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PhD THESIS

EXPERIMENTAL EVALUATION OF SOME ESSENTIAL OILS TO OBSERVE THEIR ANTITUMOR POTENTIAL

A B S T R A C T

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SUMMARY

RESUME

INTRODUCTION

Colorectal cancer (CRC) is one of the most encountered forms of cancer, ranking third in terms of mortality rate. Current therapeutic protocols, especially those for advanced stages, are accompanied by a low survival rate and multiple adverse effects despite the progress of recent years. Therefore, in search for new therapeutic alternatives, effective, targeted at the affected cells and without side effects on the healthy cells, drew attention to medicinal plants.

This increased interest is reflected in the growing up of the number of academic research. Thus, in case of essential oils (EOs) the number of publications has been continuously increased from a few dozens in the 2000s to more than 800 in 2022, with increased preoccupation in the pharmaceutical, biological, and medical areas.

Hippophae rhamnoides L. (*H. rhamnodies*) *Cymbopogon citratus* (D.C.) Stapf (*C. citratus*), *Ocimum basilicum* L. (*O. basilicum*), *Mentha piperita* L.(*M. piperita*), and *Rosmarinus officinalis (R. officinalis)* contain important sources of essential oil (EO), with therapeutic properties known since ancient times. *H. rhamnoides* essential oil (Hr_EO) exert antioxidant, anti-inflammatory, and anti-atherosclerotic activities. Various studies suggest a broad anticancer spectrum on breast, lung, liver, and blood malignancies [1–4]. *C. citratus* essential oil (Cc_EO) possesses a multitude of pharmacological effects varying from anti-bacterial, anti-fungal, anti-amebic, and anti-malarial to anti-oxidant and anti-inflammatory properties [5]. Recent publications refer to the anti-tumor activity of lemongrass extracts against breast, prostate, liver, ovarian, and colon carcinomas [6,7]. *O. basilicum* essential oil (Ob_EO) exerts anti-microbial, anti-inflammatory, anti-diabetic, cardio-protective, and anti-cancer properties providing multiple health benefits [8]. *M. piperita* essential oil (M_EO) is commonly known to relieve symptoms in coughs and colds, treat neuralgic pain and muscle pain, and ameliorate gastrointestinal disorders (relief of abdominal flatulence, pain, obstipation, repletion, and diarrhea). *R. officinalis* essential oil (R_EO) is used to treat rashes and minor wounds, headache and circulation problems, and disorders such as anti-dyspeptic, diuretic, and antispasmodic problems [9]. The anticancer effect of rosemary has been confirmed in breast, lung, liver, prostate, and leukemia cancer cells [10].

All five EOs are widely used among the population and all have been shown to be active on various types of cancer, but at the level of CRC, they have been less studied. In addition, it is important to analyze commercially available form of oils, to which the entire population has access, in order to establish efficiency or inefficiency in a certain field.

The aim of the present thesis was to evaluate the phytochemical composition and investigate the in vitro anti-tumor capacity of five comercal oils (Hr_EO, Cc_EO, Ob_EO, M_EO, and R_EO) as potential chemo-prophylactic or chemo-therapeutic alternatives in CRC management.

OBJECTIVES:

- In vitro assessment of EOs regarding the physicochemical, antioxidant, anti-migration, and cytotoxic profile;
- Antibacterial and antifungal evaluation of the commonly used EOs;
- Pre-in vivo evaluation of the potent cytotoxic EOs.

The personal contribution consists of a physicochemical evaluation of the EOs and the biological, in vitro analyze in order to identify the anticancer potential on colon cancer lines (HT-29, Caco-2 and HCT 116).

