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Vepris macrophylla Essential Oil Produces Notable Antiproliferative Activity and Morphological Alterations in Human Breast Adenocarcinoma Cells

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Abstract: Medicinal plants contain numerous bioactive molecules that synergistically provide therapeutic benefits. We have devoted our attention to various EOs without toxicity to normal cells, studying their activities against human cancer cells. In particular, we have studied the cytotoxicity of *Vepris macrophylla* (Baker) I. Verd. EO. *V. macrophylla* is an evergreen tree of Madagascar where is much appreciated as a source of traditional remedies. Its major volatile components are citral, i.e., a mixture of neral and geranial, citronellol and myrcene. The antiproliferative activities of *V. macrophylla* EO were studied against human breast adenocarcinoma cell line SKBR3. Cellular metabolism was analyzed by MTT assay at different concentrations of EO and at different times of incubation (24, 48 and 72 h). Moreover, morphological and ultrastructural analyses were performed to study its antiproliferative effects against human adenocarcinoma cells, demonstrating the ability of *V. macrophylla* EO, stored inside numerous intracellular vesicles, to damage both plasma membranes and disorganize the cytoskeleton protein as actin filaments.

Keywords: *Vepris macropylla*; essential oil; citral; antiproliferative activity; fluorescence and scanning electron microscopy; human breast cancer cell line

1. Introduction

Breast cancer is a major public health problem, representing the highest incidence rate of death for women [1,2]. The interest in products of natural origin from plants with different pharmacological activities that can be used in chemotherapy is increasingly broad. Many natural compounds have shown anticancer activities [3]. The most famous antineoplastic drug, paclitaxel, used for the treatment of breast cancer, was isolated from the bark of *Taxus brevifolia* Nutt. [4]. Furthermore, among the natural products, there are aloe-emodin, which is isolated from the root of *Rheum palmatum* L. and the leaves of aloe vera. Numerous in vitro studies have demonstrated that aloe-emodin is able to reduce the viability and proliferation of different human cancer cell lines, inducing apoptotic cell death and inhibiting adhesion and the migration process [5,6]. Another compound is curcumin, the active ingredient of turmeric, which is a polyphenolic compound with a broad range of medicinal properties, such as antibreast-cancer activity. Curcumin is able to enhance the effects of chemotherapeutic agents such as paclitaxel [3]. A natural stilbene and non-flavonoid polyphenol, resveratrol is present in grapes, peanuts and red wine. This natural product possesses anti-inflammatory, antioxidant, cardioprotective and anticancer properties [7]. Moreover, a synergistic effect has been reported by the combination of genistein

and doxorubicin [8]. Additionally, rosemary (*Rosmarinus officinalis* L.) extract can increase the activity of the anti-breast-cancer agents tamoxifen, trastuzumab, and paclitaxel [9]. Currently, one of the most interesting approaches in the fight against this tumor comprises new therapeutic strategies using natural products in combination with synthetic drugs in order to increase the therapeutic index of the drug and reduce the many undesirable side effects caused by the doses used in typical chemotherapy protocols. After chemotherapy or radiotherapy, a number of adverse effects in patients occur [10]. A natural flavonoid, quercetin has anti-inflammatory and antioxidative effects. After high dose chemotherapy, quercetin, administered in capsules of 250 mg (twice daily for four weeks), can reduce oral mucositis events in patients with blood malignancy [11]. A combination of curcumin and α-tocopherol regulates rat liver enzymes via inhibition of oxidative stress, revealing protection against cisplatin-induced hepatotoxicity [12]. Naringenin, a natural flavanone isolated from *Thymus vulgaris* L., has growth inhibitory and chemosensitization effects on human breast and colorectal cancer [13]. Naringenin can reduce the nephrotoxicity induced by daunorubicin treatment in rats [14]. Several drugs derived from natural products have already received clinical approval, and many are currently undergoing in clinical trials. To date, the Food and Drug Administration (FDA) has approved the administration of resveratrol and quercetin [15].

