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Antitumor Potential of *Lippia citriodora* Essential Oil in Breast Tumor-Bearing Mice

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Abstract: *Lippia citriodora* is a flowering plant cultivated for its lemon-scented leaves and used in folk medicine for the preparation of tea for the alleviation of symptoms of gastrointestinal disorders, cold, and asthma. The oil extracted from the plant leaves was shown to possess antioxidant potential and to exert antiproliferative activity against breast cancer. The aim of this study was to further investigate potential antitumor effects of *L. citriodora* oil (LCO) on breast cancer. The in vitro antiproliferative activity of LCO was examined against murine DA3 breast cancer cells by the sulforhodamine B assay. We further explored the LCO's pro-apoptotic potential with the Annexin-PI method. The LCO's anti-migratory effect was assessed by the wound-healing assay. LCO was found to inhibit the growth of DA3 cells in vitro, attenuate their migration, and induce apoptosis. Finally, oral administration of LCO for 14 days in mice inhibited by 55% the size of developing tumors in the DA3 murine tumor model. Noteworthy, in the tumor tissue of LCO-treated mice the apoptotic marker cleaved caspase-3 was elevated, while a reduced protein expression of survivin was observed. These results indicate that LCO, as a source of bioactive compounds, has a very interesting nutraceutical potential.

Keywords: *Lippia citriodora*; essential oil; antiproliferative effect; antimigratory activity; breast cancer; oral administration

1. Introduction

Being stationary autotrophs, plants need to overcome a variety of challenges such as herbivores, pathogens, and the need for a steady flow of nutrients despite the environmental changes. In order to cope with the various stresses and damages induced by a dynamic environment, plants have evolved to synthesize secondary metabolites that allow them to interact with their environment [1,2]. These low-molecular-weight substances, which can be species- or genera-specific [2], represent a huge and heterogeneous group of bioactive compounds, used as high-value chemicals [3].

Plant secondary metabolites are a unique source for pharmaceuticals [4], insecticides [5], flavoring agents, dyes, etc. [3]. Noteworthy, various bioactivities were identified in different phytochemicals such as antibacterial [6], antiviral [7], antifungal [8], antioxidant [9], and anti-inflammatory [10] activities. Most importantly, several plant extracts exert significant antitumor effects, namely, antiproliferative, redox regulating, cell cycle arrest-inducing, anti-migratory, and pro-apoptotic [11–14]. Carcinogenesis and tumor growth are complex processes that involve various mechanisms and the recruitment of a diverse range of molecules. Phytochemicals, being functionally pleiotropic, may lead to simultaneous targeting of the various events/pathways involved in cancer development and progression [15]. Certain dietary phytochemicals, besides their direct antitumor effects, were shown to

enhance the therapeutic potency of chemotherapeutic agents [16]. Crucially, the anticancer properties of various phytochemicals were linked to their antioxidant activity [17].

Lippia citriodora is a flowering subshrub/shrub, native in South America, that was introduced in Europe at the end of 17th century [18]. The plant is also known as *Lippia triphylla*, *Verbena triphylla*, *Aloysia triphylla*, *Aloysia citriodora*, *Verbena citriodora,* or *Lemon verbena*. *L. citriodora* is mostly cultivated for its lemon-scented leaves, traditionally used in folk medicine for the alleviation of gastrointestinal symptoms, fever, cold, asthma, diabetes, spasms, insomnia, and anxiety [19,20]. Plant extracts were shown to exert significant antioxidant activity that was associated with their flavonoid and phenolic content [21–24]. Antioxidant properties of *L. citriodora* were evaluated in various in vitro, ex vivo, or in vivo systems, employing different biochemical assays. *L. citriodora* infusion was shown to act as a free radical scavenger against superoxide, hydroxyl radicals, and hypochlorous acid [23]. Aqueous extracts of the plant were shown to inhibit the ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) and DPPH (2,2-Diphenyl-1-picrylhydrazyl) radicals and protect against protein carbonylation and lipid peroxidation in Wistar rat brain homogenates [25], while alcoholic and aqueous extracts, upon oral administration, were shown to elevate the blood glutathione levels in the alloxan-induced diabetic rat model [26]. Noteworthy, both the essential oil investigated in this study as well as citral, a mixture of two terpenoid isomers that was identified as the oil's main constituent, were shown to inhibit the H_2O_2-induced oxidative damage in human Jurkat T-leukemia cells [27]. Besides the plant's well-documented antioxidant potential, recent studies demonstrate that various extracts from *L. citriodora* have the potential to inhibit the growth of cancer cells in vitro [27–30]. Moreover, citral, the main constituent of the essential oil from *L. citriodora* [27], exerts antitumor activity in murine breast cancer models upon oral administration [31,32].

Breast cancer is the most frequent cancer and the leading cause of cancer death in women [33]. Because of the heterogeneity of breast cancer as a disease, treatment protocols and prognosis vary greatly between different patients [34]. Several important risk factors for breast cancer such as menopause, aging, genetic predisposition or estrogens, have been associated with oxidative stress [35]. Consequently, it has been proposed that compounds with antioxidant potential such as the various extracts from *L. citriodora* discussed above could prevent certain biochemical processes that lead to breast cancer development [36].

Therefore, the aim of this work was to examine the potential antitumor effects against breast cancer of the essential oil extracted from the leaves of *L. citriodora* (LCO) as a plant-specific mixture of various bioactive compounds. Specifically, we assayed the potential growth inhibitory, anti-migratory, and pro-apoptotic activities of the essential oil in vitro. Moreover, we examined whether oral administration of the oil attenuates growth of breast cancer in vivo in the experimental DA3 mouse tumor model.

2. Materials and Methods

2.1. Plant Material and Essential Oil Extraction

Collection and identification of plant material was described in a previous study by our team regarding the biological properties of *Lippia citriodora* oil [27]. Briefly, plant samples of small shrubs were purchased by Vioryl S.A. from an herbal market (Afidnes, Athens, Greece), identified as *Lippia citriodora* by a botanist, and planted and maintained in pots until their first inflorescence in May/June when the leaves and stems were collected. The collected parts of the herb were subjected to hydrodistillation in a laboratory-scale, water-steam distillation apparatus as described in our team's previously published study [27]. The composition of the extracted oil was analyzed by Gas chromatography–mass spectrometry (GC-MS) (GC: 6890 A, Agilent Technologies, USA; MSD: 5973, Agilent Technologies, Santa Clara, CA, USA) using an HP-1 ms column (25 m, 0.2 mm i.d., 0.33 μm film thickness). The detailed analysis process and chemical composition of the oil are described in Fitsiou et al. [27].

2.2. Cell Line

DA3 cells is a well-characterized tumor cell line that was derived from the D1-DMBA-3 mammary tumor, syngeneic to BALB/c mice [37]. The cells were maintained under sterile conditions at 37 °C in a humidified atmosphere of 5% CO_2 in DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL).

2.3. Animals

Mice (BALB/c, female, *n* = 18) that were 6–8 weeks old and weighted 20–25 g, were purchased from the Animal Facility of Pasteur Institute (Athens, Greece). Animals were housed in polycarbonate cages (maximum of 5 mice per cage) and maintained at room temperature, on a 12 h light-12 h dark cycle at the Animal House of Medical School at the University of Ioannina (Greece). Tap water and a commercial, pelleted diet (Mucedola) were provided *ad libitum* to all animals. Animal experiments were approved by the Animal Care and Use Committee of the Veterinary Department of Ioannina Prefecture (license number EL20BIO02). All animal procedures were carried out in accordance with the principle of the 3 Rs (replacement, refinement, reduction) and all mice used were not subjected to any pain or discomfort.