The research was divided in five parts:

- Physicochemical (using Gas Chromatography method) and antioxidant (performing the 2,2′-diphenyl-1-picrylhydrazyl assay) analyze of Hr_EO, Cc_EO, Ob_EO, M_EO and R_EO.
- In vitro study on the human immortal keratinocyte cell line (HaCaT), in order to identify the EOs' safety level. The morphological and confluence modification was observed, and cell viability was analyzed (using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay).
- In vitro assay for cytotoxic effect analysis on three CRC cell lines: HT-29, Caco-2, and HCT 116 lines. Cells were observed in terms of morphological and confluence changes, viability was analyzed (by MTT method), migratory capacity of cells was watched closely (using the wound healing assay) and the potential apoptotic amplitude was highlighted (by Hoechst assay).

- The microbiological assay of M_EO and R_EO, ones the most consumed essential oils (using the disk diffusion method) in order to confirm the antimicrobial and antifungal potential of commercial oils.
- In ovo evaluation of the most in vitro potent EOs, Hr_EO, Cc_EO and B_EO (by means of Chorioallantoic Membrane Assay and Hen's Egg Chorioallantoic Membrane assay) in order to analyze irritant effect and the tolerance profile in the vascular plexus.

RESULTS

Using Gas-Chromatography coupled with Mass Spectrometry equipment, it was observed that Hr_EO contained 13 compounds, the most abundant being estragole representing 63.1% of the total oil composition, in Cc_EO, the most abundant compounds among the 33 detected were alpha- and beta-citral, accounting for 66.2% of the total oil composition, in case of Ob_EO, similar to Hr_EO, estragole was the main abundant compound among the 31 identified, with a total percentage of 45.9 %, M_EO counted 27 compounds, and the main composite was menthone, and in R_EO 23 compounds were identified with eucalyptol as the major one (33.592%).

Analyzing the antioxidant capacity, it can be noticed from the graphs (Figure 1), all five essential oils have antioxidant activity compared to the standard (methanol solution of ascorbic acid). Regarding the highest concentration tested (0.2 mg/mL), the antioxidant potential of each essential oil respect the linearity Cc_EO> M_ EO> Ob_ EO> Hr_EO> R_ EO.

