In Vitro Pharmaco-Toxicological Characterization of Melissa officinalis Total Extract Using Oral, Pharynx and Colorectal Carcinoma Cell Lines

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Abstract: Melissa officinalis is a medicinal herb with an extensive pharmacological profile that has been proven to have beneficial effects in oral and gastrointestinal disorders. However, the effects of this plant in oral, pharyngeal, and colorectal malignancies, types of cancer with an increased incidence in recent years, are less investigated. The present study aims to evaluate the pharmacological profile of a Melissa officinalis total extract for potential benefits in oral, pharynx and colorectal carcinoma. The LC-MS profile of MO total extract (MOte) indicated a rich content in polyphenols, data that support the potent antioxidant capacity exhibited and the antimicrobial activity against both Gram-negative and Gram-positive bacteria. In addition, MOte triggered a dose-dependent and selective decrease in the viability of tumor cells (tongue and pharynx squamous cell carcinomas, and colorectal adenocarcinoma), with the most significant effect being recorded at 100 µg/mL. At the same concentration, MOte exhibited an antiangiogenic effect by inhibiting the process of angiogenesis in ovo. Overall, our findings support the potential benefits of Melissa officinalis leaf total extract as a valuable candidate for the prophylaxis of oral, pharyngeal and colorectal neoplasms.

Keywords: oral and pharynx squamous cell carcinoma; colorectal carcinoma; Melissa officinalis total extract; antioxidant; antimicrobial; antiangiogenic; cytotoxic

1. Introduction

Despite the notable interest concerning research in the field of oncology, cancer remains one of the leading causes of death globally [1], with a multitude of subtypes (more than 277 were identified so far) [2], all sharing the uncontrolled proliferation and invasion as common features [3].

Head and neck squamous cell carcinoma (HNSCC) is a highly aggressive and heterogenous epithelial neoplasm affecting different anatomical regions, including the oral cavity and oropharynx [4,5]. Despite the advances made in cancer treatment, the patients’ survival rate remains low [6,7], while its incidence is dramatically rising [8]. Colorectal cancer (CRC) is one of the most commonly diagnosed malignancies worldwide, with the highest incidence rates being recorded in Western countries [9]. The majority of CRCs
are adenocarcinomas arising due to the cancerous transformation of mucosal epithelial cells [10]. As regards the risk factors that contribute to the carcinogenesis of HNSCC and CRC, similarities were identified. In addition to the non-modifiable risk factors (e.g., age, hereditary mutations), reactive oxygen species (ROS) generated by external contributors, such as alcohol or smoking, have been strongly linked to the initiation of oral, pharyngeal and colorectal carcinomas [9,11,12]. Additionally, altered microbiome or dysbiosis is considered a risk factor for HNSCC, as well as for the tumors of gastrointestinal tract [13–15].

Chemotherapeutic protocols are routinely applied in cancer treatment. However, chemotherapy is not without its disadvantages, since its therapeutic benefits are generally accompanied by toxic events [16]. The possibility of obtaining new therapeutic agents with reduced side effects, which can improve the patients’ quality of life while undergoing chemotherapy, is currently intensively researched [17]. Herbal medicines have been widely embraced as an alternative or an adjuvant to the conventional chemotherapy in the fight against cancer, novel plant-derived compounds being discovered annually [18]. Unfortunately, there is evidence asserting the limited efficiency of monotherapy using compounds derived from natural sources in cancer prevention and treatment [19]. Therefore, the use of plant extracts as mixtures of natural compounds acting synergistically became a promising option [3,17]. Additionally, plant extracts or their main components can be co-administered together with the classic chemotherapeutic agents, combating the development of drug-resistance and the toxic effects such as nausea and vomiting [3,17].

Melissa (M.) officinalis L., also known as lemon balm, is a medicinal herb belonging to the Lamiaceae family. Lemon balm is well-known for its wide range of therapeutic effects (e.g., sedative, antimicrobial, spasmyolytic, antioxidant), determined by its biologically active constituents such as flavonoids, polyphenolic compounds, triterpenes, sesquiterpenes, and tannins [20]. Its benefits have been already recognized in the prevention of oral conditions (e.g., dental plaque) [21] and gastrointestinal conditions (e.g., irritable bowel syndrome) [22]. Moreover, several studies confirm that M. officinalis extracts elicit a strong antiproliferative activity on different cancer types [23–25]. However, data regarding the anticancer potential of M. officinalis extracts against oropharyngeal squamous cell and colorectal carcinomas are rather scarce.