2.4. DA3 Experimental Tumor Model

Mice were separated into two independent groups (nine mice per group) and a previously described treatment protocol was followed [9,11,12,38,39]. Briefly, LCO was administered *per os* by oral gavage at a daily dose of 0.552 g/kg of animal body weight diluted in a final volume of 150 μL of corn oil (vehicle), for 14 days. Animals in the control group received an equal volume of plain corn oil. Mice were monitored daily for weight fluctuations and signs of discomfort or disease. DA3 cells (5×10^6) were injected subcutaneously, in the scruff of mice's necks on the 12th day of LCO administration. Animals were euthanized by cervical dislocation on the 7th day post cancer cells inoculation. Developed tumors, spleens, and livers were surgically removed from all animals. The organs were weighed and tumor dimensions were defined by an electronic micrometer. Tumor volume was calculated by the modified ellipsoid formula (mm^3):

$$Tumor\ volume = 0.5 \times (width^2 \times length) \tag{1}$$

2.5. Chemicals and Reagents

Acetic acid, dimethyl sulfoxide (DMSO), trichloroacetic acid (TCA), sulforhodamine B (SRB) and Trizma base were purchased from Sigma-Aldrich (St. Louis, MO, USA); Annexin V-PI kit was purchased from BD Biosciences (Franklin Lakes, NJ, USA); Dulbecco's Modified Eagle's Medium (DMEM) and phosphate-buffered saline (PBS) were purchased from Gibco (Thermo Fisher Scientific, Waltham, MA, USA); trypsin, fetal bovine serum (FBS) and penicillin/streptomycin, were purchased from Biosera (Boussens, France). Formalin and Hematoxylin were purchased from Merck (Merck Millipore, Billerica, MA, USA) and eosin, xylole and paraffin were purchased from Diapath (Martinengo BG, Italy). DAB (3,3'-Diaminobenzidine) was purchased from Dako (Agilent Technologies, Santa Clara, CA, USA). All antibodies were purchased from Cell Signaling (Danvers, MA, USA).

2.6. Cell Growth Assay

Cell growth rate was analyzed by the Sulforhodamine B (SRB) assay [40], as previously described [11]. Briefly, 8000 DA3 cells were seeded per well in a 96-well plate. Cells were exposed to a wide range of increasing LCO concentrations (0–0.92 mg/mL) dissolved in DMSO (1:1 *v/v*), for 24, 48, or 72 h. Control cells were incubated in DMSO-containing DMEM (DMSO concentration ≤ 0.1% *v/v*). After treatment, cells were fixed with TCA for 1 h and stained with SRB dissolved in acetic acid for 30 min. Tris base was added in SRB-stained cells in order to dissolve the SRB cell-bound stain before measuring the samples' absorbance (optical density, OD) at 492 nm. For the OD measurement, a microplate reader

(Enspire, Perkin Elmer) was employed. Results presented are representative of at least three independent experiments. The IC$_{50}$ values were calculated using the Sigma Plot software v.11. The % inhibition of cell growth was calculated by the following formula:

$$\% \text{ growth} = (\textit{mean OD sample})/(\textit{mean OD control} \times 100) \tag{2}$$

2.7. Wound-Healing Assay

DA3 cells were seeded in 12-well plates at a seeding density of 3×10^5 cells/well. After 24 h, when a confluent monolayer was formed, a 10 µL pipette tip was used to scratch the monolayer across the well. Next, monolayers were washed with PBS so that the detached cells could be removed and either DMSO (DMSO concentration $\leq 0.1\%$ *v/v*) for control cells or LCO-containing medium for treated cells was added to the wells. To exclude the growth inhibitory effect of LCO as a potential mechanism of wound-healing inhibition, non-toxic concentrations were used (as determined by the SRB assay) for the treatment of the cells. Specifically, cells were treated with 46 µg/mL of LCO. Cells were photographed with a light microscope (Zeiss, Göttingen, Germany) that was equipped with a digital camera. Multiple photographs per time point were analyzed with ImageJ software (NIH, Bethesda, MD, USA) from three independent experiments and the average % wound area (% open image area) was calculated.

2.8. Flow Cytometric Analysis of Apoptosis by Annexin V and Propidium Iodide Staining

Detection of apoptotic cells was performed using the Annexin V-PI double staining method [41]. A commercially available kit was used according to the manufacturer's instructions. Briefly, cells were seeded in 6-well plates at a seeding density of 2×10^5 cells/well. After an overnight incubation, cells were treated with 89 or 184 µg/mL of LCO for 24, 48, or 72 h. Cells treated with appropriate DMSO (vehicle) concentrations were used as control. Following collection, cells were stained and analyzed on an Attune NxT flow cytometer (Thermo Fisher Scientific, Waltham, MA, USA). Data obtained were analyzed using FlowJo V10 software (Tree Star, Inc., Ashland, OR, USA).

2.9. Immunohistochemical Analysis

Excised tumors were fixed in 10% formalin before being dehydrated in graded concentrations of ethanol, xylole, and embedded in paraffin. Paraffin tissue blocks were cut into 3 µm thick sections with a microtome. Next, the sections were mounted onto charged glass slides. An hematoxylin and eosin stained section was obtained from each tissue block. Immunostaining was performed based on streptavidin-biotin peroxidase labeled method. Deparaffinization and hydratation were done by immersing the tissue sections into graded concentrations of ethanol to deionized water. Primary antibodies, against survivin (#2808 Cell Signaling, dilution 1:50) or cleaved caspase 3 (#9661 Cell Signaling, dilution 1:1000), were then applied to the tissues and incubated overnight at 4 °C. Immunoreactivity was visualized with 3,3′-Diaminobenzidine (DAB) followed by counterstaining with hematoxylin. For negative controls, the primary antibody was omitted. An image analysis system composed of the Olympus BX43 upright microscope, digital camera Olympus Cam-SC30 (Olympus Europa, Hamburg, Germany) and soft analysis (analySISH) was used. Two observers selected at least 10 representative regions from all the sections of each case, where immunopositive tumor cells were counted by using the ×40 objective lens, adopting a continuous score system. The expression was defined as the percentage of positive cells in the total number of the counted cells (number of immunopositive cells/total number of the counted cells).

2.10. Statistical Analysis

Data are representative of at least three independent experiments and are presented as mean $\pm$ SD. For statistical analysis and graphing, the Sigma Plot v.11 software (Systat Software Inc., San José, CA, USA) was used unless stated otherwise. For statistical comparisons between groups Student's *t*-test or Mann-Whitney test for non-parametric variables

were performed. Differences between groups were considered significant when $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3. Results and Discussion

3.1. Chemical Composition of LCO

Volatile profile analysis by GC-MS conducted by our team identified the composition of LCO, which was described in detail in a previous published study [27]. Briefly, 43 compounds representing the 87% of the total chromatographic area were identified. The two major compounds were determined to be geranial (*trans*-citral) and neral (*cis*-citral), reported as citral, which is the sum of the two isomers (Table 1). Neral and geranial accounted for 17.2% or 26.4%, respectively, of the total chromatographic area, which is in accordance with the literature where citral was previously reported as one of the main components of the oil extracted from plants of the citriodora species [18]. Noteworthy, the *trans*-isomer geranial which is 1.5 times more abundant in LCO than neral, was reported to exert more potent antitumor properties as compared to the *cis*-isomer, neral [42]. Interestingly, *L. citriodora* oil with a similar composition was previously shown to possess antioxidant properties [22], while our team investigated the antioxidant potential of LCO and reported its ability to protect Jurkat cells against H_2O_2-induced oxidative damage as well as LCO's moderate direct antioxidant activity analyzed by the DPPH and ABTS assays [27].