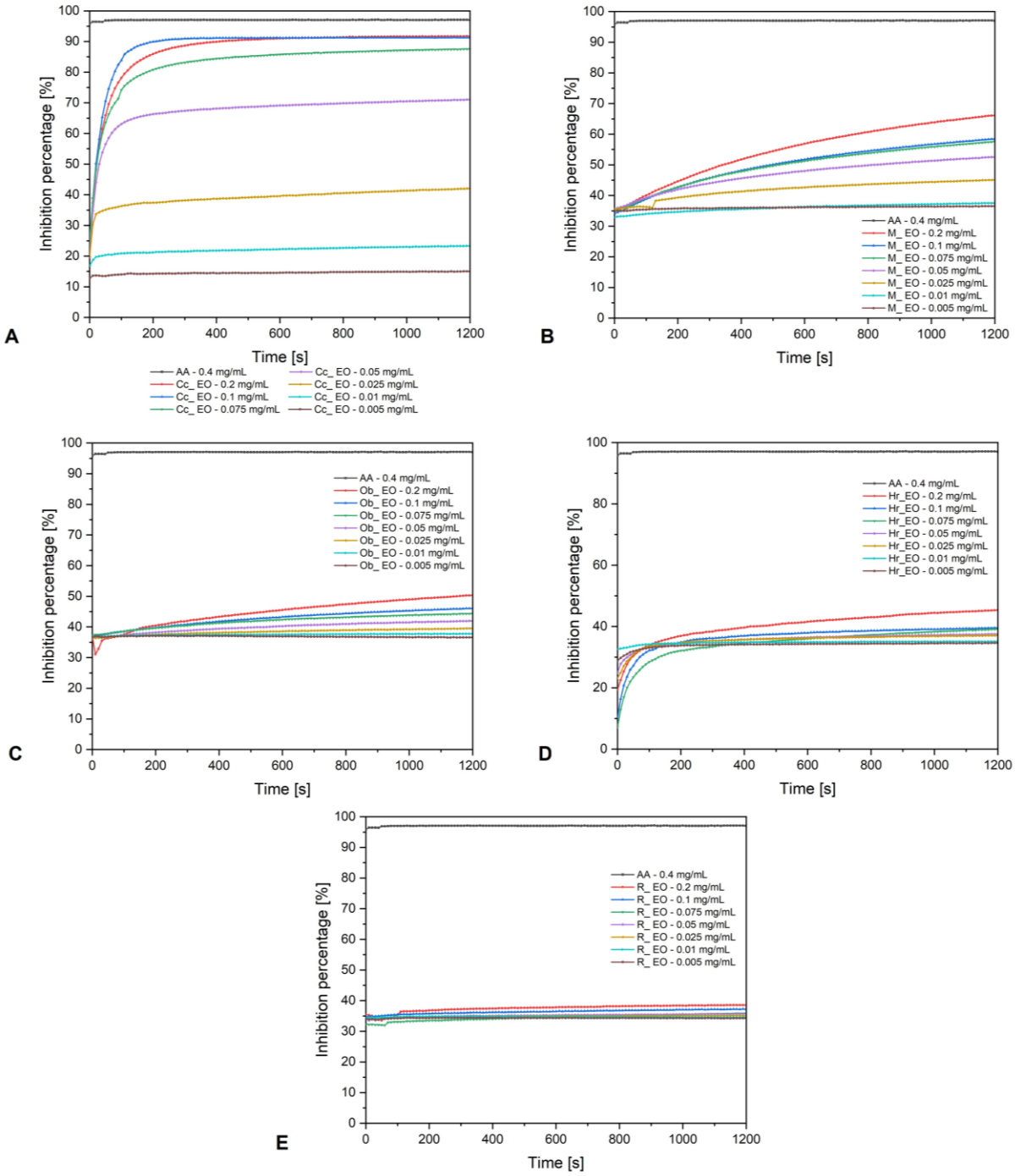

Figure 1. The time dependent inhibition percentage of essential oils tested A – Cc_ EO *vs.* AA; B – M_ EO *vs.* AA; C – Ob_ EO *vs.* AA; D – Hr_EO *vs.* AA and E – R_ EO *vs.* AA.

In the next phase, the physico-chemically tested oils were tested on HaCaT and the changes in morphology, confluence and viability were monitored. Thus, it was observed that 5,10, 25, 50 and 75 µg/mL of EO tested had no significant impact on the cells' shape and confluence. Moreover, peppermint and rosemary EOs have been tested up to 500 µg/mL (100, 150, 200, 250 and 500 µg/mL), due to the

resistance shown also on cancer cell lines, with the same insignificant results on HaCaT. The MTT assay highlighted that even at the highest concentrations, viability did not drop below 90% (Figure 2).

