https://doi.org/10.31925/farmacia.2022.4.14 **ORIGINAL ARTICLE**

ANTIOXIDANT AND *IN VITRO* CYTOTOXIC ACTIVITY OF COMMERCIAL LEMONGRASS, SEA BUCKTHORN AND BASIL ESSENTIAL OILS, AGAINST COLORECTAL CANCER CELL LINE HCT 116

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Abstract

Colorectal cancer is one of the most common forms of cancer with a high mortality rate. Classical medication with chemotherapy based on fluorouracil and leucovorin have the disadvantage of non-selectivity, so they act on both cancerous and healthy cells. Natural compounds present a high interest due to their high safety profile, low cost and promising results. For this reason, the present study aimed to investigate the cytotoxic effect of *Cymbopogon citrates* (lemongrass), *Hippophae rhamnodies* (sea buckthorn) and *Ocimum basilicum* (basil) essential oils, on HCT 116 colorectal cancer cell line. In addition, the antioxidant activity of the three essential oils was also determined. Thus, the outcomes showed that of the three oils, Lemongrass essential oil (LGEO) presents a strong antioxidant potency, comparable with the antioxidant potency of ascorbic acid, used as standard (91.68% *vs*. 97.04%). The morphology analysis revealed the fact that the oils slightly change the shape and cell confluence, the most visible effect being observed at LGEO 75 µg/mL. Our results confirmed the potency of LGEO to inhibit cell viability from 84.6% - at the lowest concentration (5 µg/mL) to 7.8% at the highest (75 µg/mL). The other two oils did not affect significantly cell growing. Hoechst assay confirmed the tendency of all the three oils to modify the shape and to fragment the cell nuclei.

Rezumat

Cancerul colorectal este una dintre cele mai frecvente forme de cancer cu o rată ridicată a mortalității. Medicația clasică cu chimioterapie pe bază de fluorouracil și leucovorin prezintă dezavantajul neselectivității, deci acționează atât asupra celulelor canceroase, cât și asupra celulelor sănătoase. Compușii naturali prezintă un interes ridicat datorită profilului lor ridicat de siguranță, costului scăzut și rezultatelor promițătoare. Din acest motiv, în cercetarea de față au fost studiate uleiurile esențiale *Cymbopogon citrates* (citronelă), *Hippophae rhamnodies* L.(cătină) și *Ocimum basilicum* (busuioc) pe linia celulară de cancer colorectal HCT 116. Testul DPPH a fost efectuat pentru a analiza potența antioxidantă. Astfel, uleiul esențial de lemongrass (LGEO) prezintă o puternică proprietate antioxidantă, comparabilă cu potențialul acidului ascorbic (91,68% față de 97,04%). Analiza morfologică a relevat faptul că uleiurile modifică ușor forma și confluența celulară, cel mai vizibil efect fiind observat la LGEO 75 µg/mL. Testul MTT a fost efectuat pentru a detecta proliferarea celulară. Rezultatele noastre au confirmat potența LGEO de a inhiba viabilitatea celulară de la 84,6% la concentrația cea mai scăzută (5 µg/mL) la 7,8% la cea mai mare (75 µg/mL). Celelalte două uleiuri nu au afectat semnificativ creșterea celulelor. Testul Hoechst a confirmat tendința tuturor uleiurilor de a modifica forma și de a fragmenta nucleii celulari.

Keywords: colorectal cancer, essential oil, viability, lemongrass

Introduction

Colorectal cancer is the third cause of deaths from cancer worldwide and the fourth most common [1]. According to GLOBOCAN data, almost a half of people involved in this disease end with death (with an incidence from 9.0 to 19.5). For this reason, it is very important to analyse the treatment schemes and to find innovative solutions for its improvement.

Fluorouracil by intravenous administration remains the standard systemic treatment for colorectal cancer. This medicine influences primarily the inhibition of thymidylate synthetase, and it is commonly administered with leucovorin, a reduced folate that is known to stabilize fluorouracil's interaction with this enzyme [2]. The major advantage of the classic medication is the lack of selectivity and the damage to healthy cells, in addition to the damaged ones.