Table 1. The 10 most abundant volatile compounds identified in *Lippia citriodora* essential oil (LCO) by GC-MS analysis. Data reproduced from Fitsiou et al. [27].
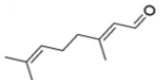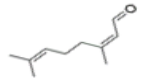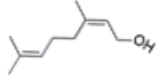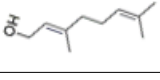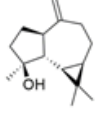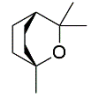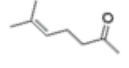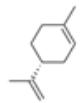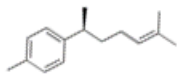
KRI [a]	Compounds	% Area	Structure	Formula	MW [b] (g/mol)
1246	Geranial (trans-citral)	26.404		$C_{10}H_{16}O$	152.23
1215	Neral (cis-citral)	17.16		$C_{10}H_{16}O$	152.23
1212	Nerol	8.047		$C_{10}H_{18}O$	154.25
1241	Geraniol	5.72		$C_{10}H_{18}O$	154.25
1551	Spathulenol	3.279		$C_{15}H_{24}O$	220.35
1008	1,8-Cineol	3.15		$C_{10}H_{18}O$	157.27
954	6-Methyl-5-hepten-2-one	2.278		$C_8H_{14}O$	126.2
1010	Limonene	2.166		$C_{10}H_{16}$	136.23

KRI [a]	Compounds	% Area	Structure	Formula	MW [b] (g/mol)
1464	Ar-curcumene	2.098		$C_{15}H_{22}$	202.33
1473	Bicyclogermacrene	1.75		$C_{15}H_{24}$	204.35

[a] KRI: Kovats Retention Indices, [b] MW: Molecular Weight.

3.2. Antiproliferative Activity of LCO

LCO exhibited a dose- and time-dependent growth inhibitory activity against DA3 murine mammary adenocarcinoma cells (Figure 1). The IC_{50} values for 24, 48, or 72 h were determined to be 96.4 ± 8.9, 77.8 ± 1.5, or 70.7 ± 5.5 µg/mL, respectively (Table 2). The higher IC_{50} values for 24 h compared to 48 and 72 h and for 48 h compared to 72 h implied that the growth inhibitory effect exerted by LCO against DA3 cells was time dependent. Cytotoxicity of LCO was previously reported by our team against human melanoma A375, hepatic HepG2, breast MCF-7, and colonic Caco2 cancer cell lines. We also observed a moderate cytotoxic activity of LCO against THP-1 human leukemic cells' line ($EC_{50} > 100$ µg/mL) [27], which is in agreement with the results reported by Escobar et al. [28]. LCO was also shown to inhibit the growth of murine P815 mastocytoma [29], human breast adenocarcinoma MDA-MB-231 cells, as well as chronic myelogenous erythroleukemia K562 and neuroblastoma SH-SY5Y cells [30]. Besides the essential oil extracted from the plant, various other *L. citriodora* extracts were reported to exert antiproliferative activity against cancer cell lines as well [43,44]. Noteworthy, *L. citriodora* extracts appear to exert cancer-specific, growth-inhibitory effects. Escobar et al. described that LCO did not significantly affect the proliferation rate of normal Vero monkey kidney cells [28]. The potential selective antiproliferative activity of *L. citriodora* extracts against cancer cells was also indicated by the cytotoxic effect of the herbal substance acteoside, isolated from *L. citriodora* leaves, against A5 murine metastatic spindle carcinoma cells. Interestingly, acteoside did not affect to the same extent the viability of C5N normal mouse keratinocytes [45]. Moreover, LCO exhibited moderate antiproliferative activity against human normal ESCs (endometrial stromal cells) [30]. Thus, even though there are only a few studies investigating the in vitro antiproliferative potential of LCO, these studies suggest that the oil extracted from *L. citriodora* exerts potent, diverse, and selective antitumor activities.
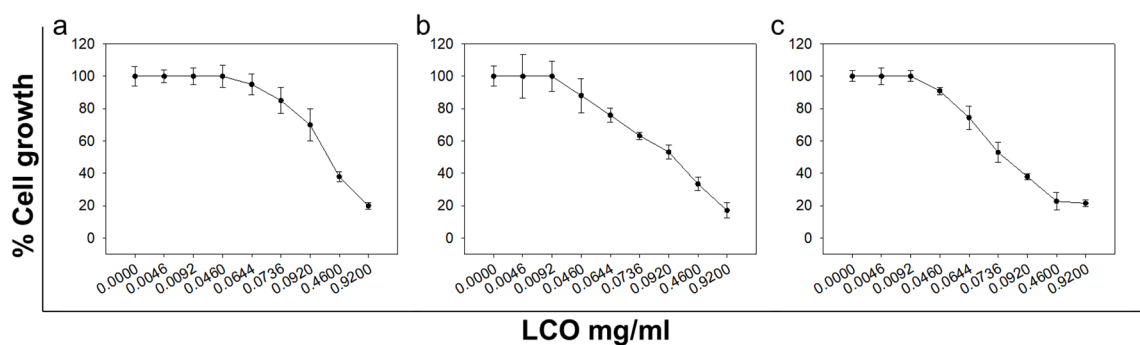
Figure 1. In vitro anticancer activity of LCO. Antiproliferative effect of increasing doses of LCO at (**a**) 24 h, (**b**) 48 h, or (**c**) 72 h on DA3 murine breast cancer cells. Percentage (%) of cell growth was analyzed by the SRB assay. All data shown are representative of at least three independent experiments. Values represent mean ± SD.

Table 2. IC$_{50}$ values (concentration that causes a 50% inhibition in cell growth) of LCO against DA3 cells determined by the SRB assay after a 24-, 48-, or a 72-h treatment. Data are representative of at least three independent experiments and are presented as mean $\pm$ SD.

IC$_{50}$ (24)	IC$_{50}$ (48)	IC$_{50}$ (72)
96.4 $\pm$ 8.9 µg/mL	77.8 $\pm$ 1.5 µg/mL	70.7 $\pm$ 5.5 µg/mL

### 3.3. Evaluation of the In Vitro Pro-Apoptotic Potential of LCO

The interesting observation of the time-dependent, growth inhibitory effect of LCO led us to further investigate the potential mechanism involved. Interestingly, it has been reported in the literature that both the oil as a mixture [29] and acteoside, an herbal substance isolated from *L. citriodora* [45], may induce apoptosis in cancer cells. Moreover, citral, which has been identified as the major component of LCO [27], was reported to induce apoptosis in breast cancer cells in vitro and in vivo in murine tumor models [31,46]. Thus, we investigated the potential pro-apoptotic activity of LCO against DA3 cells by the flow cytometric analysis of Annexin V and propidium iodide (PI) double staining method for the identification of early apoptotic cells. Our results are in accordance with the literature and indicate that LCO induces morphological changes in cells that are typical during apoptosis in DA3 cells. Specifically, cells treated with 0.089 or 0.184 mg/mL of LCO for 24 h exhibited, compared to control, a 12% or 55% rise, respectively, in the ratio of early apoptotic to viable cells (Figure 2). Cells treated for 48 h exhibited an increase of 30% or 517%, while cells treated for 72 h exhibited an increase of 10% or a decrease of 1% (as progressively, an increasing number of cells undergoes cell death) in the ratio of early apoptotic to viable cells for 0.089 or 0.184 mg/mL of LCO, respectively.