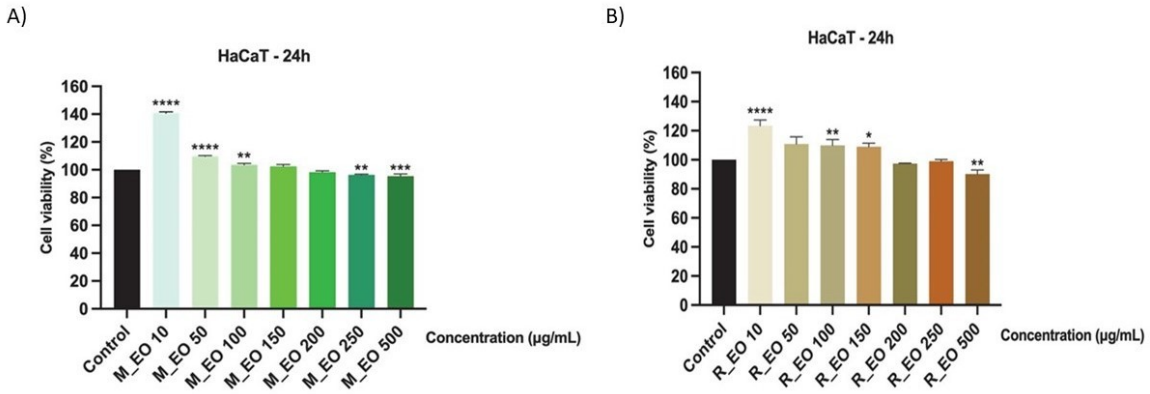

Figure 2. Viability assessment of A) *Mentha piperita* L. and B) *Rosmarinus officinalis* L. essential oil—R_EO (10, 50, 100, 150, 200, 250 and 500 µg/mL) in HaCaT cells at 24 h post-stimulation by MTT assay. The results are presented as cell viability percentage (%) normalized to control (non-stimulated) cells. These data represent the mean values ± SD of three independent experiments performed in triplicate. One-way ANOVA test was performed to determine the statistical differences in rapport with control group followed by Dunnett's multiple comparisons post-test (* $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$).

When analyzing on cancer cells, results present another profile. The lowest (5 µg/mL) and the highest (75 µg/mL) concentrations were selected for morphological and confluence analysis. Hr_EO, Cc_EO, Ob_EO induced a significant loss in the cells' confluence and adherence at 75 µg/mL, while at 5 µg/mL no changes can be noted when compared to control, on both HT-29 and Caco-2 cell lines. On HCT 116, Cc_EO induced modification at the lowest concentration. M_EO and R_EO did not affect the morphology and confluence at 75 µg/mL on all three cell lines. On HCT, several roundish and detached cells, but unmodified adherence was noticed at 150 and 200 µg/mL, but the highest concentrations—250 and 500 µg/mL induced significant morphological changes as round cells floating, loss of cell–cell adhesions, reduced confluence, loss of adherence and cellular debris, specific signs of cytotoxicity.

Following the MTT assay, the viability percentages of all the oils varied in a concentration-dependent manner. On the Caco-2 cell line, it was observed that Hr_EO exerted an important effect at the concentration of 75 µg/mL, when the viability percentages reached the values of 87.83%. Cc_EO decreased the viability of Caco-2 cells to 62.69% (at 75 µg/mL). Ob_EO significantly reduced the cell viability (53.36%) at 75 µg/mL, while at lower concentrations a stimulatory effect was

noticed. When about M_EO and R_EO, at the tested concentrations, the viability wasn't significantly modified. On HT-29 results were related, with the exception of Cc_EO which significantly reduced cell viability starting with 50 µg/mL (64.09%), followed by 75 µg/mL (46.58%). Ob_EO in case of all the five concentrations, stimulated HT-29 cell viability (Figure 3).