Essential oils (EOs) are products obtained from vegetable raw material [16]. They are complex, multicomponent systems composed of volatile small molecular weight components, mainly terpenes and non-terpene components. EOs have been used for a long time by various traditional medicine systems as antiseptic agents [17]. Nowadays, scientific evidence is available showing that certain EOs have antimicrobial, antimycotic, antiviral, antioxidant, immunomodulant and anticancer properties [18,19]. Some of these characteristics are related to their functions in plants. *Vepris macrophylla* (Baker) I. Verd. (Rutaceae) is a tree endemic to Madagascar, where it is known by many names, such as itampody, ampodiberavina, mampodifotsy, mampody (evoking its euphoric properties) (Figure 1) [20]. Indeed, the roots of *V. macrophylla* are traditionally used to manufacture euphoric alcoholic beverages. In Malagasy ethnomedicine, each part of the plant has a specific use. The root bark, grated and macerated in water, is administered as a drink to treat nervous depression or apathetic states. Fruits are used to prepare steam baths during convalescence periods following infectious diseases. The leaves are used to regulate the coronary blood flow. [21–25]. The EO obtained from *V. macrophylla* leaves showed antimicrobial activities for the treatment of infectious diseases [26]. Furthermore, it was effective against phytopathogenic fungi such as *Phytophthora cryptogea* and *Fusarium avenaceum* [27].

In our previous study, the *V. macrophylla* EO showed notable effects on human tumor cells, notably breast adenocarcinoma and colon carcinoma cell lines, highlighting its cytotoxicity [26]. These results pushed us to further explore the effects of this EO on tumor cells. Thus, in the present study, we evaluated the antiproliferative activity of *V. macrophylla* EO, at different times and concentrations, on human breast cell line SKBR3.

Furthermore, the ultrastructural morphological alterations of SKBR3 cells were evaluated by fluorescence and scanning electron microscopy analyses.

Figure 1. Vepris macrophylla tree.

2. Materials and Methods

2.1. Plant Material and Essential Oil (EO) Extraction and Composition

The EO was obtained by hydrodistillation of *V. macrophylla* leaves, collected in the east coast of Madagascar (Sahamamy/Analalava), using a portable alembic as reported in Maggi et al. [26]. Following GC and GC-MS analysis the following major components were detected in the EO chemical profile: geranial (33.2%), neral (23.1%), citronellol (14.5%), and myrcene (8.3%).

2.2. Cell Culture

SKBR3 cell line from human breast cancer was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and was grown in Dulbecco's Modified Eagle's Medium (DMEM) medium with 10% fetal bovine serum (HyClone™ FBS (U.S.A. origin), Characterized), 1% nonessential amino acids, 1% L-glutamine, 100 IU per mL penicillin, 100 IU per mL streptomycin, in a humidified atmosphere at 37 °C with 5% CO_2.

2.3. Cell Viability Assay

MTT assay was utilized to evaluate cell viability. Briefly, cells were seeded for 24 h in a 96-well plate (NunclNunclonTM, NuncGmbH & Co., Wiesbaden, Germany) with a density of 1.2×10^4 cells/well. Then were treated with *V. macrophylla* EO at concentration of 1.25; 2.5; 5 and 10 µg/mL for 24, 48 and 72 h. After the incubation period, 0.5 mg/mL of MTT (Sigma, Deisenhofen, Germany) was added to each well for 2 h at 37 °C and the cells were dissolved with 200 µL/well of dimethylsulfoxide (Merck, Darmstadt, Germany). The absorbance of formazan was read at 570 nm on a scanning microtiter spectrophotometer plate reader. The results were calculated as the percentage of viability in relation to the untreated cells standardized to 100%. They are the mean ± SD of three separate experiments done in triplicate.

2.4. Immunfluorescence Microscopy

SKBR3 cells were grown for 24 h on 12 mm diameter coverslips and treated with *V. macrophylla* EO for an incubation time of 24 and 48 h. Cells were then fixed with 4% paraformaldehyde for 30 min and were permeabilized with 0.5% Triton X-100 (Sigma Chemicals Co., St. Louis, MO, USA) for 5 min. For actin detection, cells were stained with FITC-phalloidin (Sigma) at room temperature for 30 min. For nuclei detection, cells were stained with Hoechst 33258 (Sigma-Aldrich, St. Louis, MO, USA; #861405) at 37 °C for 15 min. After the washing with PBS coverslips were mounted with glycerol-phosphate and images were acquired with a Nikon Microphot fluorescence microscope (Nikon Instruments, Melville, NY, USA) equipped with a Zeiss CCD camera (Carl Zeiss, Oberkochen, Germany).