Natural sources from herbal plants including polyphenols and carotenoids have the potency to reduce/inhibit the oxidation of lipids, proteins, and nucleic acids and as much preventing the initiation of oxidizing chain reactions, essential reaction in the induction of cancers [3]. Essential oils (EOs) from plants were proved to be significant sources of bioactive compounds, with a wide range of therapeutic effects – antibacterial, anti-fungal, anti-inflammatory, antioxidant and cancer chemo-preventive activities [4].

Thus, diverse types of malignancies like colon cancer, glioma, human liver tumour, gastric cancer, breast cancer, pulmonary tumours and leukaemia are reported to be lowered after treatment with plant essential oils (EOs) from herbs [5]. Polyphenols as well terpenoids as essential constituents of herb's oils may prevent tumour cell proliferation through induction of apoptosis or necrosis [6]. *Cymbopogon citratus* sp. (*C. citratus*) named lemongrass (LG) is a representative from *Poaceae* family and it is primarily cultivated for their essential oils. The major compound of the oil is citral, which is well known as possessing strong activity against Gram-positive and Gram-negative bacteria as well as fungi [7]. The natural mixture of bioactive present in LGEO is known to represent a promising solution for ovarian and colon cancer, by regulation of multidrug resistance and P-glycoprotein efflux pump inhibition [8].

Hippophae rhamnodies L. (*H. rhamnodies*), named Sea Buckthorn, is included in the *Elaeagnaceae* family and is good known for its antioxidant, antidiabetic anti-viral, antibacterial, anti-inflammatory, cardioprotective, vasodilating, hepatoprotective, antiatherogenic, anti-carcinogenic, immunomodulating and hypocholesterolaemic effects [9, 10]. Sea buckthorn essential oil (SBEO) is an important source of mainly unsaturated fatty acids (mainly linoleic and linolenic acids) and presents important inhibitory potency against human liver cancer cells, human acute myeloid leukaemia, prostate cancer and glioma cells [11, 12]. The particularity of this oil is that it contains rare palmitooleic acid (omega-7) which is an important component of skin lipids and stimulates regeneration in the epidermis and wound healing [13].

Ocimum Basilicum L. named basil, is a medical herb of the family *Lamiaceae*, useful in the treatment of kidney, cough, malfunction, diarrhoea, worms and headache. The major compounds of the oil are linalool, citral, methyl cinnamate, methyl eugenol and estragol [14]. Basil essential oil (BEO) is well known to be active against different human's cancer cell lines: the human cervix adenocarcinoma cells, human melanoma cells, human ovarian cells, human chronic myelogenous leukaemia cells and colorectal cells [15].

The aim of the present study it undertakes the assessment of the antioxidant activities of LGEO, SBEO and BEO alongside with the cytotoxic effect and the changes induced by commercial oils against HCT 116 colon cancer cell line. Moreover, it was investigated the type of the cell death induced by commercial EOs, using the Hoechst assay.

Materials and Methods

Materials

LGEO was purchased from SC Bionovativ SRL (Bucharest, Romania), SBEO from SC Hofigal Export Import SA (Bucharest, Romania) and BEO was acquired from Adams Vision SRL, respectively (Bucharest, Romania). Phosphate buffer saline (PBS), dimethyl sulfoxide (DMSO), trypsin-EDTA solution, foetal bovine serum (FBS), penicillin/streptomycin, Hoechst and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) reagents were acquired from Sigma Aldrich, Merck KgaA (Darmstadt, Germany). 2,2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma Aldrich (Steinheim, Germany), the ascorbic acid used as standard was acquired from Lach-Ner Company (Prague, Czech Republic) and methanol 99% was acquired from Chimopar (Bucharest, Romania). The cell culture media, McCoy's 5A Medium (ATCC® 30-2007™) was purchased from ATCC (American Type Cell Collection, Lomianki, Poland). All the used reagents were of analytical standard purity and were applied according to the manufacturers' recommendations.