This observation indicates that LCO induces concentration-dependent apoptotic effects in DA3 cells.

Apoptosis is a mechanism that comprises a very significant target for anticancer therapy [47]. There have been previous reports on the pro-apoptotic activity of essential oils extracted from different plant species against various cancer cell lines [11,48,49]. These extracts, due to their diverse chemical composition, comprise a valuable source of bioactive compounds [50]. Our results indicate that LCO could be a good candidate for the identification of apoptosis-promoting phytochemicals.

3.4. LCO Exerts Antimigratory Effects

Furthermore, we investigated whether LCO affects migration of DA3 cells in vitro. Migration of cancer cells is a crucial step in cancer invasion and metastasis [51]. Identification of novel therapeutic compounds that have the potential to attenuate cancer cell migration could lead to the development of novel or combination anticancer strategies. Noteworthy, various phytochemicals have been reported to block migration of cancer cells in vitro [11,12,52]. Despite the lack of studies regarding the potential antimigratory effect of LCO, citral, the most abundant phytochemical in LCO, was shown to inhibit the migration of both human and murine breast cancer cells [32,53]. Thus, we employed the wound-healing assay in order to investigate whether LCO attenuates migration of DA3 cells. Noteworthy, the cells were treated with a non-toxic concentration of LCO (46 µg/mL) that, according to our SRB results (Figure 1), did not significantly affect cell growth. Our results indicate that it took more time for the LCO-treated cells to fill the open area compared to control cells (Figure 3). Specifically, the mean ratio of open area closure in control cells reached 94% after only 2 h, while the LCO-treated group exhibited only a 58% of open-area-closure ratio in the same time point. Wound closure occurred after 3 h in control cells while the closure was not complete in LCO-treated cells that exhibited a remaining 15% of open image area. Considering the reported antimigratory activity of citral, LCO's observed migration-attenuating effect might be attributed to its major constituent.
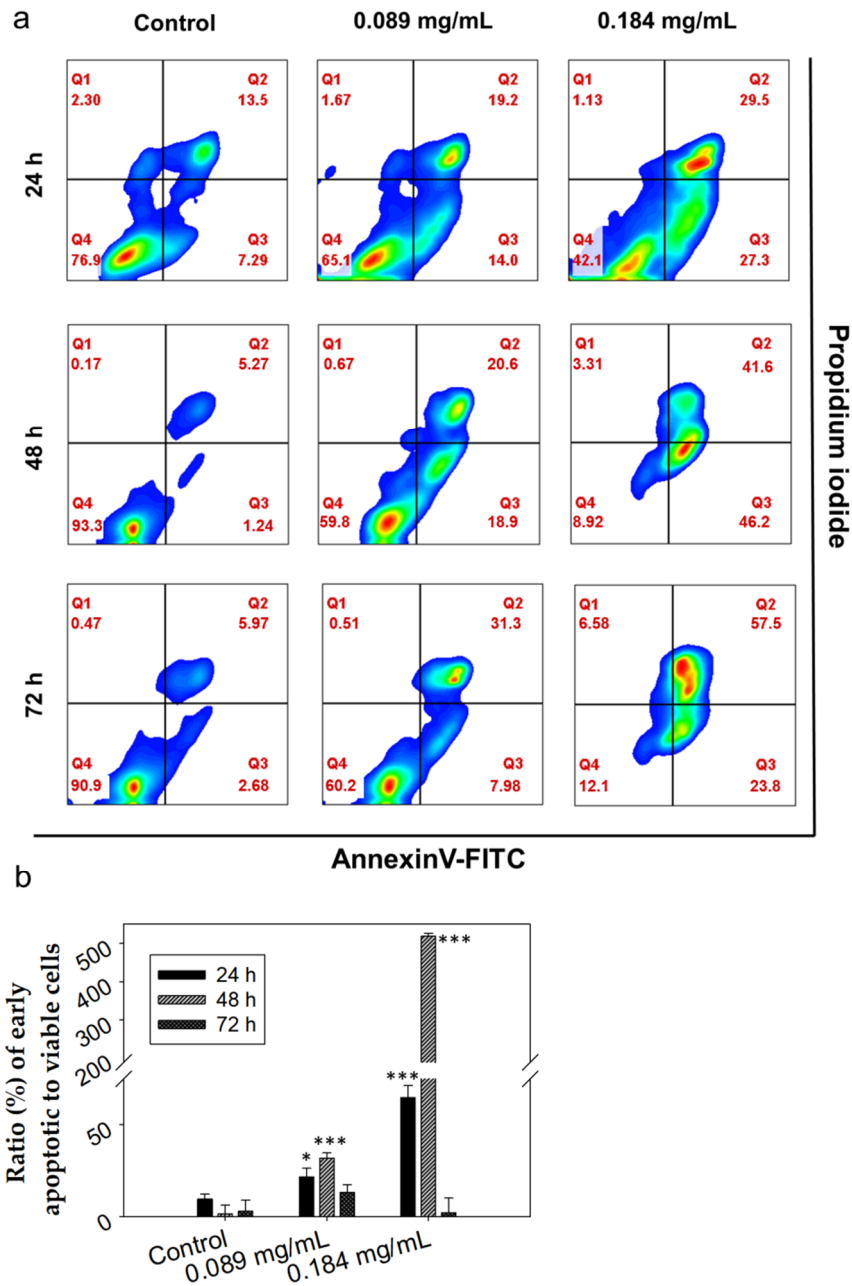
Figure 2. LCO induces apoptotic cell death in DA3 murine breast adenocarcinoma cells detected by flow cytometry. Cells were treated with 0.089 or 0.184 mg/mL of the LCO for 24, 48, or 72 h and stained with Annexin V-FITC and PI before being analyzed by flow cytometry. (**a**) Density plots of control and treated cells. The percentages of viable (Annexin V-FITC and PI negative), early apoptotic (Annexin V-positive and PI-negative), and late apoptotic/necrotic (Annexin V/PI double-positive) cells are indicated on the plots. (**b**) Percentage (%) ratio of early apoptotic to viable cells. At least 10,000 cells were analyzed per sample. Results are presented as the mean ± SD of three independent experiments. Asterisks indicate statistically significant difference between control and treated cells (* $p < 0.05$, *** $p < 0.001$ Student's *t*-test).
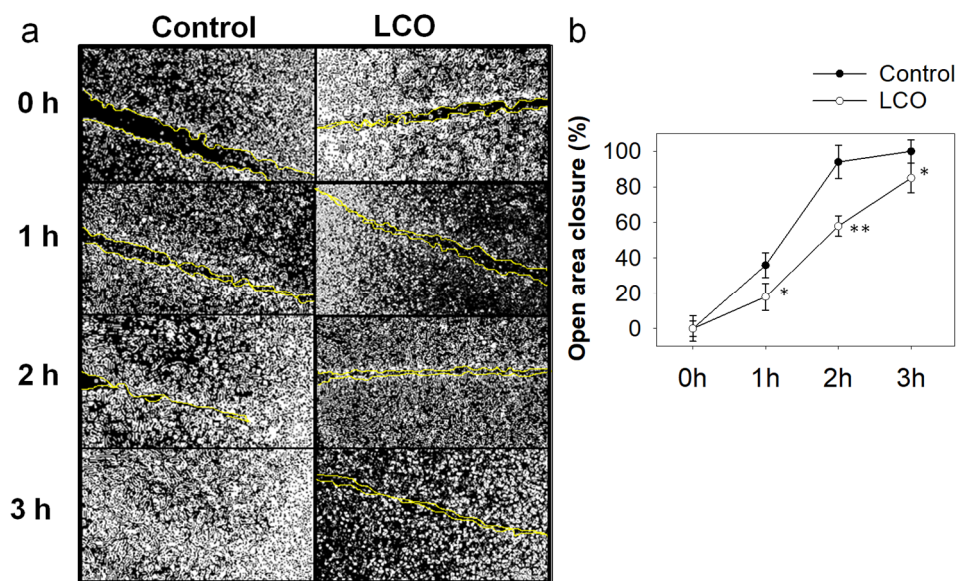
Figure 3. Effect of LCO on migration of cancer cells. Wound-healing assay for DA3 cells treated with 46 µg/mL LCO or DMSO for control. Migration rate of cells was monitored with an optical microscope equipped with a digital camera. (**a**) Photographs of the wounds captured at the indicated time points. (**b**) Quantification of the percentage of wound closure was estimated by ImageJ software analysis. Data are presented as the mean ± SD of three independent experiments. Asterisks indicate statistically significant differences (* $p < 0.05$, ** $p < 0.005$, Student's *t*-test).