Taking into consideration the aforementioned data, the present investigation aims to determine the pharmacological profile of M. officinalis total extract (MOte) obtained from leaves as potential alternative for the prophylaxis of oral, pharyngeal, and colorectal carcinoma. In this regard, the following objectives were pursued: (i) characterization of M. officinalis total extract in terms of chemical constituents; (ii) evaluation of the MOte antioxidant capacity; (iii) investigation of the MOte antimicrobial activity; (iv) assessment of MOte cytotoxic potential in human squamous cell carcinoma (FaDu and SCC-4) and colorectal adenocarcinoma (Caco-2 and HT-29) cell lines in comparison to healthy cells (primary human gingival keratinocytes (PGK)), and (v) control of MOte impact on the in ovo angiogenesis process.

2. Materials and Methods

2.1. Chemicals and Reagents

The reagents utilized to obtain and to characterize the total extract were: ethanol absolute (puriss. p.a., ≥99.8%) from Merck (Darmstadt, Germany); double-distilled water; 2,2-diphenyl-1-picrylhydrazyl (DPPH) from Sigma Aldrich (Darmstadt, Germany); ascorbic acid from Lach-Ner Company (Neratovice, Czech Republic); methanol (99.9% purity) and acetic acid (99.9% purity) from Merck (Darmstadt, Germany); analytical standards of caftaric acid, gentisic acid, chlorogenic acid, caffeic acid, coumaric acid, ferulic acid, rosmarinic acid, hyperoside, isoquercitrin, rutin, myricetin, fisetin, luteolin, astragalin, kaempferol and apigenin from Sigma-Aldrich (Darmstadt, Germany); and ultrapure deionized water obtained with a MiliQ system Milli-Q® integral water purification system (Merck Millipore, Darmstadt, Germany).
The culture media and the specific reagents for cell culture, such as Dulbecco's modified Eagle Medium (DMEM):F12, Eagle's Minimum Essential Medium (EMEM), Dermal Cell Basal Medium (DCBM), Keratinocyte Growth Kit, McCoy's 5a Medium Modified, trypsin/EDTA solution, phosphate saline buffer (PBS), penicillin/streptomycin solution, fetal bovine serum (FBS), Trypan blue and Alamar blue solutions, were supplied by Sigma Aldrich (Darmstadt, Germany), Thermo Fisher Scientific (Waltham, MA, USA) and ATCC (American Type Culture Collection).

2.2. Cell Lines

One healthy cell line (primary gingival keratinocytes (PGK, ATCC® PCS-200-014™)) and four human tumor cell lines (two of squamous cell carcinoma (FaDu and SCC-4) (ATCC® HTB-43™ and ATCC® CRL-1624™, Manassas, VA, USA) and two of colorectal adenocarcinoma (Caco-2 and HT-29) (ATCC® HTB-37™ and ATCC® HTB-38™, Manassas, VA, USA)), purchased from ATCC (American Type Culture Collection), were selected for this study.

2.3. Preparation of Melissa officinalis Total Extract

The plant material (Melissa officinalis leaves) used in this study was collected from Western Romania, Timis County and certified at the Pharmaceutical Botany Department (voucher herbarium specimen no. MO/S7/2019), Faculty of Pharmacy, “Victor Babes” University of Medicine and Pharmacy. The harvested leaves were dried at room temperature and stored in proper conditions (in dark flasks at laboratory temperature and normal atmosphere).

In order to obtain the Melissa officinalis total extract, the maceration method was employed. In brief, the following protocol was applied: leaves (5 g, crushed and homogenized) were placed in the flask and 25 mL of EtOH 50% was used for extraction for 7 days. The final extract was filtered using filter paper, the solvent was removed by a rotary evaporator (Heidolph Hei-VAP Advantage Rotary Evaporator package) under vacuum, and the pellet was lyophilized and stored in a dark glass tube at 2–8 °C until further analysis.

2.4. Liquid Chromatography–Mass Spectrometry Analysis

Liquid chromatography (LC) coupled with mass spectrometry (MS) analysis was conducted using an Agilent analytical system (6120 from Santa Clara, CA, USA) composed of: (i) HPLC system: 1260 Infinity HPLC with degasser (G1322A), quaternary pump (G1311B), column thermostat (G1316A), detector (G1365C MWD) and manual injector (G1328C) and (ii) quadrupolar mass spectrometer with ESI (electrospray ionization source). The analytical system is linked to a computer equipped with specific software (OpenLAB CDS ChemStation Workstation) for control, analysis, and data processing.