Methods

The antioxidant potential. The antioxidant potential of the essential oils was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) free-radical scavenging test and the results were presented by EC_{50} value, defined as the half maximal inhibitory concentration of the antioxidants contained in each essential oil, needed to scavenge 50% of DPPH radical present in the test solution. According to the method of Lopes-Lutz *et al.* [16] with some modifications, it was prepared a methanol solution of DPPH 0.1 mM which was kept at 4°C until further use. In parallel, each essential oil (LGEO, SBEO and BEO) was diluted in methanol until a final concentrations (200; 100; 75; 50; 25; 10 and 5 µg/mL) was obtained. A precise volume, of each oil, was added into a quartz test cuvette (10 x 10 mm) with 2.7 mL DPPH 0.1 mM methanol solution. The absorbance values were read at a wavelength of 517 nm, continuously for 20 minutes, using an UviLine 9400 spectrophotometer from SI Analytics (Mainz, Germany). As standard for comparison, a methanol solution of ascorbic acid 0.4 mg/mL was prepared and evaluate as well. The inhibition percentage of DPPH

free radical, expressed as (IP%), was calculated with the following equation:

$$IP\ (\%) = 100 - ((A_{oil} - A_{DPPH}) * 100), \quad \text{Eq. (1)}$$

where: A_{oil} is the absorbance of each essential oil in the presence of DPPH free radical and A_{DPPH} is the absorbance of DPPH free radical, measured at 517 nm without oil.

The half maximal inhibitory concentration (EC_{50}) was determined by linear regression analysis curve plotting, using OriginLab 2020b software, between inhibition percentages (IP%) obtained and concentrations of the methanol solutions of each essential oil.

Cell culture. The present study was conducted using human colorectal adenocarcinoma cell line HCT 116 purchased from American Type Cell Collection (ATCC) as frozen vials. Cells were cultured in their specific McCoy's 5A Medium supplemented with 10% FCS, completed with 1% mixture containing antibiotics (100 U/mL penicillin *per* 100 µg/mL streptomycin) to prevent microbial contamination. The cells were maintained under standard conditions, 5% CO_2 and a temperature of 37°C in a humidified incubator.

Cell Morphology Evaluation. To assess the changes induced by commercial oils in terms of morphology, the HCT 116 cells were examined (after 48h of EOs treatment) with Cytation 1 (BioTek Instruments Inc., Winooski, VT, USA) under bright field illumination. EOs were solubilized in DMSO, at 5, 10, 25, 50 and 75 µg/mL. The pictures were analysed using the Gen5 Microplate Data Collection and Analysis Software (BioTek Instruments Inc., Winooski, VT, USA).

Viability Assay. The cell viability was analysed using the MTT assay. Briefly, cells were cultured in 96-well plates (10^4 cells/200 µL/well) and treated with 5, 10, 25, 50, 75 µg/mL EOs diluted in DMSO, followed by 48 h of incubation, at 5% CO_2 and 37°C. Following the treatment period with EOs, 10 µL/well of MTT solution (5 mg/mL) was added and the plate was incubated for 3 h, the formazan crystals formed were dissolved during 30 min in the dark in 100 µL of solubilisation buffer provided by the manufacturer. The reduced MTT was measured spectrophotometrically at 570 nm, using the Cytation 5 (BioTek Instruments Inc., Winooski, VT, USA) microplate reader. All experiments were performed in triplicate.

Hoechst assay. To determine the type of cell death induced by EOs, the Hoechst method was applied. The protocol followed the manufacturer's instructions. Briefly, cells were cultured at 1×10^5/well in 12-well plates. After reaching a confluence of 80 - 90%, the cells were treated with two concentrations of EO: 5 - the lowest and 75 µg/mL, the highest. After 24 h of treatment, the medium was removed and 100 µL of staining solution was added to each well diluted 1:2000 in PBS. After incubation for 6 - 10 min at room temperature and protected from light, the staining solution was removed and washed three times with PBS. The pictures were taken using Cytation 1 (BioTek Instruments Inc., Winooski, VT, USA) and processed using the Gen5 Microplate Data Collection and Analysis Software (BioTek Instruments Inc., Winooski, VT, USA).

