	0.17
Camphor	0.03
Thymol	0.25
Linalool oxide	0.79
p-Menth-8-ene-1,2-diol	0.31
Methyl N-methyl anthranilate	13.17
α-Humulene	0.05
5-Methylene-3-methylhexan-1,3-diol	0.04
Caryophyllene oxide	0.52
Total identified compounds	99.38

Table 2: Chemical composition of geranium essential oil

Compound	Peak area (%)
α-Pinene	0.27
Limonene	0.16
Linalool oxide	0.15
Linalool	5.13
Trans-Rose oxide	0.34
p-Menthone	1.47
Isomenthone	5.44
p-Menth-1-ene-8-ol	0.14
Citronellol	29.90
Trans-Geraniol	18.03
Geranyl acetate	4.52
Citronellyl acetate	0.81
α-Copaene	0.65
Neryl acetate	0.63
β-Bourbonene	1.46
trans-Caryophyllene	1.51
α-Cubebene	0.15
Citronellyl propionate	0.54
Aristolene	0.45
α-Humulene	1.00
Geranyl propionate	1.55
Germacrene D	2.05
Germacrene B	1.25
γ-Cadinene	2.89
Geranyl butyrate	2.53
Phenyl ethyl tiglate	1.93
10-epi-γ-eudesmol	8.27
Agarospirol	1.14
Globulol	1.67
Geranyl tiglate	2.50
Geranyl hexanoate	0.44
Geranyl butyrate	0.26
Total identified compounds	99.23

Our results agree with those reported by Melendreras *et al.*[21], Lota *et al.*[22], Verzera *et al.* [23] and Pedruzzi *et al.*[24].

Thirty two compounds constituting 99.23% of geranium essential oil have been identified. The major components were citronellol (29.90%), trans-geraniol (18.03%), 10-epi-γ-eudesmol (8.27%), isomenthone (5.44%), linalool (5.13%), geranyl acetate (4.52%), γ-Cadinene (2.89%), Geranyl butyrate (2.53%), Geranyl tiglate (2.50%) and gemacrene D (2.05%). All other components represent in amount lower than 2%. Oxygenated compounds are the main group in geranium essential oil. These results are similar to that obtained by Rao *et al.*[25] who reported that geranium essential oil was rich in oxygenated components and commercial rhodinol (linalool + citronellol + geraniol) fraction.

From the results of the two essential oils, it can be concluded that petitgrain mandarin is considered as the major source of hydrocarbons, while geranium is the major source of oxygenated compounds.

Antioxidant Activities of Essential Oils: The DPPH free radical scavenging activities of petitgrain mandarin and geranium essential oils at various concentrations were determined and compared with that of the standard antioxidant ascorbic acid (Table 3). All the tested samples showed lower DPPH radical scavenging activity when compared with the standard. The highest antioxidant scavenging effect (%) was obtained with ascorbic acid (93.60%) for concentration of 100 µg/ml, while it was 76.80% for 200 µg/ml concentration of petitgrain mandarin essential oil, and 89.60% for 200 µg/ml concentration of geranium essential oil when recorded after 60 min. The essential oils of both plants reduced the concentration of DPPH free radical with an efficacy near to that of standard antioxidant. Both plants essential oils were able to reduce the stable, purple-colored radical DPPH into yellow-colored DPPH reaching 50% of reduction with EC_{50} values as follows: EC_{50} (petitgrain mandarin) = 79.84 µg/ml; EC_{50} (geranium) = 66.45 µg/ml and EC_{50} (ascorbic acid) = 38.49 µg/ml. The EC_{50} values indicated that the antioxidant activity of the geranium essential oil was higher than that of the petitgrain mandarin essential oil. The quantity of petitgrain mandarin and geranium essential oils required were about 2.07 and 1.73 fold, respectively, when compared with the standard antioxidant ascorbic acid. The antiradical scavenging activity of oils might be attributed to the replacement of hydroxyl groups in the aromatic ring systems of the phenolic compounds as a result of their hydrogen donating ability [16]. Yi *et al.*[26] showed antioxidant activities of *Citrus reticulate* by various antioxidant assays, including DPPH scavenging, hydroxyl radical scavenging, superoxide anion radical scavenging and hydrogen peroxide scavenging. Few authors have reported antioxidant and radical scavenging properties of petitgrain mandarin essential oil [27,28]. Lalli *et al.*[29] investigated the antioxidant properties of *Pelargonium* species by DPPH assay. The author found that EC_{50} of *Pelargonium graveolens* was 14.49 µg/ml (acetone extract). In other studies, the methanolic extract of *Pelargonium* species (Geraniaceae) were found to exert pronounced antioxidant activities [30]. The essential oil of geranium has shown active antioxidant capacities [31].