Polyphenols quantification on LC was performed on a reverse phase column (Zorbax Eclipse Plus C18; 3.0 × 100 mm × 3.5 µ). The applied analytical method required the use of gradient, as it was adapted according to the method previously described in the literature [26]. The solvents used for separation consisted of 0.1% acetic acid in water (mobile phase A) and methanol (mobile phase B). A linear gradient elution was applied, as follows: the first five minutes 5% mobile phase B (methanol) in linear elution, continuing until 35 min in gradient elution reaching 90% mobile phase B (methanol), proportion kept until 38 min and ending with 5% mobile phase B (methanol) until 40 min. Other analysis conditions were: (i) flow rate 1 mL/min, (ii) elution time 40 min, (iii) injection volume 10 µL, (iv) column temperature 40 °C, and (v) UV detection at 280 and 320 nm. MS analysis was performed under the following conditions: (i) negative ion mode, (ii) ESI in the SIM (single ion monitoring) mode, (iii) capillary voltage 3500 V, (iv) dry gas flow 12 L/min at 350 °C, (v) nebulizer pressure 55 psig, and (vi) fragmentor 70. Calibration curves were generated to quantify the individual polyphenols by the external standard method (0.05–2.5 µg/mL range) and, to establish the m/z scale of the mass spectrum, an external calibration standard ESI Tuning Mix was used.
2.5. DPPH Assay

The antioxidant activity (AOxA) test was conducted to evaluate the radical scavenging ability of different MOte concentrations by DPPH assay. In brief, 500 µL of total extract was mixed with $2 \times 10^3$ µL solvent (ethanol 50%) and treated with $5 \times 10^2$ µL of DPPH 1 mM. The absorbance was continuously measured for 900 s at 517 nm wavelength with a T70 UV/VIS Spectrophotometer (PG Instruments Ltd., Lutterworth, UK) to notice the modifications related to AOxA values. As positive control was used, ascorbic acid and the final AOxA were expressed as a percentage (%), calculated with the formula presented in the literature [27].

2.6. Antimicrobial Evaluation

Both Gram-positive (G+) and Gram-negative (G−) bacteria and three types of Candida (presented in Table 1) were treated with different concentrations of Melissa officinalis total extract. All bacterial and fungal strains were purchased from American Type Culture Collection (Manassas, VA, USA). The disk diffusion method for susceptibility testing was applied, in accordance with the Standard Rules for Antimicrobial Susceptibility Testing using Impregnated Disks.

Table 1. Bacterial and fungal strains used to evaluate the antimicrobial potential of Melissa officinalis total extract.

| Bacterial and Fungal Strains          | Code          |
|--------------------------------------|---------------|
| **Gram-positive**                    |               |
| Bacillus cereus                       | ATCC® 11778™  |
| Enterococcus faecalis                | ATCC® 51299™  |
| Staphylococcus aureus                | ATCC® 25923™  |
| Streptococcus pyogenes               | ATCC® 19615™  |
| **Gram-negative**                    |               |
| Escherichia coli                     | ATCC® 25922™  |
| Haemophilus influenzae               | ATCC® 49144™  |
| Klebsiella pneumonia                 | ATCC® 700603™ |
| Pseudomonas aeruginosa               | ATCC® 27853™  |
| Salmonella typhimurium               | ATCC® 14028™  |
| Shigella flexneri                    | ATCC® 12022™  |
| **Fungi**                            |               |
| Candida albicans                     | ATCC® 10231™  |
| Candida glabrata                     | ATCC® 90030™  |
| Candida parapsilosis                 | ATCC® 22019™  |

In vitro testing was conducted on sterile paper disks (6 mm) which were impregnated with 20 µL/concentration of MOte (5, 25 and 50 µg/mL). The method was performed according to the one presented in the literature [28], adapted to our laboratory conditions, and it consisted of the following steps: (i) preparation of standard dilutions (10$^{-2}$ fresh fungi cultures and a 10$^{-3}$ fresh bacteria cultures), (ii) cultivation and stimulation on Petri plates with the MOte, and (iii) incubation (at 30 °C for fungi and 37 °C for bacteria) for 24–48 h. Gentamicin (Oxoid™ commercial disks, ThermoFischer Scientific, Waltham, MA, USA) and Nystatin (Oxoid™ commercial disks, ThermoFischer Scientific, Waltham, MA, USA) were used as positive controls and distilled water was used as a negative control.