Thus, we suggest that a comparative study regarding the antiproliferative and antimigratory activities of citral and LCO be carried out in order to investigate whether citral or a combination of other phytochemicals present in LCO exert the biological reactivity of the oil.

3.5. Oral Administration of LCO Attenuates Tumor Growth in DA3 Breast Cancer Model in Mice

Oral administration of LCO for 14 days (Figure 4a) induced a statistically significant inhibition in the growth of DA3-tumors in BALB/c mice compared to control animals (Figure 5), while no indications of adverse or toxic effects were observed. Specifically, the daily oral administration of 0.552 g/kg of body weight of LCO in female BALB/c mice for 14 days did not affect animal body weight (Figure 4b) or liver or spleen indexes (Figure 4c,d), which are sensitive indicators for the detection of toxicity in BALB/c mice [54]. Moreover, while animal health was daily monitored for signs of disease or discomfort, no signs of pain or behavioral deviations from normal were detected. Unfortunately, there are no previous studies regarding the in vivo toxicity of the essential oil extracted from *L. citriodora*. Etemad et al. studied the in vivo toxicological profile of the intraperitoneally administered aqueous extract from the plant and reported that the LD_{50} in BALB/c mice is 5 g/kg of body weight [55], a dose nine times lower than the dose used in our experiments. Moreover, several oral studies on citral, which constitutes 43.6% of LCO (Table 1), reported no adverse effects for a dose of up to 1.0 g/kg animal weight in rodents for an administration period of 14 days to 13 weeks [56,57].
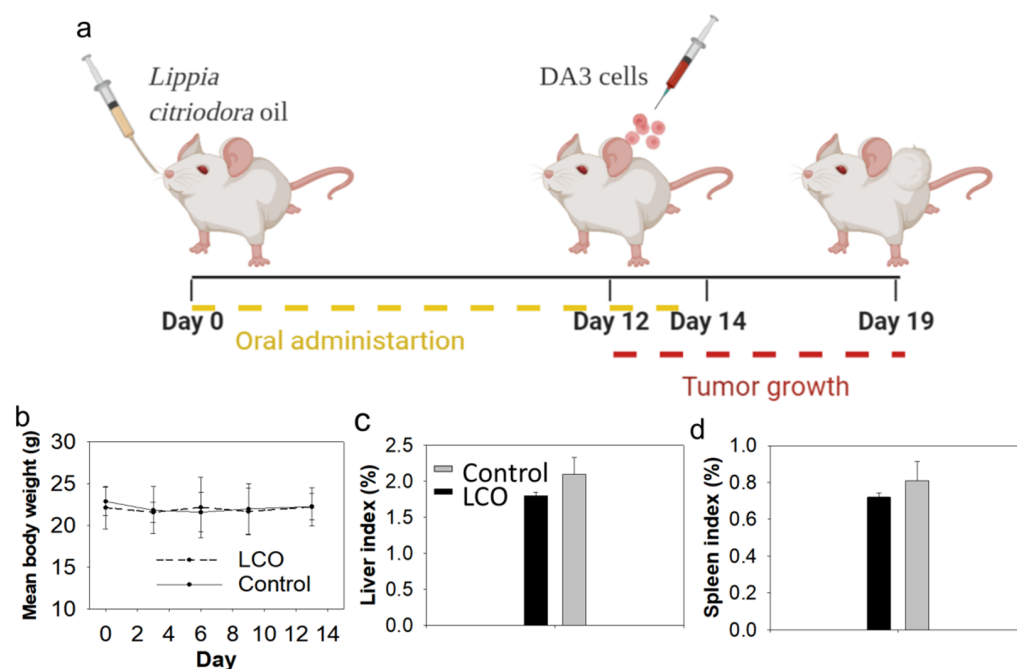
Figure 4. Orally administered LCO in mice is well tolerated. (**a**) LCO was administered daily to BALB/c mice by oral gavage for 14 days (0.552 g/kg body weight/day). On the 12th day, mice were inoculated subcutaneously with DA3 cancer cells (5×10^6 cells per mouse) and, 7 days later, animals were euthanized. (**b**) No significant change was observed in animal body weight between LCO-treated and control mice during the LCO administration period. No significant differences were observed between (**c**) liver- or (**d**) spleen-weight-to-body-weight ratios between control and LCO-treated mice.

Noteworthy, the administration scheme of LCO, described in Figure 4a, induced a 55% inhibition in tumor volume in the DA3 breast adenocarcinoma model. In specific, 7 days post cancer cells' inoculation, control animals developed tumors of an average size of 377 mm^3, while LCO-treated animals developed tumors of an average size of 170 mm^3. To the best of our knowledge, this is the first report on the antitumor potential of orally administered LCO.