Geranium and also petitgrain mandarin essential oils are possible sources of antioxidant compounds since these extracts were relatively non-toxic [28,29]. These observations prompt the necessity for further studies, focusing on the isolation and structure elucidation of their antioxidant compound/s, since they have potential use as therapeutic agents in managing diseases associated with free radicals and also have the potential employed as additives in the food or cosmetic industries.

Anticancer Activities of Essential Oils: In order to understand the effect of petitgrain mandarin and geranium essential oils on human promyelocytic leukemia cells, experiments were conducted using cultured HL-60 and NB4 cell lines. Results of the viability were measured using trypan blue assay. The results found that the incubation of HL-60 cells with petitgrain mandarin and geranium essential oils at all concentrations (25 – 200 µg/ml) for 24 hour reduced the viability of these cells. The dead cells were increased by increasing the concentration of both plants essential oils (Table, 4). The highest HL-60 dead cell (%) was recorded by geranium essential oil (79.27%) for concentration of 200 µg/ml, while it was 68.87% for 200 µg/ml concentration of petitgrain mandarin essential oil.

The same trend was observed in NB4 cells as similar as in HL-60 cells. The highest NB4 dead cells (%) was recorded by geranium essential oil (79.8%) for concentration of 200 µg/ml, while it was 58.90% for 200 µg/ml concentration of petitgrain mandarin essential oil. It must be noticed that, there were no significant difference in dead cell (%) between the concentrations 100 and 200 µg/ml of petitgrain mandarin or geranium essential oils (Table, 5). The LC_{50} values were determined from the graphs of the essential oils on HL-60 and NB4 cell lines. Geranium essential oil showed potent cytotoxic effects with the LC_{50} values of 62.50 µg/ml in NB4 cell line and 86.5 µg/ml in HL-60 cell line, whereas petitgrain mandarin essential oil gave the LC_{50} values of 85.05 µg/ml in NB4 cell line and 105.73 µg/ml in HL-60 cell line. The LC_{50} values indicated that the anticancer activity of geranium essential oil was higher than petitgrain mandarin essential oil against both cell lines. Tian *et al.*[32] investigated the antiproliferative activity of

Table 3: The DPPH free radical scavenging activities of petitgrain mandarin and geranium essential oils

Sources	Concentration (µg/ml)	Scavenging effect (%)
Petitgrain mandarin		
	25	6.20 ± 0.42 h
	50	21.00 ± 1.21 g
	75	46.80 ± 3.11 d
	100	58.70 ± 3.90 c
	200	76.80 ± 4.05 b
Geranium		
	25	25.00 ± 1.53 fg
	50	38.20 ± 2.62 e
	75	56.70 ± 3.19 c
	100	78.00 ± 4.17 b
	200	89.60 ± 3.92 a
Ascorbic acid	25	31.43 ± 1.60 ef
	50	63.30 ± 2.37 c
	75	80.90 ± 3.71 b
	100	93.60 ± 3.13 a
LSD		8.79

The values are means ± SE.
The mean values with different small letters within a column indicate significant differences ($P < 0.05$).

Table 4: Effect of petitgrain mandarin and geranium essential oils on cells viability of HL-60 cells after 24 h of treatment

Sources	Concentration (µg/ml)	Dead cells (%)	Viable cells (%)
Control	0	0.00 g	100
Petitgrain mandarin			
	25	12.30 ± 3.70 f	87.70
	50	15.60 ± 3.22 ef	84.40
	75	37.00 ± 3.79 d	63.00
	100	46.83 ± 3.45 d	53.17
	200	68.87 ± 3.81 b	31.13
Geranium			
	25	9.60 ± 3.52 f	90.40
	50	22.90 ± 2.98 e	77.10
	75	39.40 ± 3.49 d	60.60
	100	57.60 ± 3.95 c	42.40
	200	79.27 ± 1.28 a	20.73
LSD		9.49	

The values are means ± SE.
The mean values with different small letters within a column indicate significant differences ($P < 0.05$).

limonoids, found in high concentrations in mandarin against HL-60 cell lines. Mak *et al.* [33] isolated anti-leukemia compounds from *Citrus reticulate* peels. Extracts of *Citrus reticulate* roots has been reported as anticancer [34], antibacterial [35] as well as antioxidant activity [27]. Many authors reviewed that *Pelargonium graveolens* has potential antitumor activity against uterine cervical neoplasia [36-38].