2.7. Cell Culture Conditions and Viability Assessment

The cells were cultured in specific medium dependent on cell type, as follows: Dermal Cell Basal Medium for PGK, Eagle’s Minimum Essential (EMEM) for FaDu and Caco-2 cells, DMEM:F12 for SCC-4 cells, and McCoy’s 5a Medium for HT-29 cells. The media
utilized for tumor cells cultivation were supplemented with fetal bovine serum to a final concentration of 10% (in the case of FaDu, SCC-4 and HT-29 cells) or 20% (in the case of Caco-2 cells). All the steps necessary to obtain confluent cell cultures for the optimal development of the experiments were carried out according to the protocols specified by the manufacturer. During the experiment, the cells were maintained in the incubator in a humidified atmosphere at 37 °C and 5% CO₂.

To evaluate the impact of Mote on cell viability, the following protocol was applied: (i) a number of $1 \times 10^4$ cells (PGK, FaDu, SCC-4, Caco-2 and HT-29)/well/200 µL culture media were seeded in 96-well plates and left overnight to adhere to the plate; (ii) the cells were treated for 24 and 48 h with five increasing concentrations of total extract (5, 10, 25, 50 and 100 µg/mL); (iii) after the treatment period (24 and 48 h), the cells were incubated with the Alamar Blue reagent for 3 h; and (iv) the absorbance was measured spectrophotometrically (570 and 600 nm) using a microplate reader (xMark™ Microplate Absorbance Spectrophotometer, Biorad, Tokyo, Japan). The percentages of viable cells were calculated according to a previously published formula [29].

2.8. Chorioallantoic Membrane Assay

Chick embryo chorioallantoic membrane (CAM) assay was employed to assess the antiangiogenic capacity of Melissa officinalis total extract. The protocol, described in the literature [30], involves the following steps: (i) incubation with humidity control of fertilized chicken eggs at 37 °C; (ii) aspiration of 5–6 mL albumen in order to detach the chorioallantoic membrane from the inner shell on day three (EDD3, the third embryonic day of development); (iii) a window was cut on the upper side of the egg on EDD 4; (iv) application of 10 µL total extract in double distilled water inside an inert plastic ring previously applied onto the CAM surface on EDD 7. We selected the most representative concentrations based upon the data from prior in vitro assessment (25, 50, 100 µg/mL). The control was represented by the solvent in distilled water. Stereomicroscopic assessment was conducted on Discovery 8 Stereomicroscope (Zeiss, Göttingen, Germany), analyzing live modifications on the vascular plexus of the chorioallantoic membrane using higher magnification, and subsequently recording relevant images displaying the entire treated area, by means of Axio CAM 105 color (Zeiss digital camera, Zeiss, Göttingen, Germany). Images were analyzed by Zeiss ZEN software and processed using ImageJ and GIMP software.

2.9. Statistical Analysis

GraphPad Prism version 8.3.0 for Windows (GraphPad Software, San Diego, CA, USA) was used for statistical analysis. The data are represented as the mean values ± standard deviation (SD) of three independent experiments performed in triplicate. Statistical test one-way ANOVA followed by Tukey’s post-test were utilized and the differences between the test samples were noted with * (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$).

3. Results

3.1. LC-MS Assessment of Melissa officinalis Total Extract

The total extract was subjected to LC-MS analysis and screened for more than 20 polyphenols. The amount (µg/g dry material) of the identified phytocompounds is presented in Table 2, and only the compounds that had a concentration greater than 1 µg/g dry material (d.m.) were shown.

The detected compounds were mainly flavonoids (rutin, fisetin, kaempferol, myricetin, apigenin and luteolin) and acids (abscisic, quinic, chionic, caffeic, caftaric, gentisic, chlorogenic, coumaric, ferulic and rosmarinic). Some flavonoids were identified in their glycosylated forms: myritricin (myricetin rhamnoside), isoquercitrin (quercetin glucoside), hyperoside (quercetin galactoside) and astragalin (kaempferol glucoside). Rosmarinic acid was observed in its monomer from [M-H] at 359.077 m/z, as well as in its dimer form [2M-H] at 719.157 m/z. The LC conditions used for qualitative analysis were optimized using different gradient curves. Initially, the method included an elution profile which
started at 5% B and then increased to 60% B over 40 min. However, we found that a steeper gradient from 5 to 90% B over 40 min allowed for the identification of more compounds with better confidence.