Statistical analysis

The experimental data are presented as means ± standard deviation. The differences between results were analysed by performing the one-way ANOVA analysis and Dunett's multiple comparisons post-test. The used software was GraphPad Prism V. 6.0.0 for Windows (GraphPad Software, USA, www.graphpad.com). The statistically significant differences between data were labelled: * $p < 0.1$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Results and Discussion

Antioxidants play a significant role in health preservation by inhibiting oxidation processes. DPPH scavenging is through the most often used methods and offers the first approach for evaluating antioxidant activity [17]. In Figure 1 are depicted the values corresponding to the inhibition percentage of the essential oils tested (LGEO, SBEO and BEO), each at 7 concentrations (200; 100; 75; 50; 25; 10 and 5 µg/mL) as compared with ascorbic acid (AA) methanol solution of 0.4 mg/mL.

As it can be noticed from the graph, all three essential oils have antioxidant activity compared to the standard (methanol solution of ascorbic acid). Regarding the highest concentration tested (0.2 mg/mL), the anti-oxidant potential of each essential oil respect the linearity LGEO > BEO > SBEO. Moreover, the antioxidant potential of lemongrass is almost comparable with antioxidant potential of the standard (ascorbic acid), 91.68% *vs*. 97.04% (Figure 1A).

Regarding the reduction rate of DPPH free radical, generally it can be observed that the antioxidants contained in essential oils consumes the entire amount of free radical in the first 200 seconds, subsequently the reaction reaches equilibrium. Of course, there is an exception, the highest tested concentration (0.2 mg/mL) from sea buckthorn essential oil (Figure 1B). In the case of this sample, the reaction does not reach equilibrium even after 20 minutes, which means that, the antioxidants contained in essential oils react with DPPH free radical throughout the recording time of the analysis. Anyway, in all the cases it can be noted that the antioxidant potency of essential oils tested are concentration-dependent.

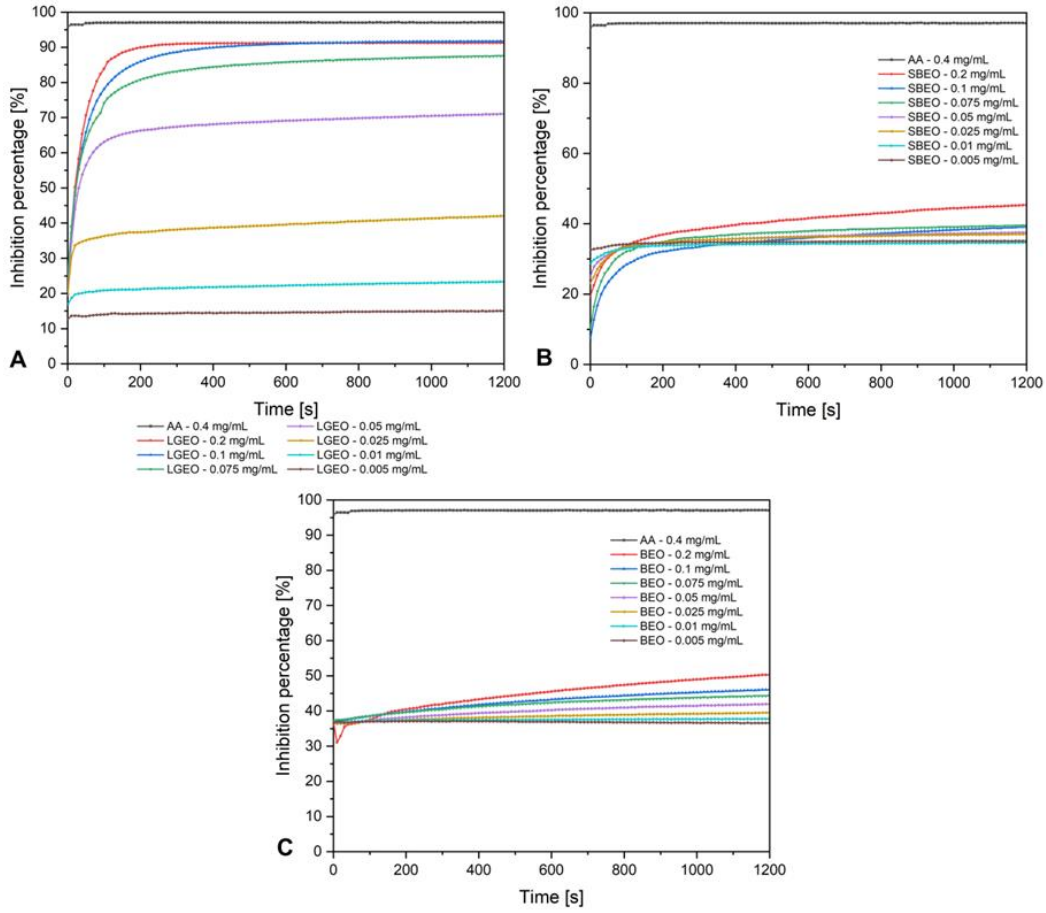

Figure 1.