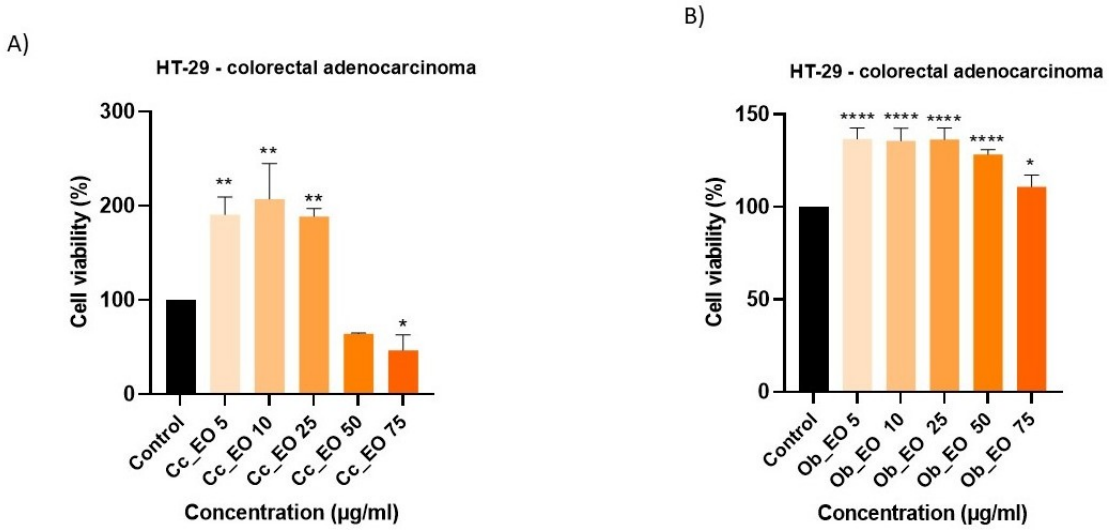

Figure 3. In vitro assessment of the effect Cc_EO and Ob_EO exerts on the viability of HT-29 colorectal adenocarcinoma cells after 48 h of treatment by applying the MTT assay. The data are presented as viability percentages (%) normalized to control (untreated cells) and expressed as mean values ± SD of three independent experiments performed in triplicate. The statistical differences between the control and the treated group were identified by applying the one-way ANOVA analysis followed by the Dunett's multiple comparisons post-test (* p < 0.1; ** p < 0.01; *< 0.001; ****< 0.0001).**

On HCT 116, Cc_EO at the 5 µg/mL modified the viability with 16% (84.14%), and down regulation since it tended to decrease with increasing concentration, so at the highest concentration, the viability was 7.8%. On the other hand, Hr_EO and Ob_EO oils do not affect the cell viability, more than that, it seems to stimulates cell proliferation (Figure 4). When analyzing M_EO, it was noticed a decrease in the cell viability percentages starting with the concentration of 150 µg/mL, and the highest decrease was calculated for the highest concentration tested—500 µg/mL (81.15%). These results indicate a mild/low cytotoxicity induced by M_EO in colorectal cancer cells. R_EO induced a stronger reduction of cell viability percentage in cancer cells as compared to M_EO. R_EO induced a dose-dependent decrease of cell viability percentage, the lowest percentage of viable cells (50.25%) being calculated for the highest concentration tested—500 µg/mL.