Table 2. The main phenolic compounds identified in Melissa officinalis total extract by LC-MS.

| Standard Compound | Rt (min) | Monoisotopic Mass (Da) | Detected Mass (Parent Ion) | Conc (µg/g d.m.) |
|-------------------|---------|------------------------|---------------------------|-----------------|
| Caftaric acid     | 1.93    | 312.048126             | 311 [M-H]−               | 41.23           |
| Gentisic acid     | 2.64    | 154.026611             | 153 [M-H]−               | 27.44           |
| Chlorogenic acid  | 6.71    | 354.095093             | 353 [M-H]−               | 1.45            |
| Caffeic acid      | 6.98    | 180.042252             | 179 [M-H]−               | 223.47          |
| Coumaric acid     | 12.09   | 164.047348             | 163 [M-H]−               | 1.44            |
| Ferulic acid      | 14.10   | 194.057907             | 193 [M-H]−               | 12.83           |
| Hyperoside        | 20.49   | 464.095490             | 463 [M-H]−               | 2.06            |
| Isoquercitrin     | 21.96   | 464.095490             | 463 [M-H]−               | 36.49           |
| Rutin             | 22.65   | 610.153381             | 609 [M-H]−               | 1.81            |
| Rosmarinic acid   | 24.11   | 719 [2M-H]−            |                           |                 |
| Myricetin         | 24.77   | 318.037567             | 317 [M-H]−               | 11.24           |
| Astragalin        | 25.32   | 448.100555             | 447 [M-H]−               | 457.33          |
| Fisetin           | 25.91   | 286.047729             | 285 [M-H]−               | 20.55           |
| Luteolin          | 33.26   | 286.047729             | 285 [M-H]−               | 325.26          |
| Kaempferol        | 36.09   | 286.047729             | 285 [M-H]−               | 99.57           |
| Apigenin          | 37.42   | 270.052826             | 269 [M-H]−               | 1.26            |

3.2. Antioxidant Activity (AOxA) Assessment

The findings regarding the chemical composition of the MOte led us to the investigation of its antioxidant capacity. The AOxA of the tested total extract dilutions was assessed by DPPH radical scavenging assay. As presented in the Materials and Methods section, three dilutions were evaluated (5 mg/mL, 2.5 mg/mL and 0.5 mg/mL), and ascorbic acid was used as positive control. Ascorbic acid at the standard concentration tested (2.5 mg/mL) presented an AOxA of ~95%, as can be seen in Figure 1. All the samples showed an increased AOxA, in a time-dependent manner over 15 min. The reaction speed in the first three minutes was high and led to values that exceeded 80%. Over time, the reaction rate decreased, being in inverse proportion to the value of antioxidant activity, so that after 15 min of reaction, the activity was ~93% (5 mg/mL), ~94% (2.5 mg/mL) and ~47% (0.5 mg/mL). However, the equilibrium was not reached after 15 min, compared to ascorbic acid, which indicates a manifestation over time of antioxidant activity by the continuous release of molecules responsible for capturing free radicals (see Figure 1).

Figure 1. Time-dependent antioxidant activity of the Melissa officinalis leaves total extract.
3.3. Antimicrobial Activity

The total extract obtained from the leaves of *Melissa officinalis* L. was evaluated for its in vitro antimicrobial activity. The data pointed out that the total extract exerted an antimicrobial activity against all tested microorganisms in a concentration and type-dependent manner (Figure 2). In the case of Gram-positive bacteria, the highest activity was observed against *S. aureus* at the concentration of 50 µg/mL, while in the case of Gram-negative bacteria, the most susceptible strain was *K. pneumoniae* at the same concentration. The antifungal effect of MOte exhibited on *Candida* species was noticeably lower compared to positive control, as can be observed in Figure 2.

![Figure 2. Antimicrobial activity of *Melissa officinalis* total extract (MOte) (5, 25, and 50 µg/mL) in Gram-positive bacteria, Gram-negative bacteria and fungi assessed by disk diffusion method. The results are expressed as diameter zone of inhibition. The data represent the mean values ± SD of three independent experiments performed in triplicate. One-way ANOVA analysis was applied to determine the statistical differences in rapport with control cells followed by Tukey post-test (* p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001).](image)

3.4. Melissa Officinalis Total Extract Influences Cells Viability in a Cell-Type Dependent Manner

Based on previous findings regarding the antiproliferative effect of *Melissa officinalis* extracts, in the present study the impact of a *Melissa officinalis* leaves total extract in four different tumor cell lines was investigated: squamous cell carcinoma (FaDu and SCC-4), and human colorectal adenocarcinoma (Caco-2 and HT-29), by the means of the Alamar blue assay. In addition, the potential cytotoxic effect of the total extract was assessed in a healthy cell line; primary gingival keratinocytes (PGK).