The time dependent inhibition percentage of essential oils tested A – LGEO *vs.* AA; B – SBEO *vs.* AA; C – BEO *vs.* AA

Table I presents the inhibition percentage of all the samples tested, presented clearer, as an average of three measurements ± standard deviation (SD). The values were calculated with equation (1) and further used for the establishment of EC_{50} values. EC_{50}, characterizing the antioxidant potency as evaluated by the DPPH assay, were as follows:

$$AA - EC_{50} = 0.9 \pm 0.05\ \mu g / mL,$$
$$LGEO - EC_{50} = 31.3 \pm 1.7\ \mu g / mL,$$
$$BEO - EC_{50} = 60.7 \pm 4.5\ \mu g / mL,$$
$$SBEO - EC_{50} = 84.7 \pm 11.6\ \mu g / mL.$$

Table I

The inhibition percentage of essential oils at all seven concentrations tested (200; 100; 75; 50; 25; 10 and 5 µg/mL)

LGEO		BEO		SBEO	
Concentration (mg/mL)	**% IP**	**Concentration (mg/mL)**	**% IP**	**Concentration (mg/mL)**	**% IP**
0.2	91.69 ± 0.03	0.2	50.19 ± 0.1	0.2	45.24 ± 0.08
0.1	91.26 ± 0.02	0.1	45.98 ± 0.08	0.1	39.51 ± 0.05
0.075	87.51 ± 0.05	0.075	44.29 ± 0.05	0.075	39.04 ± 0.07
0.05	70.99 ± 0.05	0.05	41.91 ± 0.04	0.05	37.51 ± 0.03
0.025	41.95 ± 0.06	0.025	39.46 ± 0.04	0.025	37.04 ± 0.03
0.01	23.30 ± 0.04	0.01	37.76 ± 0.02	0.01	35.11 ± 0.03
0.005	14.99 ± 0.05	0.005	36.59 ± 0.02	0.005	34.61 ± 0.03

Our results are in accordance with literature's reports and in some cases even better. For example, Anggraeni *et al*. [18] examined the antioxidant potency of *C. citratum* oil and they found that the inhibition average was 61.76 µg/mL using DPPH scavenging method. Their results were lower than ours (91.69 ± 0.03 µg/mL), the difference being printed by the oil extraction method. Another study realized by Hartatie *et al.* found an anti-oxidant activity up to 72.72% inhibition [19]. Even in the case of SBEO we obtained similar results to those of other researchers, *e.g.*, Rosch *et al*. [20] investigate the antioxidant potency of *H. rhamnodies*

essential oil and they found higher inhibitory potency, 75% to total antioxidant activity.

The next step is to identify the effects of the commercial oils on the HCT cell. Thus, the morphological changes induced by oils at 5 concentrations (5, 10, 25, 50 and 75 µg/mL), on HCT cells were analysed (Figure 2). For the *in vitro* assays, HCT 116 was chosen as colorectal cell line due to its essential properties: i) fast growing and ii) frequently used in colon cancer investigations [21-23].

In case of SBEO and BEO, there were not significant differences observed between untreated cells (control) and those who were 48 h treated with EOs. However, cells treated with 75 µg/mL BEO tend to be more rounded and lose their elongation. Instead, in the case of lemongrass oil (LGEO), the changes were visible from the lowest concentration, with modifications in shape and confluence. Similar morphological changes on HCT 116 were also observed in the study of Ruvinov *et al*. [24] by treating cells with lemongrass extract at 0.01 mg/mL.

