Supercritical CO_2 as a Potent Solvent to Isolate Nutraceuticals from *Lavandula* and *Mentha* Species Targeting Cancer

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ABSTRACT
The knowledge of the healing power of some species of plants is ancient. Nowadays, the preference for natural products has increased, and with it, the interest in recover and isolate bioactive phytochemicals with potential health benefits.
In this study, supercritical CO_2 was explored as a reliable solvent to extract anticancer compounds, namely terpenes and fatty acids, from different aromatic plants. The extractions were performed in two distinct species of the genus *Lavandula* (*L. angustifolia* and *L. stoechas*) and three species of the genus *Mentha* (*M. spicata*, *M. piperita* var. *citrata* and *M. pulegium*). All extractions were performed at 323K and 25MPa for 60 min, at a CO_2 flow rate of 20 g/min. The resulting samples were screened for the presence of terpenes, by thin layer chromatography (TLC) and then analyzed by gas chromatography coupled with mass spectrometry detection (GC-MS). Furthermore, two different assays for the determination of antioxidant activity were conducted, namely ORAC and HOSC. Cell-based assays in human HT-29 colorectal cancer cells were also performed, aiming at evaluating the potential of the extracts as cancer cell-growth inhibitors.
The extract of *M. piperita* var. *citrata* presented the highest antioxidant activity on HOSC assay, whereas the extract of *L. angustifolia*, presented the highest antioxidant activity on ORAC assay. The *L. angustifolia* extract also revealed to be the most promising colorectal cancer cell-growth inhibitor together with the extracts of *M. spicata* and *L. stoechas*. The main anticancer compounds identified in samples included linalool, camphor, fenchone, carvone and linoleic acid, among others.

INTRODUCTION
Colorectal cancer is the third most common cancer among men and the second with respect to women. [1] Among the environmental factors that are involved in the risk of contracting cancer, there is extensive evidence that nutritional factors can influence several fundamental processes which are related with disease development and progression. In particular, colorectal cancer is generally pointed to be greatly affected by food and nutrition factors, as well as other types of cancer that affect the gastrointestinal tract. [2]
Plants and foods have been used for centuries for their medicinal properties, to prevent and treat all kind of diseases, including cancer. [3] All species of plants produce a large number of organic compounds, which are imperative for their normal development and function. These

chemicals include the primary metabolites namely amino acids, carbohydrates, fats, nucleic acids etc.; and the secondary metabolites, which are specific to some taxonomic groups, such as terpenes, phenolics, carotenoids, alkaloids, saponins, glucosinolates, cyanogenic glycosides, etc. [4] *Lamiaceae* species, and in particular the genus *Lavandula* and *Mentha*, are considered to be very important because of their use in traditional medicine, culinary, cosmetics, perfumery, flavoring and production of essential oils throughout the world. [5] Nowadays, it is believed that the phytochemicals that compose the different groups of metabolites are, among other things, responsible for the health promoting effects shown by plants and foods over the centuries. [6] In particular, medicinal plants are known for being rich in terpenes and fatty acids. The herbal terpenoids have been proved to have strong anticancer potential by inhibition of tumor proliferation and induction of apoptosis. [7] Regarding to fatty acids, there have been many clinical and epidemiologic studies that report that some of them are effective in reducing the incidence of cancer, cardiovascular diseases, inflammation, autoimmune disorders, hypertension, hypotriglyceridemic effect, etc.. [8]

In the last years, the interest in the recovery of bioactive compounds from natural sources has increased. In this field, supercritical fluid extraction (SFE) is a particularly interesting technology that is innovative, clean and environmental friendly, with special interest for the extraction of plant and herb products. [9] It uses clean, safe, inexpensive, nonflammable, nontoxic, environment-friendly, nonpolluting solvents, such as CO_2, in supercritical conditions. [10] Moreover, previous works reported that SFE is a reliable technology to extract bioactive compounds from natural sources and, in particular, terpenes and fatty acids. [11, 12]

In this study, supercritical CO_2 was explored as a reliable solvent to extract anticancer compounds from different *Lavandula* and *Mentha* plants, namely terpenes and fatty acids.