The results indicated that the treatment of PGK cells for 24 h with MOte led to an increased cell proliferation (at the lowest concentrations tested: 5, 10 and 25 µg/mL), and even at the highest dose tested (100 µg/mL) no toxicity was observed on cell viability status. A similar trend was observed after the 48 h stimulation (Figure 3).
Figure 3. Cell viability assessment of *Melissa officinalis* total extract (MOte) (5, 10, 25, 50 and 100 µg/mL) in primary gingival keratinocytes at 24 and 48 h post-stimulation by Alamar blue assay. The results are expressed as cell viability percentage (%) normalized to control (untreated cells). The data represent the mean values ± SD of three independent experiments performed in triplicate. One-way ANOVA analysis was applied to determine the statistical differences in rapport with control cells, followed by Tukey post-test (**** p < 0.0001).

The MOte stimulation induced a dose-dependent decrease in cell viability percentage in squamous and colorectal carcinoma cell lines, with the most denotive effect being noticed at the highest concentrations tested (25, 50, and 100 µg/mL). The results were different compared to healthy keratinocytes, as a significant decrease was noticed at the concentration of 100 µg/mL at both time intervals, as follows: (i) for FaDu cells, the viability reached the percentage of 35% after 24 h and 41% after 48 h (Figure 4); (ii) in the case of SCC-4 cells, the viability values were 40% and 44% for 24 and 48 h, respectively (Figure 4); (iii) the viability of Caco-2 cells was reduced to 70% after 24 h, and to 60% after 48 h (Figure 4); and (iv) for the HT-29 cell line, the viability results were 48% at 24 h and 44% at 48 h (Figure 4). The FaDu pharynx carcinoma cells showed the highest response to the MOte treatment, while the Caco-2 colorectal adenocarcinoma cells were the least responsive. The effect of MOte on SCC-4 and HT-29 cell lines was similar.

3.5. Chorioallantoic Membrane Assay

The total extract was tested on the chorioallantoic membrane in order to evaluate its potential implications in the process of angiogenesis (Figure 5). The application of the samples started on the seventh embryonic day of development, considering that between 7–11 EDD is the period when the endothelial cells are highly mitotic, therefore testing in this interval is indicative for a potential effect on the inhibition of angiogenesis. The most active concentrations in terms of cell viability were selected for the evaluation on the CAM assay, and a dose-related effect was observed for samples in concentrations higher than 25 µg/mL on the inhibition of the blood vessels compared to the solvent that was used as a control, which did not influence normal angiogenesis.

After 24 h of contact with the samples, inside the application ring, MO total extract in concentrations of 25 and 50 µg/mL showed a significant effect on the formation of new capillaries, decreasing the number of thin capillaries, compared to the control sample. The highest concentration 100 µg/mL induced the most visible reduction in blood vessels inside the application ring and a reduced number of interconnecting points. As observed in Figure 5, at 24 h after application, the control sample induced a higher number of capillaries, while for MOte samples, the number of fine vessels was reduced, especially in the case of 100 µg/mL extract. No signs of toxicity upon the blood vessel network were noted during the experimental process.
Figure 4. Cell viability assessment of *Melissa officinalis* total extract (MOte) (5, 10, 25, 50 and 100 µg/mL) in: (a) FaDu—pharynx squamous cell carcinoma; (b) SCC-4—tongue squamous cell carcinoma; (c) Caco-2—colorectal adenocarcinoma and (d) HT-29—colorectal adenocarcinoma at 24 and 48 h post-stimulation by Alamar blue assay. The results are expressed as cell viability percentage (%) normalized to control (untreated cells). The data represent the mean values ± SD of three independent experiments performed in triplicate. One-way ANOVA analysis was applied to determine the statistical differences in rapport with control cells followed by Tukey post-test (**p < 0.01 and ****p < 0.0001).
Figure 5. Stereomicroscopic images regarding the effects of Melissa officinalis total extract on the chorioallantoic membrane assay. Evaluation was assessed at first application contact (0 h) and after the application (24 h) of samples (three concentrations of MOte extract 25, 50, and 100 µg/mL) and control.