Statistical analysis indicated that, there are linear positive correlation between antioxidant activity and anticancer activity of petitgrain mandarin essential oil against both human promyelocytic leukemia cells, HL-

Table 5: Effect of petitgrain mandarin and geranium essential oils on cells viability of NB4 cells after 24 h of treatment

Sources	Concentration (µg/ml)	Dead cells (%)	Viable cells (%)
Control	0	0.00 [g]	100
Petitgrain mandarin	25	11.23 ± 2.32 [f]	88.77
	50	19.83 ± 2.89 [f]	80.17
	75	43.77 ± 3.41 [d]	56.23
	100	54.63 ± 2.81 [c]	45.37
	200	58.90 ± 3.35 [bc]	41.10
Geranium	25	19.73 ± 3.12 [f]	80.27
	50	32.53 ± 3.79 [e]	67.47
	75	66.17 ± 2.49 [b]	33.83
	100	74.50 ± 3.12 [a]	25.50
	200	79.80 ± 2.02 [a]	20.20
LSD		8.31	

The values are means ± SE.
The mean values with different small letters within a column indicate significant differences ($P < 0.05$).

60 (Pearson correlation=0.983, $P \leq 0.001$) and NB4 (Pearson correlation=0.985, $P \leq 0.001$) cell lines. Also, antioxidant activity of geranium essential oil are positively correlated with its anticancer activity against HL-60 (Pearson correlation=0.991, $P \leq 0.000$) and NB4 (Pearson correlation=0.963, $P \leq 0.004$) cell lines.

Essential oils and their main components possess a wide spectrum of biological activity, which may be of great importance in several fields, from food chemistry to pharmacology and pharmaceutics [39]. The main advantage of essential oils is that they can be used in any foods and are considered generally recognized as safe (GRAS), as long as their maximum effects is attained with the minimum change in the organoleptic properties of the food [40].

Essential oils rich in monoterpenes are recognized as food preservatives [41-43], and monoterpenic essential oils are natural antioxidants [44] that are active against certain cancers [45]. Indeed, a number of dietary monoterpenes have antitumoral activity that can prevent the formation or progress of cancer and cause tumor regression.

Most of the principle components present in petitgrain mandarin and geranium essential oils are monoterpenes. Monoterpenes have shown prevention of mammary, lung, skin, liver and forestomach cancers in rat models [46]. In the present study, the inhibition of human promyelocytic leukemia cells (HL-60 and NB4) may be due to the presence of monoterpenes.

The difference in antioxidant and anticancer effects between the two essential oils is attributable to the chemical composition of each essential oil. Analysis of petitgrain mandarin essential oil showed γ-terpinene, methyl N-methyl anthranilate and α-terpinene as the major constituents. Also, citronellol and trans-geraniol were the major constituents in geranium essential oil. These compounds are known to possess antioxidant and anticancer properties. The anticancer and antioxidant activities of petitgrain mandarin essential oil may be due to its content of γ-terpinene, α-terpinene [47] and methyl N-methyl anthranilate which showed *in vitro* growth inhibitory properties against human tumor cell lines [48]. On the other side, the antioxidant and anticancer activities of geranium essential oil may be attributed to the major contents of citronellol and trans-geraniol. Zhuang *et al.*[49] reported that citronellol, an oil soluble compound derived from the geranium, has anticancer and anti-inflammatory properties. Burke *et al.*[50] investigated the anticancer activity of geraniol. The author found that significant (60-90%) inhibition of the anchorage-independent growth of human MIA PaCa2 pancreatic tumor cells was attained with geraniol.

There are many reports in the literature concerning the biological activities of petitgrain mandarin and geranium essential oils, but there are few studies on the antioxidant and anticancer activities of these essential oils.

In conclusion, the present study shows that essential oils of petitgrain mandarin and geranium may be potentially used as good sources of antioxidants and anticancer (HL-60 and NB4). The overall results obtained from geranium essential oil were better than those obtained from petitgrain mandarin essential oil. In addition, the petitgrain mandarin essential oil is more effective on HL-60 cell line than NB4 cell line, while geranium essential oil is more effective on NB4 cell line than HL-60 cell line. Consumption of foods prepared with geranium and petitgrain mandarin or their essential oils may have significant health benefits. The observed antioxidant and anticancer properties of the two essential oils may have useful implications for detailed studies of their natural antimicrobial and antiviral agents. However, studies *in vivo* are needed to assess the true antioxidant and anticancer activities of these essential oils and to determine the metabolic pathways involved in their degradation.

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