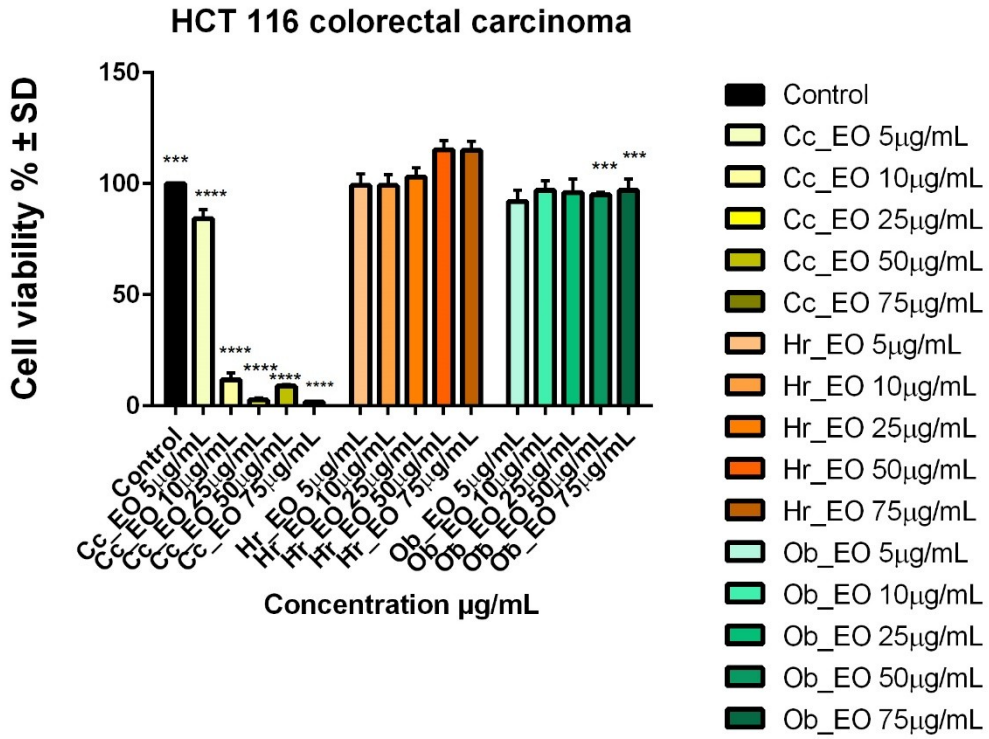

Figure 4. In vitro assessment of the effect Cc_EO, Hr_EO and Ob_EO (5, 10, 25, 50 and 75 µg/mL) exerts on the viability of HCT 116 cell line after 48 h of treatment by applying the MTT assay. The statistical differences between the control and the treated group were analysed by applying the one-way ANOVA analysis followed by the Dunett's multiple comparisons post-test (*p < 0.001; **** p <0.0001)**

The Hoechst experiment was performed to identify whether cell death occurred by apoptosis or necrosis. Hence, HT-29 and Caco-2 cells were stimulated for 48 h with two different concentrations (5 and 75 µg/mL) of Hr_EO, Cc_EO, and Ob_EO, and several changes in the aspect of the cellular nuclei were observed only at the highest concentration tested—75 µg/mL. Hr_EO induced nuclear fragmentation in Caco-2 cells, and membrane blebbing in HT-29 cells. Cc_EO induced visible dysmorphology only in HT-29 cells (nuclear condensation and fragmentation). In Caco-2 cells, Ob_EO caused nuclear fragmentation, chromatin condensation, and massive nuclear growth, while in HT-29 cells chromatin condensation and nuclei fragmentation were noticed. On HCT 116, highest concentrations of all three EOs tend to induce modifications in the cellular shape, with signs of fragmentation, especially in case of Cc_EO, where cells seem to be the most affected. M_EO and R_EO were tested at 10 and 150 µg/mL. The nuclei of the HCT 116 cells were not affected by the lowest concentration of both EOs, still the 150 µg/mL concentration triggered some apoptotic specific signs (nuclear shrinking

and fragmentation) as the ones described for Staurosporine solution (control positive for apoptosis).

After wound healing assay, significant inhibition in the cells' migration was observed in the case of Hr_EO, following their treatment with a concentration of 50 µg/mL with wound healing rates of 11.78% (HT-29) and 27.61% (Caco-2) which are lower when compared to control (25.20%—HT-29 and 40.73%—Caco-2). The same tendency was observed in the case of Cc_EO, but at highest tested concentration (50 µg/mL) it presented a much better capacity for migratory inhibition, especially on HT-29 cells, with a wound healing rate (WHR) of 3.40%. Ob_EO didn't inhibit cell migration. Following M_EO and R_EO treatment, inhibition in the cells' migration was tallied their treatment with M_EO for 5 µg/mL on HT-29 and Caco-2 and with R_EO for 50 µg/mL on HCT 116. The other concentrations present stimulator tendency, especially M_EO 50 µg/mL on HCT 116 (35% stronger that control).

The Disk diffusion method was performed to assess the antimicrobial potential of M_EO and R_EO and data showed that M_EO, used in concentration of 10 µg/mL (M_EO_1), 5 µg/mL (M_EO_2), and 2.5 µg/mL (M_EO_3) exerted significant antimicrobial activity compared to R_EO (tested at the same concentrations), especially in Gram-positive bacteria. The most notable antibacterial effect exerted by M_EO was against Gram positive bacteria *Streptococcus pyogenes* [inhibition zone (IZ) = 33.33 mm, MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) 1.25 µg/mL, at 10 µg/mL)]. R_EO, used in concentration of 10 µg/mL, exhibited a more pronounced effect than the positive control against *Pseudomonas aeruginosa* (IZ = 20.33 mm, MIC, and MBC 5 µg/mL at 10 µg/mL).