4. Discussion

This study aimed to determine the effects induced by a Melissa officinalis total extract on several biological processes (cell viability, angiogenesis, and microbial growth) associated with the development of squamous cell carcinoma of the tongue and pharynx, and colorectal adenocarcinoma. The leading hypothesis was based on the MOte pharmacological profile being considered as a potential candidate for the prophylaxis of these cancer types, as it suppresses cell proliferation and angiogenesis, as well as acting as an antioxidant and antimicrobial agent. The major findings in this regard are: (i) MOte significantly reduced the viability of tongue and pharyngeal squamous cells, as well as the viability of the colorectal carcinoma cells in a concentration-dependent trend, the highest sensitivity being noticed in the case of FaDu pharyngeal SCC; (ii) at the highest concentration tested (100 µg/mL), MOte regressed angiogenesis; and (iii) MOte elicited a dose-dependent antibacterial effect on Gram-positive and Gram-negative bacteria.

A concise review written by Lan and colleagues portrays the advantages that traditional herbal medicine extracts have in the treatment of HNSCC due to their ability to suppress important carcinogenic processes, such as cell proliferation, cell survival, motility, and angiogenesis [6]. Similarly, Aiello and collaborators highlighted the benefits of medicinal plants in colon cancer therapy and prevention [31]. Therefore, in the present study, Melissa officinalis leaves total extract was prepared to be tested as a potential candidate for the chemoprevention of oral and pharyngeal squamous cell carcinoma and colorectal adenocarcinoma. A key step in characterizing a newly obtained total extract consists of the determination of its chemical composition.

A number of factors might influence the chemical composition of medicinal plants. Therefore, the classes of bioactive compounds found in M. officinalis extracts vary sharply from region to region. Several phenolic acids, both benzoic acid and cinnamic acid derivatives, have been identified in Melissa officinalis extracts and in most of the analyzed samples; the predominant compound was rosmarinic acid [32,33]. In addition, a higher amount of rosmarinic, chlorogenic and gallic acids were detected in the samples from Bosnia and Herzegovina compared to those from Turkey [34]. Flavonoids (compounds that contain a benzopyran heterocycle bound to a benzene ring) are also present in significant numbers in M. officinalis extracts [33]. The LC-MS profile of the MO total extract revealed the presence of several polyphenolic compounds (Table 2). In agreement with the published data, our findings indicate that rosmarinic acid was present in MOte in the highest concentration (36,837.82 µg/g d.m.), whereas apigenin in the lowest (1.26 µg/g d.m.) in terms of com-
pounds that quantitatively exceeded 1 µg/g d.m. The agronomic conditions represent a factor associated with variations in the phenolic composition and the antioxidant capacity, but it is generally accepted that rosmarinic acid is the predominant compound in all varieties of *Melissa officinalis*.

The antioxidant capacity of *M. officinalis* L. is directly correlated with its chemical composition. The significant amounts of phenolic compounds detected in plant composition are known for their role in scavenging free radicals and they contribute to the antioxidant effect [35]. The role of oxidative stress in various pathologic processes, including carcinogenesis, has been well documented [36]. Moreover, antioxidants proved to be useful in cancer prophylaxis [37] and treatment [38]. In the light of these facts, the antioxidant potential of the *Melissa officinalis* total extract was evaluated. The analysis of the antioxidant activity using DPPH is frequently used to test the ability of free radicals to be captured by biologically active compounds in medicinal extracts and beyond [39]. Despite the fact that it is a classical method, it continues to be frequently used due to its main advantages, such as simplicity, rapidity, reliability, safe performance conditions, and reproducible results [40].

In the present study, the total extract obtained from *M. officinalis* leaves showed increased values of antioxidant activity (~93% at 5 mg/mL and ~94% at 2.5 mg/mL), indicating an important potential as a free radical scavenger. Our data regarding the antioxidant activity values are consistent with the studies described in the literature. The use of suitable solvents leads to the extraction of biologically active molecules in significant amounts, and the scavenging capacity of free radicals is significantly improved. Values of antioxidant activity around 90% for ethanolic extract from MO leaves are mentioned in the literature [41], emphasizing the importance of solvent polarity for obtaining adequate antioxidant effects. According to a study developed by Kandem et al., the MO ethanolic extract scavenged the DPPH radical in a dose-dependent manner \( IC_{50} = 48.76 \pm 1.94 \mu g/mL \) [42]. The antioxidant capacity of *Melissa officinalis* preparations has also been proven in clinical studies; it is important to mention the study that highlighted the beneficial effects of *M. officinalis* infusion in the reduction of oxidative stress in radiology staff [43].