3.6. LCO Increases Cleaved Caspase 3 Levels and Reduces Protein Expression of Survivin (BIRC5a) in DA3 Tumors

In accordance with our in vitro results (Figure 2), the apoptotic marker cleaved caspase 3 (c-cas3) was elevated in the tumor tissue of LCO-treated animals. In specific, an average of 30% of cells was positive for active caspase 3 in mice that had been receiving LCO compared to 19% in control animals (Figure 5d). These results, along with the elevated pro-apoptotic-to-live cells' ratios that were detected in vitro in DA3 cells exposed to LCO, indicate that apoptosis might be among the mechanisms initiated by LCO in breast cancer cells. Moreover, protein expression of survivin, a proliferation marker and negative regulator of apoptosis that is highly expressed in most human tumors [58], was reduced by 20% in the tumor tissue of LCO-treated mice (Figure 5e).
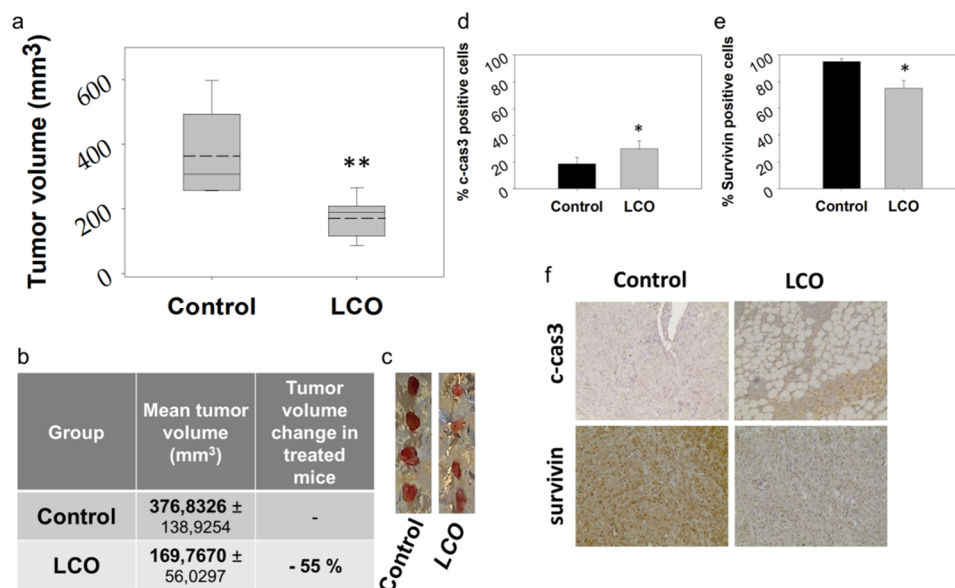
Figure 5. Oral administration of LCO attenuates tumor growth in DA3 breast cancer model in mice. LCO was administered daily to BALB/c mice by oral gavage for 14 days and, on the 12th day, mice were inoculated subcutaneously with DA3 cancer cells (5×10^6 cells per mouse). Seven days later, animals were euthanized and the developed tumors were harvested. A statistically significant reduction of ≈55% in tumor volume ($p = 0.001$, Student's *t*-test) was observed in LCO-treated animals compared to the control group. (**a**) Graphical representation of tumor volume distribution (solid lines indicate median and dashed lines indicate mean values), (**b**) mean tumor volume, and (**c**) photographic observation of excised tumors. (**d**–**f**) Immunohistochemical detection of (**d**) cleaved caspase-3 and (**e**) survivin in tumor tissue from LCO-treated or control mice. (**f**) Representative images showing the effect of LCO administration on survivin andc-cas3 protein levels. The differences in the expression of cleaved cas-3 or survivin in tumor tissue from treated versus control mice are statistically significant ($p = 0.023$ or $p = 0.024$, respectively), as calculated using the SigmaPlot 11.0 statistical software; (* $p < 0.05$, ** $p < 0.005$).

4. Conclusions

Our results provide compelling evidence for the antitumor potential of LCO against breast cancer. We demonstrated that LCO, as a mixture of various phytochemicals with antioxidant activity, inhibits the proliferation of breast cancer cells in vitro and attenuates tumor growth in vivo in a murine breast adenocarcinoma model. In line with the traditional use of the plant in tea infusion preparations, we decided to use oral exposure as the route of LCO administration. Despite the restricted bioavailability due to pre-systemic metabolism, LCO, as a mixture of phytochemicals, induced a 55% inhibition in tumor volume. Noteworthy, LCO attenuated the migration and proliferation of DA3 breast cancer cells in vitro, partially by inducing apoptotic cell death as evidenced by the Annexin V/PI assay. Moreover, orally administered LCO induced the activation of caspase 3 and down-regulated the protein expression of the anti-apoptotic protein survivin in mouse tumor tissue. Taken together, these results confirm the hypothesis that the essential oil extracted from the plant *L. citriodora* exerts antitumor effects against breast adenocarcinoma and highlight the interest of LCO as a source of bioactive compounds with great nutraceutical potential. LCO's most abundant phytochemical, citral, is a good candidate for further investigation in future studies.

References

1. Harborne, J.B. *Introduction to Ecological Biochemistry*; Elsevier: Amsterdam, The Netherlands, 1993; Volume 6, ISBN 9780080918587.
2. Kennedy, D.O.; Wightman, E.L. Herbal extracts and phytochemicals: Plant secondary metabolites and the enhancement of human brain function. *Adv. Nutr.* **2011**, *2*, 32–50. [CrossRef]
3. Thirumurugan, D.; Cholarajan, A.; Raja, S.S.S.; Vijayakumar, R. An Introductory Chapter: Secondary Metabolites. In *Secondary Metabolites—Sources and Applications*; IntechOpen Limited: London, UK, 2018.
4. Hussein, R.A.; El-Anssary, A.A. Plants Secondary Metabolites: The Key Drivers of the Pharmacological Actions of Medicinal Plants. In *Herbal Medicine*; IntechOpen Limited: London, UK, 2019.
5. Senthil-Nathan, S. A Review of Resistance Mechanisms of Synthetic Insecticides and Botanicals, Phytochemicals, and Essential Oils as Alternative Larvicidal Agents Against Mosquitoes. *Front. Physiol.* **2020**, *10*, 1591. [CrossRef]
6. Mitropoulou, G.; Fitsiou, E.; Spyridopoulou, K.; Tiptiri-Kourpeti, A.; Bardouki, H.; Vamvakias, M.; Panas, P.; Chlichlia, K.; Pappa, A.; Kourkoutas, Y. Citrus medica essential oil exhibits significant antimicrobial and antiproliferative activity. *LWT Food Sci. Technol.* **2017**, *84*, 344–352. [CrossRef]
7. Yarmolinsky, L.; Huleihel, M.; Zaccai, M.; Ben-Shabat, S. Potent antiviral flavone glycosides from Ficus benjamina leaves. *Fitoterapia* **2012**, *83*, 362–367. [CrossRef] [PubMed]
8. Khan, M.R.; Omoloso, A.D. Antibacterial, antifungal activities of Barringtonia asiatica. *Fitoterapia* **2002**, *73*, 255–260. [CrossRef]
9. Tiptiri-Kourpeti, A.; Fitsiou, E.; Spyridopoulou, K.; Vasileiadis, S.; Iliopoulos, C.; Galanis, A.; Vekiari, S.; Pappa, A.; Chlichlia, K. Evaluation of Antioxidant and Antiproliferative Properties of Cornus mas L. Fruit Juice. *Antioxidants* **2019**, *8*, 377. [CrossRef]
10. Owolabi, O.O.; James, D.B.; Sani, I.; Andongma, B.T.; Fasanya, O.O.; Kure, B. Phytochemical analysis, antioxidant and anti-inflammatory potential of FERETIA apodanthera root bark extracts. *BMC Complement. Altern. Med.* **2018**, *18*, 1–9. [CrossRef] [PubMed]
11. Spyridopoulou, K.; Fitsiou, E.; Bouloukosta, E.; Tiptiri-Kourpeti, A.; Vamvakias, M.; Oreopoulou, A.; Papavassilopoulou, E.; Pappa, A.; Chlichlia, K. Extraction, Chemical Composition, and Anticancer Potential of *Origanum onites* L. Essential Oil. *Molecules* **2019**, *24*, 2612. [CrossRef] [PubMed]
12. Spyridopoulou, K.; Tiptiri-Kourpeti, A.; Lampri, E.; Fitsiou, E.; Vasileiadis, S.; Vamvakias, M.; Bardouki, H.; Goussia, A.; Malamou-Mitsi, V.; Panayiotidis, M.I.; et al. Dietary mastic oil extracted from *Pistacia lentiscus* var. chia suppresses tumor growth in experimental colon cancer models. *Sci. Rep.* **2017**, *7*, 3782. [CrossRef]

13. Fitsiou, E.; Mitropoulou, G.; Spyridopoulou, K.; Tiptiri-Kourpeti, A.; Vamvakias, M.; Bardouki, H.; Panayiotidis, M.I.; Galanis, A.; Kourkoutas, Y.; Chlichlia, K.; et al. Phytochemical profile and evaluation of the biological activities of essential oils derived from the greek aromatic plant species Ocimum basilicum, Mentha spicata, Pimpinella anisum and Fortunella margarita. *Molecules* **2016**, *21*, 1069. [CrossRef]