2.5. Scanning Electron Microscopy (SEM)

SEM analysis allowed the study of cell surface modifications induced by *V. macrophylla* EO. Samples were grown for 24 h on glass coverslips and treated for 24, 48 and 72 h with *V. macrophylla* EO with a concentration of 0.01; 0.1; 1.25 and 2.5 µg/mL. Then cells were fixed in 2.5% glutaraldehyde in 0.2 M Na-cacodylate buffer for 2 h and postfixed with 1% (*w/w*) OsO_4 for 1 h. Subsequently, cells were dehydrated using an ethanol gradient. After the passage in 100% ethanol, the samples were submitted to drying with CO_2 (Critical point dryer CPD 030, Bal-Tec AG, Lichtenstein) and gold coated by sputtering (SCD 050 Blazers device, Bal-Tec). Samples were observed with a scanning electron microscope FE-SEM Quanta Inspect F (FEI—Thermo Fisher Scientific; Eindhoven—The Netherlands).

2.6. Statistical Analysis

All data were repeated in at least three different experiments, and results are expressed as the mean ± standard deviation. Statistical differences were determined using a one-way ANOVA test and values with $p < 0.05$ being considered significant.

3. Results

3.1. Evaluation of Citotoxicity of V. macrophylla EO

MTT assay was used to study cell viability and consequently the proliferative capacity of SKBR3 adenocarcinoma cells after treatment with *V. macrophylla* EO. This assay allows to evaluate the toxicity of a substance, through the comparison of cell viability indices obtained from treated cells compared to control. Figure 2 shows the results of the MTT assay obtained from treatments at 24, 48 and 72 h at different concentrations of *V. macrophylla* EO (1.25; 2.5; 5 and 10 µg/mL) on human breast adenocarcinoma cells SKBR3.

The results showed that *V. macrophylla* EO was able to reduce cell proliferation below 60%, even at the lowest concentrations (1.25 µg/mL) after 24 h of treatment. This trend was also observed at longer times (48 and 72 h) for all other concentrations (2.5; 5, and 10 µg/mL).

3.2. Immunoflorescence Microscopy Observations

In the course of experimental analyses, we performed investigations by fluorescence microscopy that allowed us to evaluate the morphological alterations of cells after treatment with *V. macrophylla* EO at different times and concentrations.

After treatment with *V. macrophylla* EO, the SKBR3 cells were labeled both with Hoechst 33258, which is used to highlight morphological-ultrastructural alterations in the nucleus, and with FITC-phalloidin, to highlight changes in the cell cytoskeleton and actin filaments, induced by different treatments with EO (Figure 3). The micrographs obtained by fluorescence microscopy showed that at 1.25 and 2.5 µg/mL concentrations, the lowest concentrations of EO, after 24 h of treatment, the nuclei still possessed a morphology similar to those of the control cells, while the actin filaments of the cytoskeleton revealed a significant rearrangement of their ultrastructures. Moreover, in some cells, there were numerous vesicular structures, probably containing the same EO (Figure 4a,b, see arrow-

heads). Images of cells treated with the same concentration of EO for an incubation time of 48 h revealed a reduction of nuclei diameters of the cells. Moreover, the morphologies of the actin filament appeared altered, and numerous green fluorescence points were identified exclusively around the nucleus (Figure 4c,d). This result reveals that *V. macrophylla* EO is able to disorganize the actin filament network (see also Figure 3).
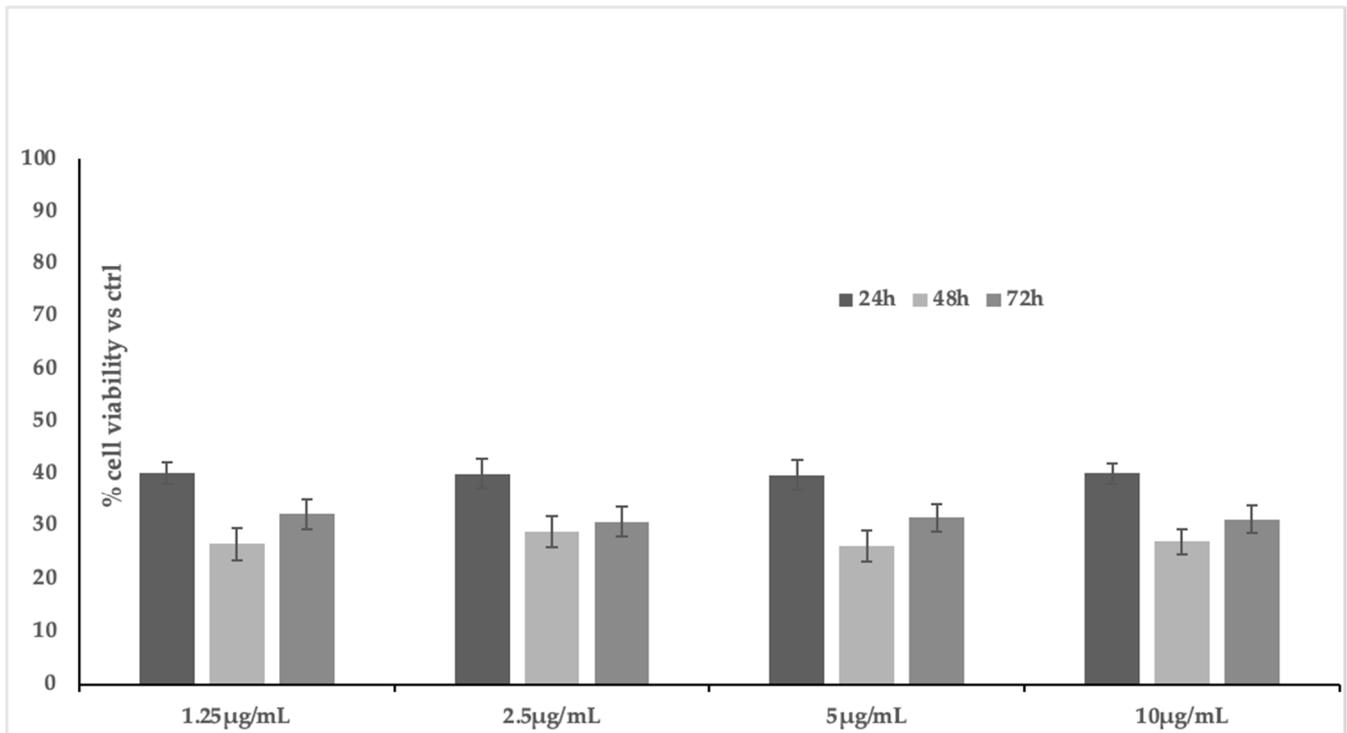