Recently, several concerns were raised regarding the link between the microbiome and oral SCC. Despite the lack of data in this area of research, it has been established that the oral flora (*Candida*, streptococci) intervenes in carcinogenesis via several mechanisms (e.g., production of pro-carcinogens or carcinogens) [44]. Likewise, the consistent changes in gut microbiota, leading to dysbiosis and development of harmful bacteria are associated with CRC development [45]. Therefore, we tested the antimicrobial activity of MOte against pathogenic and saprophytic germs (Gram-positive and Gram-negative bacteria, and fungi). MOte induced a concentration-dependent inhibition on both bacterial species, with the most active effect being observed at 50 µg/mL. The most susceptible strains were *S. aureus* and *K. pneumoniae*, with diameter zones of 20.98 mm (compared to positive control ~22 mm) and 22.98 mm (compared to positive control ~27 mm), respectively. The antifungal activity against the species of *Candida* was not as strong as the antimicrobial effect (Figure 2).

Taking into consideration that the abnormal proliferation is an important process in carcinogenesis [46], in this paper we verified the influence of the *Melissa officinalis* total extract on the viability of four cancer cell lines, covering the oral, pharyngeal and colorectal malignancies: SCC-4 (tongue squamous cell carcinoma), FaDu (pharynx squamous cell carcinoma), HT-29 and Caco-2 (colorectal adenocarcinomas). The results revealed a dose-dependent decrease in the viability of all cancer cell lines at 24 and 48 h, the response of tumor cells to MOte treatment being also cell-type dependent: FaDu > SCC-4 > HT-29 > Caco-2 (Figure 4), with the most significant decrease being recorded at the highest concentration tested (100 µg/mL). Importantly, MOte caused no cytotoxicity in healthy gingival keratinocytes (PGK) at the same concentration tested. On the contrary, a stimulatory effect was noticed (Figure 3), suggesting a good biocompatibility and a specificity for cancer cells. Similar results were previously published [47,48].

Weidner and colleagues studied the capacity of a hydroethanolic lemon balm (*Melissa officinalis*) leaves extract to inhibit the proliferation of two cancer cell lines, HT-29 and T84.
(lung metastasis of a colon carcinoma), in comparison with four optimized anticancer drugs. Their results showed that the extract inhibited the cell proliferation in a concentration-dependent manner with an IC$_{50}$ of 346 ± 19 µg/mL for the HT-29 cell line, data that are in agreement with our results. Interestingly, it was observed that the MO hydroethanolic extract, known as a free radical scavenger, manifested a pro-oxidative effect on the cancer cells, increasing the ROS formation both inside and outside the colon carcinoma cells [47]. Encalada and co-workers analyzed the cytotoxic effect of two MO extracts, ethanolic and aqueous, recording the cell proliferation inhibition at six different concentrations and three time points (after 24, 48 and 72 h). Using a colorectal carcinoma cell line (HCT-116), their results showed that the viability decreased in a dose and time-dependent manner. Regarding the aqueous extract, after 24 h of incubation, the antiproliferative effect was noticed at concentrations higher than 100 µg/mL [48]. In a study conducted by Kuo et al., it has been shown that the lyophilized water extract of MO reduced the viability of colorectal cancer cells (HCT116, HT-29, SW620, DLD-1), and induced G2/M phase cell cycle arrest and caspase-dependent apoptosis [49]. Studies regarding the activity of MO extracts on oral carcinomas are rather scarce, however the effect of rosmarinic acid on these cancer types has been investigated. Luo et al. reported that rosmarinic acid inhibits the growth of oral cancer cells SCC-15 via apoptosis [50].

Regarding the impact of MOte on cancer cells, another process that we investigated was angiogenesis, which is necessary for the progression of tumors, since the malignant cells' survival and proliferation is highly dependent on the oxygen and nutrients provided by the surrounding blood vessels [51]. The chorioallantoic membrane assay revealed concentration-dependent effects on the process of vessel formation. After 24 h of treatment with concentrations of 25, 50, 100 µg/mL, an impaired formation of new capillaries and a reduced number of blood vessels were noted in a dose-related manner. Ghiulai et al. noticed that the ethanolic (97 and 70%) and methanolic MO extracts suppressed angiogenesis and progression of breast cancer cells in ovo [23]. Lee and colleagues demonstrated the ability of an MO leaf extract to inhibit choroidal neovascularization in a rat model [52].

5. Conclusions

The results of the present study bring consistent evidence that the Melissa officinalis leaf total extract exerts multiple benefits (antioxidant, antimicrobial, antiangiogenic and cytotoxic effects) as a potential agent for the chemoprevention of tongue, pharyngeal, and colorectal cancers. Nevertheless, further studies (e.g., in vivo, clinical trials) are necessary to confirm the utility of M. officinalis total extract in preventing the evolution of oral, pharyngeal, and colorectal neoplasms.

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