14. Kumar, M.; Kaur, V.; Kumar, S.; Kaur, S. Phytoconstituents as apoptosis inducing agents: Strategy to combat cancer. *Cytotechnology* **2016**, *68*, 531–563. [CrossRef]

15. Singh, S.; Sharma, B.; Kanwar, S.S.; Kumar, A. Lead phytochemicals for anticancer drug development. *Front. Plant Sci.* **2016**, *7*, 1667. [CrossRef]

16. Banudevi, S.; Swaminathan, S.; Maheswari, K.U. Pleiotropic Role of Dietary Phytochemicals in Cancer: Emerging Perspectives for Combinational Therapy. *Nutr. Cancer* **2015**, *67*, 1021–1048. [CrossRef]

17. Wang, S.; Meckling, K.A.; Marcone, M.F.; Kakuda, Y.; Tsao, R. Can phytochemical antioxidant rich foods act as anti-cancer agents? *Food Res. Int.* **2011**, *44*, 2545–2554. [CrossRef]

18. Argyropoulou, C.; Daferera, D.; Tarantilis, P.A.; Fasseas, C.; Polissiou, M. Chemical composition of the essential oil from leaves of Lippia citriodora H.B.K. (Verbenaceae) at two developmental stages. *Biochem. Syst. Ecol.* **2007**, *35*, 831–837. [CrossRef]

19. Lee, Y.-S.; Yang, W.-K.; Kim, H.; Min, B.; Caturla, N.; Jones, J.; Park, Y.-C.; Lee, Y.-C.; Kim, S.-H. Metabolaid® Combination of Lemon Verbena and Hibiscus Flower Extract Prevents High-Fat Diet-Induced Obesity through AMP-Activated Protein Kinase Activation. *Nutrients* **2018**, *10*, 1204. [CrossRef]

20. Santos-Gomes, P.C.; Fernandes-Ferreira, M.; Vicente, A.M.S. Composition of the essential oils from flowers and leaves of vervain [aloysia triphylla (L'Herit.) britton] grown in Portugal. *J. Essent. Oil Res.* **2005**, *17*, 73–78. [CrossRef]

21. Naser Aldeen, M.G.; Mansoor, R.; AlJoubbeh, M. Fluctuations of phenols and flavonoids in infusion of lemon verbena (Lippia citriodora) dried leaves during growth stages. *Nutr. Food Sci.* **2015**, *45*, 766–773. [CrossRef]

22. Farahmandfar, R.; Asnaashari, M.; Pourshayegan, M.; Maghsoudi, S.; Moniri, H. Evaluation of antioxidant properties of lemon verbena (*Lippia citriodora*) essential oil and its capacity in sunflower oil stabilization during storage time. *Food Sci. Nutr.* **2018**, *6*, 983–990. [CrossRef] [PubMed]

23. Valentão, P.; Fernandes, E.; Carvalho, F.; Andrade, P.B.; Seabra, R.M.; Bastos, M.D.L. Studies on the antioxidant activity of Lippia citriodora infusion: Scavenging effect on superoxide radical, hydroxyl radical and hypochlorous acid. *Biol. Pharm. Bull.* **2002**, *25*, 1324–1327. [CrossRef] [PubMed]

24. Villegas-Aguilar, M.d.C.; Leyva-Jiménez, F.J.; Cádiz-Gurrea, M.d.l.L.; Segura-Carretero, A.; Arráez-Román, D. Comprehensive Analysis of Antioxidant Compounds from Lippia citriodora and Hibiscus sabdariffa Green Extracts Attained by Response Surface Methodology. *Antioxidants* **2020**, *9*, 1175. [CrossRef] [PubMed]

25. Portmann, E.; Nigro, M.M.L.; Reides, C.G.; Llesuy, S.; Ricco, R.A.; Wagner, M.L.; Gurni, A.A.; Carballo, M.A. Aqueous extracts of Lippia turbinata and Aloysia citriodora (Verbenaceae): Assessment of antioxidant capacity and DNA damage. *Int. J. Toxicol.* **2012**, *31*, 192–202. [CrossRef] [PubMed]

26. El-Hawary, S.S.; Yousif, M.F.; Abdel Motaal, A.A.; Abd-Hameed, L.M. Bioactivities, phenolic compounds and in-vitro propagation of Lippia citriodora Kunth cultivated in Egypt. *Bull. Fac. Pharm. Cairo Univ.* **2012**, *50*, 1–6. [CrossRef]

27. Fitsiou, E.; Mitropoulou, G.; Spyridopoulou, K.; Vamvakias, M.; Bardouki, H.; Galanis, A.; Chlichlia, K.; Kourkoutas, Y.; Panayiotidis, M.; Pappa, A. Chemical Composition and Evaluation of the Biological Properties of the Essential Oil of the Dietary Phytochemical Lippia citriodora. *Molecules* **2018**, *23*, 123. [CrossRef] [PubMed]

28. Escobar, P.; Leal, S.M.; Herrera, L.V.; Martinez, J.R.; Stashenko, E. Chemical composition and antiprotozoal activities of Colombian Lippia spp essential oils and their major components. *Mem. Inst. Oswaldo Cruz* **2010**, *105*, 184–190. [CrossRef] [PubMed]

29. Oukerrou, M.A.; Tilaoui, M.; Mouse, H.A.; Bouchmaa, N.; Zyad, A. Differential Cytotoxic Activity of Essential Oil of Lippia citriodora from Different Regions in Morocco. *Chem. Biodivers.* **2017**, *14*, e1600497. [CrossRef] [PubMed]

30. Najar, B.; Shortrede, J.E.; Pistelli, L.; Buhagiar, J. Chemical Composition and in vitro Cytotoxic Screening of Sixteen Commercial Essential Oils on Five Cancer Cell Lines. *Chem. Biodivers.* **2020**, *17*, e1900478. [CrossRef] [PubMed]

31. Nigjeh, S.E.; Yeap, S.K.; Nordin, N.; Rahman, H.; Rosli, R. In vivo anti-tumor effects of citral on 4T1 breast cancer cells via induction of apoptosis and downregulation of aldehyde dehydrogenase activity. *Molecules* **2019**, *24*, 3241. [CrossRef]

32. Nordin, N.; Yeap, S.K.; Rahman, H.S.; Zamberi, N.R.; Mohamad, N.E.; Abu, N.; Masarudin, M.J.; Abdullah, R.; Alitheen, N.B. Antitumor and Anti-Metastatic Effects of Citral-Loaded Nanostructured Lipid Carrier in 4T1-Induced Breast Cancer Mouse Model. *Molecules* **2020**, *25*, 2670. [CrossRef]

33. Bray, F.; Ferlay, J.; Soerjomataram, I.; Siegel, R.L.; Torre, L.A.; Jemal, A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin.* **2018**, *68*, 394–424. [CrossRef]

34. Yin, L.; Duan, J.J.; Bian, X.W.; Yu, S.C. Triple-negative breast cancer molecular subtyping and treatment progress. *Breast Cancer Res.* **2020**, *22*, 1–13. [CrossRef] [PubMed]

35. Gurer-Orhan, H.; Ince, E.; Konyar, D.; Saso, L.; Suzen, S. The Role of Oxidative Stress Modulators in Breast Cancer. *Curr. Med. Chem.* **2017**, *25*, 4084–4101. [CrossRef]

36. Griñan-Lison, C.; Blaya-Cánovas, J.L.; López-Tejada, A.; Ávalos-Moreno, M.; Navarro-Ocón, A.; Cara, F.E.; González-González, A.; Lorente, J.A.; Marchal, J.A.; Granados-Principal, S. Antioxidants for the treatment of breast cancer: Are we there yet? *Antioxidants* **2021**, *10*, 205. [CrossRef] [PubMed]

37. Yu, B.; Kusmartsev, S.; Cheng, F.; Paolini, M.; Nefedova, Y.; Sotomayor, E.; Gabrilovich, D. Effective combination of chemotherapy and dendritic cell administration for the treatment of advanced-stage experimental breast cancer. *Clin. Cancer Res.* **2003**, *9*, 285–294.