Figure 2. Evaluation of SKBR3 cell viability treated for 24, 48 and 72 h with different concentrations of EO obtained from *Vepris macrophylla* leaves. Cell viability was calculated considering the control value and standardized to 100%. Data represent mean ds (n = 3).
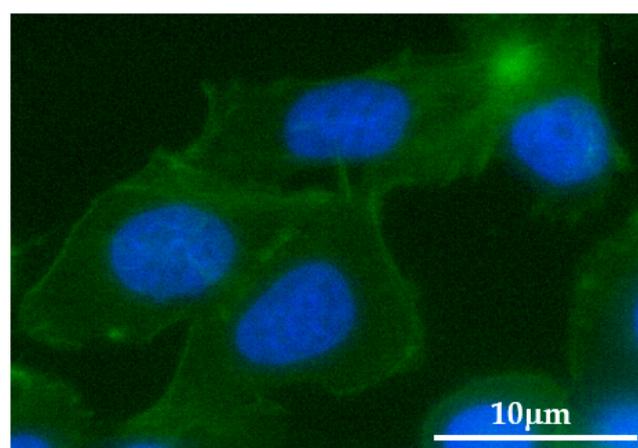

Figure 3. Images after double-cell staining with FITC-phalloidin (green) and Hoechst 33258 (blue) in SKBR3 control cells.
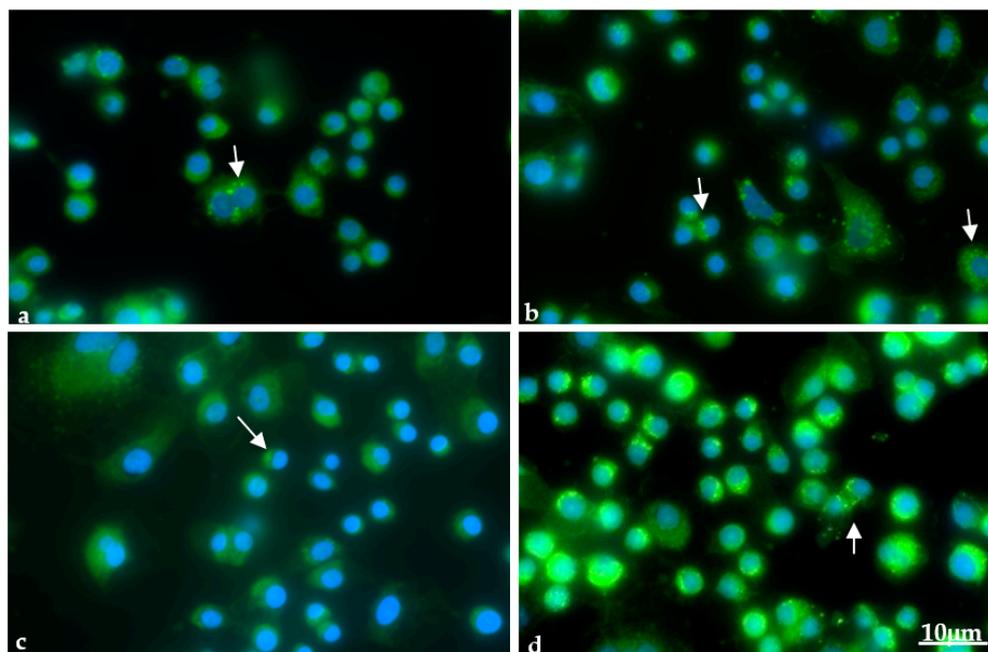
Figure 4. Immunofluorescence micrographs shown the morphological alterations on SKBR3 cells induced by *Vepris macrophylla* EO after treatment with 1.25 (**a**,**c**) and 2.5 μg/mL (**b**,**d**) concentrations at two incubation times 24 h (**a**,**b**) and 48 h (**c**,**d**). Arrowheads indicate probably vesicles filled with EO.