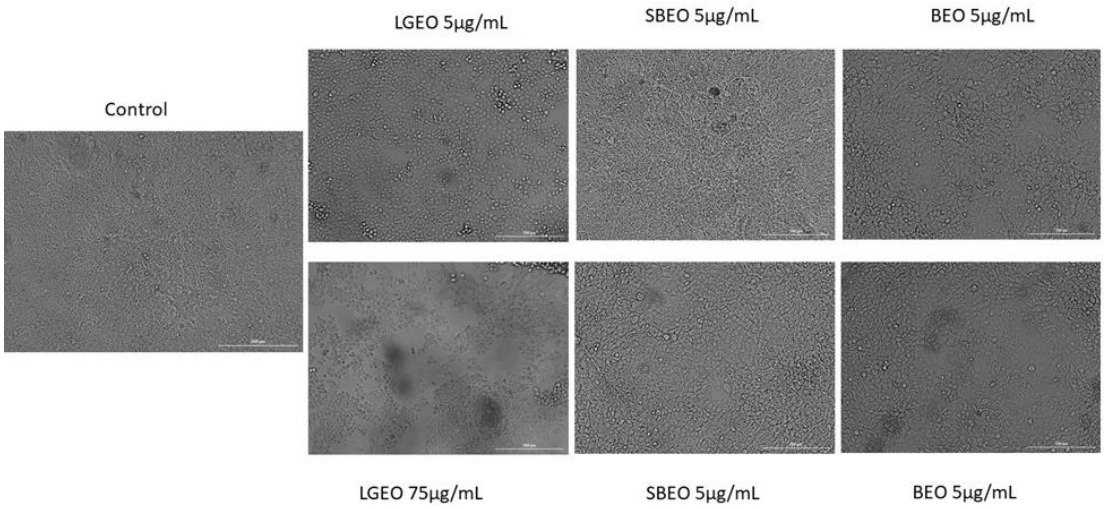

Figure 2.

Morphological aspect of HCT 116 cells after treatment for 48 h with LGEO, SBEO and BEO (5, 10, 50 and 75 µg/mL)

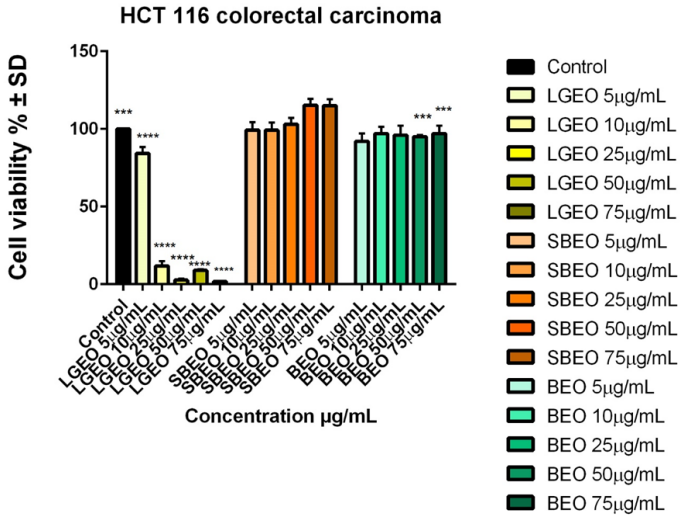

Figure 3.

In vitro assessment of the effect LGEO, SBEO and BEO (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)

Regarding the cell viability (Figure 3), this was intensely affected in case of 48 h of treatment with LGEO. Thereby, at the 5 µg/mL the viability was modified with 16% (84.14%), and down regulation since it tends to decreases with increasing concentration, so at the highest concentration, the viability was 7.8%. On the other hand, the other two oils do not affect the down regulation of cells, more than that, it seems to stimulates cell proliferation. Similar significant inhibitory properties of LGEO were observed by Piaru *et al*. [25]. Halabi

et al. analysed the potency of Lemongrass extract on different cell lines, including HCT 116. The significant inhibitory trend (above 50%) was observed at 50 µg/mL extract concentration [26]. The cytotoxic effect may be induced by citral, the most abundant component in all LGEOs, well known as cytotoxic compound [27]. In addition, citral is known to inhibit and other types of cancer cells, including A549 and H1975 cells [28]. The Hoechst assay was performed to identify whether cell death occurred by apoptosis or necrosis. Hence, HCT 116 cells were stimulated for 48 h (in 12 well plates) with two different concentrations (5 and 75 µg/mL) of all three EOs, the cell nuclei were counterstained using the Hoechst 33342 reagent, and the results were compared with unstimulated cells. So, it was observed that highest concentrations of all three EOs tend to induce modifications in the cellular shape, with signs of fragmentation, especial in case of LGEO, where cells seem to be the most affected (Figure 4).