38. Spyridopoulou, K.; Tryfonopoulou, E.; Aindelis, G.; Ypsilantis, P.; Sarafidis, C.; Kalogirou, O.; Chlichlia, K. Biogenic selenium nanoparticles produced by *Lactobacillus casei* ATCC 393 inhibit colon cancer cell growth in vitro and in vivo. *Nanoscale Adv.* **2021**, *3*, 2516–2528. [CrossRef]

39. Tiptiri-Kourpeti, A.; Spyridopoulou, K.; Santarmaki, V.; Aindelis, G.; Tompoulidou, E.; Lamprianidou, E.E.; Saxami, G.; Ypsilantis, P.; Lampri, E.S.; Simopoulos, C.; et al. Lactobacillus casei Exerts Anti-Proliferative Effects Accompanied by Apoptotic Cell Death and Up-Regulation of TRAIL in Colon Carcinoma Cells. *PLoS ONE* **2016**, *11*, e0147960. [CrossRef]

40. Vichai, V.; Kirtikara, K. Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nat. Protoc.* **2006**, *1*, 1112–1116. [CrossRef]

41. Haanen, C.; Steffens-nakken, H.; Reutelingsperger, C. A novel assay for apoptosis Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J. Immunol. Methods* **1995**, *184*, 39–51.

42. Bailly, C. Targets and pathways involved in the antitumor activity of citral and its stereo-isomers. *Eur. J. Pharmacol.* **2020**, *871*, 172945. [CrossRef]

43. Zhang, Y.; Chen, Y.; Wang, S.; Dong, Y.; Wang, T.; Qu, L.; Li, N.; Wang, T. Bioactive Constituents from the Aerial Parts of Lippia triphylla. *Molecules* **2015**, *20*, 21946–21959. [CrossRef] [PubMed]

44. Brahmi, N.; Scognamiglio, M.; Pacifico, S.; Mekhoukhe, A.; Madani, K.; Fiorentino, A.; Monaco, P. 1H NMR based metabolic profiling of eleven Algerian aromatic plants and evaluation of their antioxidant and cytotoxic properties. *Food Res. Int.* **2015**, *76*, 334–341. [CrossRef]

45. Cheimonidi, C.; Samara, P.; Polychronopoulos, P.; Tsakiri, E.N.; Nikou, T.; Myrianthopoulos, V.; Sakellaropoulos, T.; Zoumpourlis, V.; Mikros, E.; Papassideri, I.; et al. Selective cytotoxicity of the herbal substance acteoside against tumor cells and its mechanistic insights. *Redox Biol.* **2018**, *16*, 169–178. [CrossRef] [PubMed]

46. Thomas, M.L.; de Antueno, R.; Coyle, K.M.; Sultan, M.; Cruickshank, B.M.; Giacomantonio, M.A.; Giacomantonio, C.A.; Duncan, R.; Marcato, P. Citral reduces breast tumor growth by inhibiting the cancer stem cell marker ALDH1A3. *Mol. Oncol.* **2016**, *10*, 1485–1496. [CrossRef]

47. Storey, S. Targeting apoptosis: Selected anticancer strategies. *Nat. Rev. Drug Discov.* **2008**, *7*, 971–972. [CrossRef] [PubMed]

48. Pavithra, P.S.; Mehta, A.; Verma, R.S. Induction of apoptosis by essential oil from P. missionis in skin epidermoid cancer cells. *Phytomedicine* **2018**, *50*, 184–195. [CrossRef]

49. Quassinti, L.; Maggi, F.; Barboni, L.; Ricciutelli, M.; Cortese, M.; Papa, F.; Garulli, C.; Kalogris, C.; Vittori, S.; Bramucci, M. Wild celery (*Smyrnium olusatrum* L.) oil and isofuranodiene induce apoptosis in human colon carcinoma cells. *Fitoterapia* **2014**, *97*, 133–141. [CrossRef]

50. Fitsiou, E.; Pappa, A. Anticancer activity of essential oils and other extracts from aromatic plants grown in Greece. *Antioxidants* **2019**, *8*, 290. [CrossRef]

51. Van Zijl, F.; Krupitza, G.; Mikulits, W. Initial steps of metastasis: Cell invasion and endothelial transmigration. *Mutat. Res. Rev. Mutat. Res.* **2011**, *728*, 23–34. [CrossRef]

52. Ham, S.L.; Nasrollahi, S.; Shah, K.N.; Soltisz, A.; Paruchuri, S.; Yun, Y.H.; Luker, G.D.; Bishayee, A.; Tavana, H. Phytochemicals potently inhibit migration of metastatic breast cancer cells. *Integr. Biol.* **2015**, *7*, 792–800. [CrossRef] [PubMed]

53. Nordin, N.; Yeap, S.K.; Rahman, H.S.; Zamberi, N.R.; Abu, N.; Mohamad, N.E.; How, C.W.; Masarudin, M.J.; Abdullah, R.; Alitheen, N.B. In vitro cytotoxicity and anticancer effects of citral nanostructured lipid carrier on MDA MBA-231 human breast cancer cells. *Sci. Rep.* **2019**, *9*, 1614. [CrossRef] [PubMed]

54. Badgujar, P.C.; Jain, S.K.; Singh, A.; Punia, J.S.; Gupta, R.P.; Chandratre, G.A. Immunotoxic effects of imidacloprid following 28 days of oral exposure in BALB/c mice. *Environ. Toxicol. Pharmacol.* **2013**, *35*, 408–418. [CrossRef]

55. Etemad, L.; Shirvan, Z.O.; Vahdati-Mashhadian, N.; Moallem, S.A.; Zafari, R.; Hosseinzadeh, H. Acute, subacute, and cell toxicity of the aqueous extract of Lippia citriodora. *Jundishapur J. Nat. Pharm. Prod.* **2016**, *11*. [CrossRef]

56. Dieter, M.P.; Goehl, T.J.; Jameson, C.W.; Elwell, M.R.; Hildebrandt, P.K.; Yuan, J.H. Comparison of the toxicity of citral in F344 rats and B6C3F1 mice when administered by microencapsulation in feed or by corn-oil gavage. *Food Chem. Toxicol.* **1993**, *31*, 463–474. [CrossRef]

57. Hagan, E.C.; Hansen, W.H.; Fitzhugh, O.G.; Jenner, P.M.; Jones, W.I.; Taylor, J.M.; Long, E.L.; Nelson, A.A.; Brouwer, J.B. Food flavourings and compounds of related structure. II. Subacute and chronic toxicity. *Food Cosmet. Toxicol.* **1967**, *5*, 141–157. [CrossRef]

58. Malcles, M.H.; Wang, H.W.; Koumi, A.; Tsai, Y.H.; Yu, M.; Godfrey, A.; Boshoff, C. Characterisation of the anti-apoptotic function of survivin-ΔEx3 during TNFα-mediated cell death. *Br. J. Cancer* **2007**, *96*, 1659–1666. [CrossRef] [PubMed]