Hen's Egg Chorioallantoic Membrane Assay was applied for the most potent three volatile oils (Hr_EO, Cc_EO and Ob_EO) used in the highest concentration (75 µg/mL), previously evaluated in vitro in the cell viability test. The images made at the chorioallantoic membrane before and after the application of the substances can be observed (Figure 5). In the case of sodium dodecyl sulfate (SDS), in the first minute after its application, strong irritating effects, such as lysis, coagulation, and vascular hemorrhage were registered at the level of the chorioallantoic membrane. In the case of volatile oils, they did not cause major changes in the vascular plexus, the only effect recorded being a slight intravascular coagulation, recorded at the end of the five minutes.

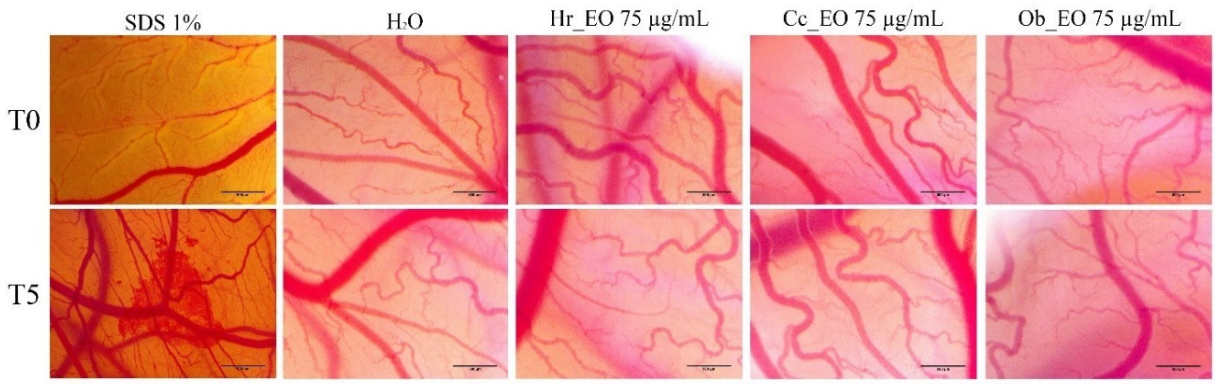

Figure 5. Stereomicroscope images of the CAMs inoculated with negative control (distilled water—H_2O), positive control (sodium dodecyl sulfate—SDS), and essential oil test samples (Hr_EO, Cc_EO, and Ob_EO).

CONCLUSIONS

The main objective of the current thesis was to investigate the chemical composition, antioxidant, antimicrobial and anti-cancer activity of essential oils bought from the trade, derived from *H. rhamnodies*, *C. citratus* , *O. basilicum*, *M. piperita*, and *R. officinalis* species.

The results indicate the presence of active phytocompounds (estragole the main compound identified in Hr_EO and Ob_EO, alpha and beta-citral the main compounds from Cc_EO, menthol and menthone in M_EO and eucalyptol in R_EO) with a significant antioxidant potency, in a concentration-dose dependent manner.

In order to identify cytotoxic potency, EOs were tested on healthy cells, to prove the safe level on non-cancer cells. Following the studies carried out it can be concluded that EOs taken in the study do not interfere significantly with the cell morphology and viability of HaCaT cells, so can be considered safe to use.

When analyzing the efficiency in human colorectal adenocarcinoma and carcinoma cells, it was observed that EOs decreased the viability, reduced the confluence, and induced apoptotic specific nuclear features in CRC cells; Hr_EO and Cc_EO exerted the most potent anti-migratory effect.

The antimicrobial assay provides evidence that the *M. piperita* EO exerted potent antimicrobial activity on *S. pyogenes*. As regards the *R. officinalis* EO, it showed low anti-microbial effects.

The results obtained on the chorioallantoic membrane indicate that these essential oils have a relatively low impact, which indicates that the analyzed compounds exhibit a high biosafety and tolerance profile in the vascular plexus.

Thereby, we can conclude that the analyzed oils have antioxidant, antibacterial and cytotoxic potential and deserve to be closely analyzed with a view to the possible improvement of classic CRC medications.