Immunofluorescence observations of SKBR3 cells treated with 5 and 10 μg/mL, at 48 h, were in agreement with results obtained by MTT assay (see Figure 2), showing both the same morphological alterations and numerous vesicles inside the cytoplasm of cancer cells (Figure 5a,b, arrowheads).
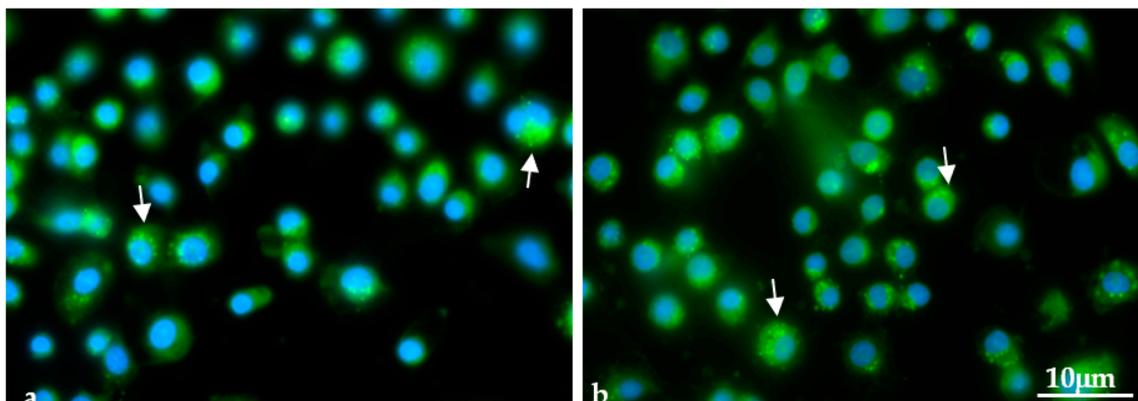

Figure 5. Morphological alterations of SKBR3 cells after treatment with 5 (**a**) and 10 (**b**) μg/mL of *Vepris macrophylla* EO at 48 h. Arrowheads indicate probably vesicles filled with EO.

3.3. SEM Analysis

SEM observations of SKBR3 control cells at 24, 48 and 72 h showed numerous microvilli and random "ruffles" on the cell surface (Figure 6).
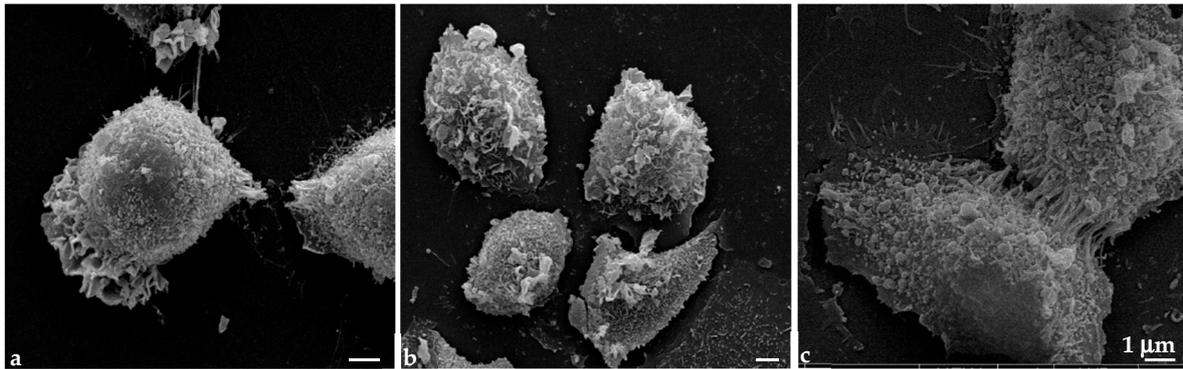
Figure 6. SEM images of SKBR3 control cells at 24, 48 and 72 h (**a**–**c** respectively).