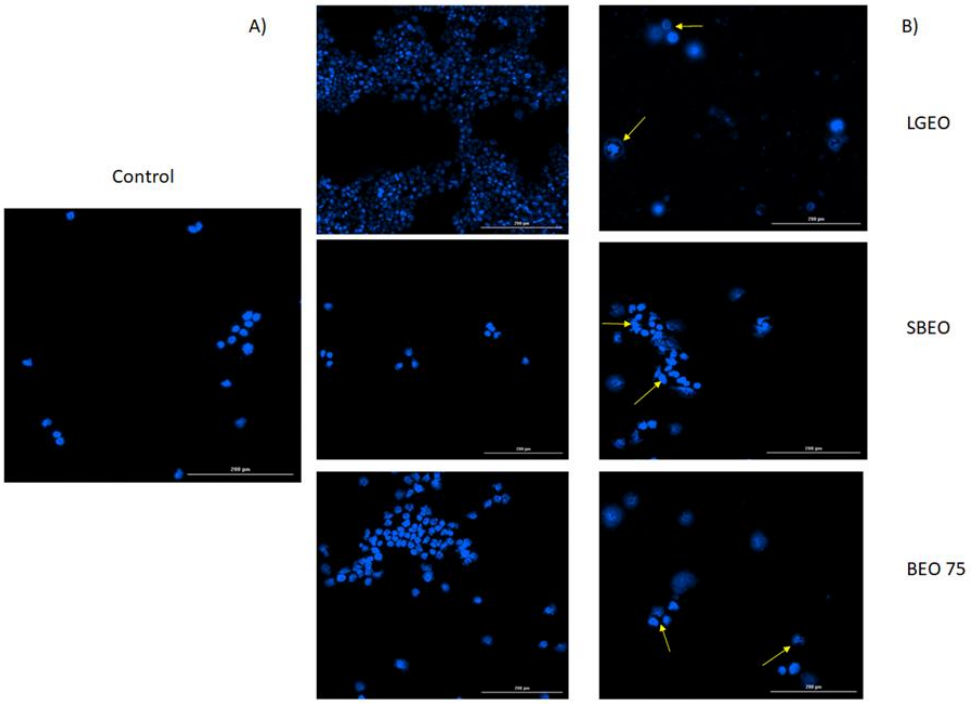

Figure 4.
Images of the cellular nuclei stained using Hoechst 33342 reagent in HCT 116 cells following the 48 h treatment with LGEO, SBEO and BEO (A-5 and B-75 µg/mL)

In their report, Ruvinov *et al*. observed the same ability of LGEO to induce apoptosis. In their study, the EO was efficient in aggressive human colorectal cancer form [25]. Wu *et al*. confirmed the potential of Sea buckthorn to induce apoptosis upon 80 and 120 µg/mL concentration *in vitro* and *in vivo* [29]. *O. basilicum* EO presented similar pro-apoptotic characteristics on human colon cancer cell lines LS174T and COLO205 [30].

Conclusions

The present study undertakes the evaluation of cytotoxic effect and the changes induced by three commercial oils against HCT 116 colon cancer cell line. The assessment of the antioxidant activities of all three commercial EOs (LGEO, SBEO and BEO) indicate that, at the highest tested concentration (200 µg/mL), LGEO showed a strong antioxidant effect (91.69%), followed by a medium one exhibited by BEO (50.19%) and SBEO (45.24%). Moreover, at 75 µg/mL, LGEO inhibit HCT 116 cell viability up to 7.8%. Thus, *C. citratus* essential oil (LGEO) was the most potent, by modifying cellular shape and confluence, inhibiting cell proliferation and inducing cell nuclei fragmentation. Furthermore, LGEO may be analysed in more detail, being able to be a promising solution for future natural treatments against colon cancers.

Conflict of interest

The authors declare no conflict of interest.

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