After treatment with EO at different concentrations, cancer cells appeared very damaged. Figures 7–9 show important ultrastructural alterations of SKBR3 cells. The morphological alterations are clearly visible after only 24 h of treatment (Figure 7) with four different concentrations of EO (0.01; 0.1; 1.25 and 2.5 μg/mL, respectively). Moreover, at higher concentrations (1.25 and 2.5 μg/mL), SKBR3 cells appeared flat and adherent to the substrate, probably due the effect on cytoskeletal actin disorganization, as also shown by fluorescence microscopy analysis (Figure 4). When cells were treated with the lowest concentrations of EO (0.01 and 0.1 μg/mL), at 48 h, the plasma membranes were entirely destroyed (Figure 8a,b). Cells showed plasma membrane alterations like those observed 24 h after treatment with the highest concentration (2.5 μg/mL) of EO. Figure 8d shows cells treated with 2.5 μg/mL for 48 h, in which the plasma membranes are completely damaged. Finally, as a dose-dependent effect, after 72 h, these morphological alterations were clearly visible in all SKBR3 cells treated with *V. macrophylla* EO (Figure 9a–c).
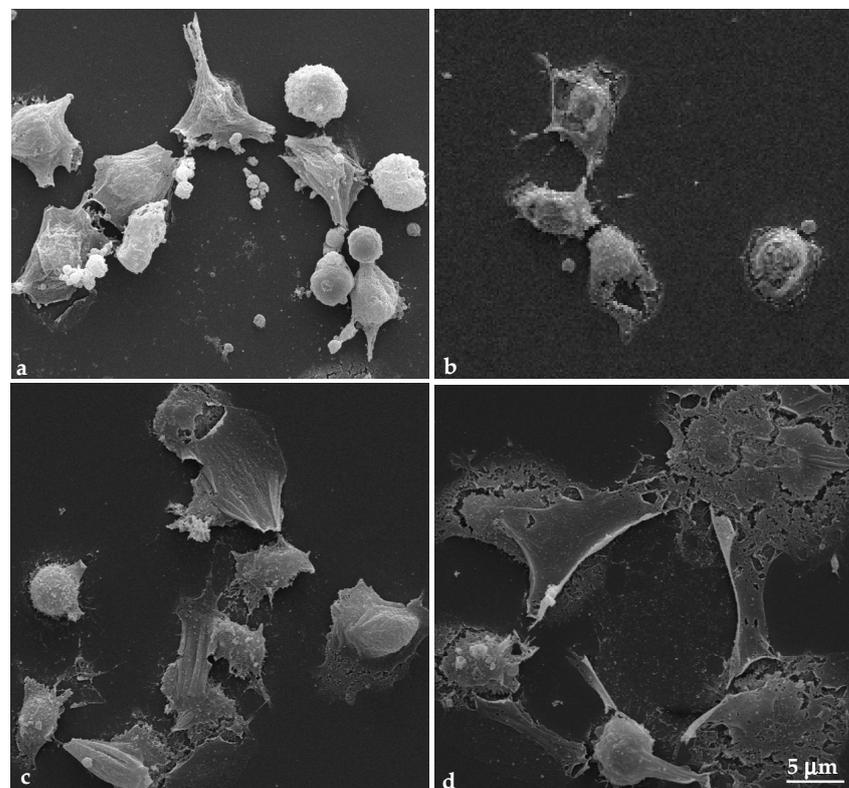

Figure 7. SEM micrographs of SKBR3 cells after 24 h of V. macrophylla EO incubation (0.01 (**a**), 0.1 (**b**), 1.25 (**c**) and 2.5 (**d**) μg/mL, respectively).
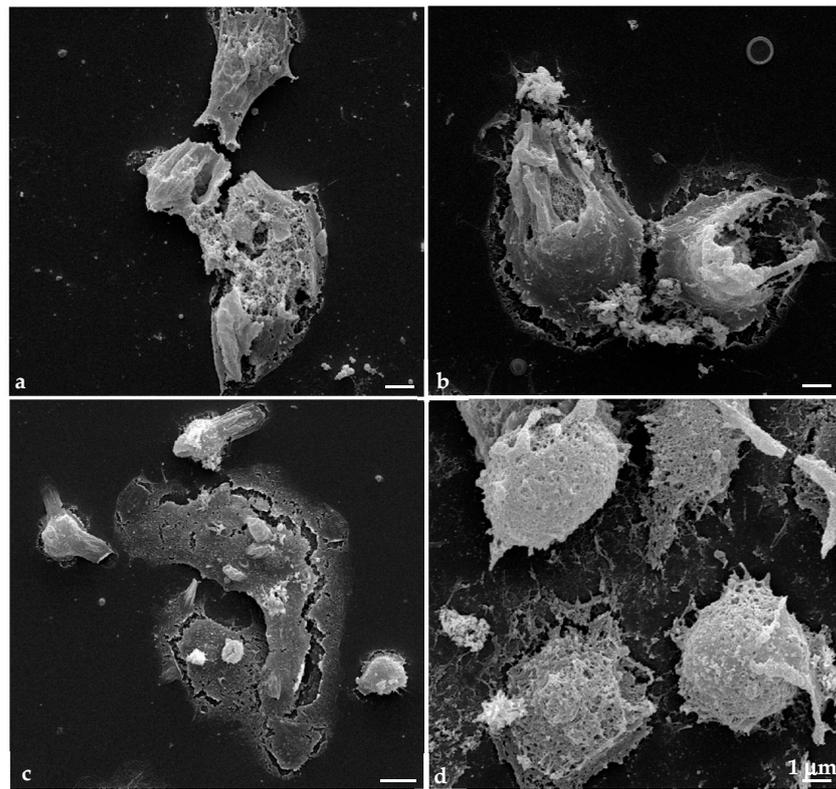
Figure 8. SEM micrographs of SKBR3 cells after 48 h of *V. macrophylla* EO incubation (0.01 (**a**), 0.1 (**b**), 1.25 (**c**) and 2.5 (**d**) µg/mL, respectively).
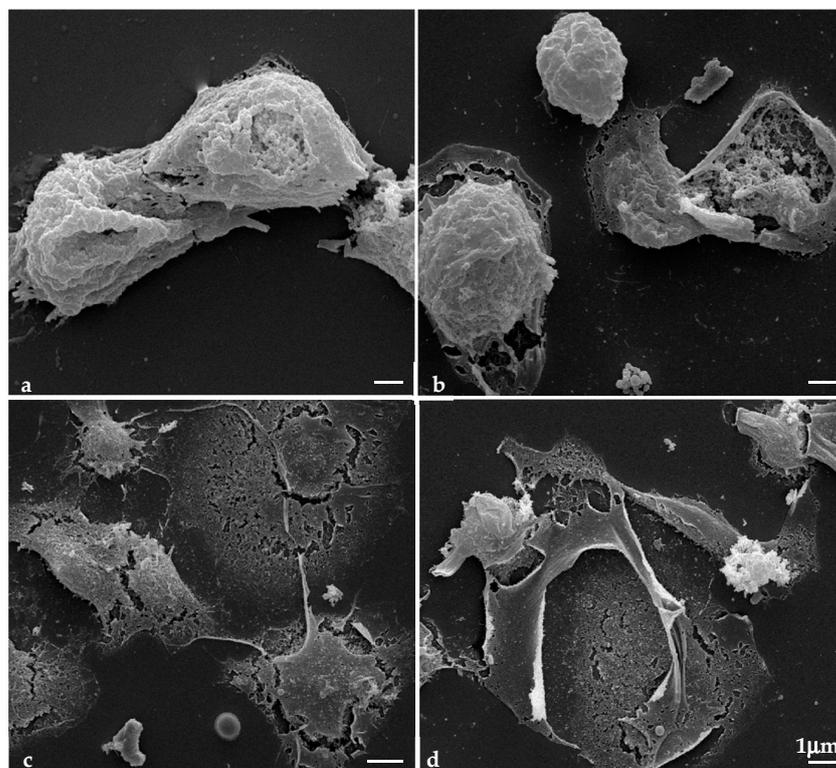

Figure 9. Micrographs of SKBR3 cells after 72 h of *V. macrophylla* EO incubation (0.01 (**a**), 0.1 (**b**), 1.25 (**c**) and 2.5 (**d**) µg/mL, respectively).

Figure 10 shows immunofluorescence and SEM images of the SKBR3 cells with some vesicles on and inside the cytoplasm (arrowheads). These structures, containing the stored EO of *V. macrophylla*, could slow the release of EOs, enhancing both the antiproliferative activity and alteration of cancer cell adhesion on the substrates by actin filaments.
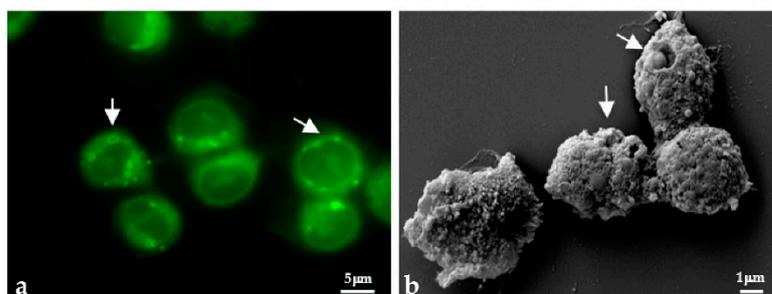

Figure 10. SKBR3 treated with *Vepris macrophylla* EO at 24 h with 1.25 µg/mL (**a**) fluorescence microscopy and (**b**) scanning electron microscopy micrographs. Arrowheads indicate probably vesicles filled with EO.

4. Discussion

EOs and some of their components exert antitumor activities against numerous cancer models, such as colon [28], lung and liver [29], by affecting multiple pathways [30–33]. Di Martile et al. [34] summarized studies showing the properties of EOs to induce in vitro and in vivo cell death in melanoma models. They also indicated the use of EOs in clinical trials with the reduction of the side effects in cancer patients. Moreover, frankincense, pine needle and geranium EOs are also able to suppress tumor progression through the regulation of the AMPK/mTOR pathway in breast cancer [35]. Several EOs also have chemopreventive properties [34]. In eukaryotic cells, EOs can cause depolarization of mitochondrial membranes and decrease their fluidity. In this way, EOs are able to induce severe damage in the mitochondria, leading to cell death. Bhakkiyalakshmi et al. demonstrated that carvacrol, a phenolic monoterpenoid, is able to cause cell death as apoptosis. This process is associated with the production of free radicals that cause the rapid consumption of the intracellular pool of antioxidants [36]. Another study by Arunasree et al. evaluated the mechanism of action of carvacrol in the MDA-MB-231 cell line. This monoterpenoid induced cell death via cytochrome C release after mitochondria permeabilization [37]. Moreover, we also demonstrated the antitumoral activity of Tea Tree Oil (TTO), with an apoptotic effect on melanoma cell lines [38]. In general, the cytotoxic effects of EOs in toto, or those of some of their components, such as monoterpenoids [39], are due to their lipophilicity, which allows them to cross membranes and destroy them [40,41].

Herein, the damage caused by *V. macrophylla* EO to the cell membranes of SKBR3 cells can be attributed to its main component, citral, which is a mixture of the two monoterpenes, neral and geranial. Citral is generally recognized as safe (GRAS) by the FDA, and is commonly used as a flavoring agent in the EU. On the other hand, this compound is endowed with antimicrotubule [42] and chemopreventive properties [43]. Several studies have shown that citral is able to cause cell death to tumor cells and severely damage cytoskeletal structures. On the other hand, other components occurring in the *V. macrophylla* EO, including both major and minor constituents, may be involved in the overall synergistic effect normally displayed by the EO [26]. Moreover, as demonstrated by other authors [44], our results show that *V. macrophylla* EO significantly changes the cytoskeletal organization of SKBR3 cancer cells, suppressing actin cytoskeletal rearrangement and destroying the cell membrane thanks in part to its monoterpenoid activities. Furthermore, this EO significantly decreased the proliferation and migration of human SKBR3 cells in a concentration and time-dependent manner.

5. Conclusions

In the present study, we examined the in vitro antitumoral activities of *V. macrophylla* EO on the SKBR3 human adenocarcinoma cell line. The results demonstrated that this EO is able to induce numerous ultrastructural alterations thanks its ability to both disorganize the cytoskeleton and damage the plasma membrane. Thus, these results suggest a potential use of *V. macrophylla* EO and its main components as a chemosensitizer in clinical practice. Future studies should clarify the mechanism of action and biochemical process activated by this EO. Other studies will be performed using fluorescence microscopy to study all cytoskeletal proteins in order to assess whether these complexes will be used to reduce the degree of invasiveness of human cancer cells by increasing their ability to adhere to the substrate.

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