MATERIALS AND METHODS

Materials. For SFE experiments carbon dioxide (CO_2) pure grade (99.95%) (Air Liquide, Algés, Portugal) was used. Determination of the moisture content, was performed using a mixture of xylene isomers (Carlo Erba Reagents, Milan, Italy). For antioxidant activity assays 2',2'-azobis (2-amidinopropane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), disodium fluorescein (FL), Iron (III) chloride ($FeCl_3$), hydrogen peroxide (H_2O_2) and acetone ≥ 99.5% from Sigma-Aldrich (St. Quentin Fallavier, France) were used. All cell culture media and supplements, were obtained from Invitrogen (Gibco, Invitrogen Corporation, Paisley, UK). Moreover, chemicals used for cell-based assays were: dimethyl sulphoxide (DMSO) (99.5%, Panreac, Barcelona, Spain) and methylthiazolyldiphenyl - tetrazolium bromide (MTT), from Sigma-Aldrich (St Quentin Fallavier, France).

Raw material. Aerial parts, including stalks, leaves and flowering tops, of two different species of the genus *Lavandula*, namely *L. angustifolia* and *L. stoechas*, along with three other species of the genus *Mentha,* namely *M. spicata*, *M. piperita* var. *citrata* and *M. pulegium,* were collected between July and August 2013 at Mafra, Portugal. Raw material was dried at room temperature, in the absence of light, and then milled in a domestic chopper. The particle size of ground material was determined using an AS 200 basic vertical vibratory sieve shaker (Retsch, Haan, Germany), with a measuring range between 250µm and 1000µm.

Moisture content determination. Moisture content was determined by conventional Dean–Stark distillation, as previously described [13]. The moisture percentage was calculated assuming that the water volume was equivalent to its weight in grams, as follows:

$$Moisture\ (\%) = \frac{Water\ volume\ (mL)}{Weight\ of\ the\ original\ plant\ sample(g)} \times 100 \quad (1)$$

SFE procedure. The extractions were carried out in a pilot-plant scale supercritical fluid extractor (Thar Technology, Pittsburgh, PA, USA, model SFE-500F-2-C50) comprising a 500 mL cylinder extraction cell and two different separators, each of them with 500 mL of capacity, with independent control of temperature and pressure. All extractions were performed at 323K and 25 MPa with supercritical CO_2. The methodology applied consisted in 15 minutes of static extraction time followed by 60 minutes of dynamic extraction time, at a CO_2 flow rate of 20 g/min. The first fraction collector was at room temperature and a pressure of 6 MPa, and the second fraction collector was at NTP conditions, in order to achieve extracts with different compositions.

Phytochemical characterization by TLC. TLC analysis was performed using silica gel plates (Merck KGaA, Darmstadt, Germany) with a 254 nm fluorescent indicator (aluminium base sheets 20x20 cm, thickness 200 μm, medium pore diameter 60 Å). Dichloromethane **(**Fisher Scientific, Loughborough, UK) was used as mobile phase and the plate was revealed with Liebermann-Burchard reagent, which reveals terpenes and steroids. After revelation, the plates were examined under a UV lamp 365 nm.

Phytochemical characterization and quantification by GC-MS. GC-MS analysis was carried out using a Shimadzu GCMS-QP2010 (Shimadzu Corporation, Kyoto, Japan) gas chromatograph with a quadrupole mass spectrometer and electronic impact source. A DB-5MS (J&W Scientific) capillary column was used to separate de compounds. GCMS Solutions software was used for data acquisition and the components of the extracts were identified by comparison with the mass spectra from libraries. Quantification results were given by the same software and were expressed as relative peak areas.

Antioxidant assays. Antioxidant activity of extracts was accessed by ORAC and HOSC assays. These assays were carried out by following the procedures previously reported [14, 15], modified for the FLx800 fluorescence microplate reader (BioTek Instruments, Winooski, VT, USA). [16, 17] The ORAC and HOSC values were calculated by a regression equation between the Trolox concentration and the net area under the FL decay curve, taking into account that the results of antioxidant capacity depend on sample concentration. These results were expressed as Trolox equivalents per gram of extract (μmol TE/g of extract).

Cytotoxicity assays. Cytotoxicity of extracts was accessed in human Caco-2 cells, which are a good model of the human intestinal barrier as previously described. [18] After 24h of incubation with the extracts, cell viability was determined using the colorimetric MTT assay.

Antiproliferative assays. Antiproliferative activity of extracts was accessed in human colorectal cancer cells HT-29 as previously described. [11] After 24h of incubation with the extracts, cell viability was determined using the colorimetric MTT assay. The results were expressed as effective concentration values (EC50 – concentrations that inhibit HT-29 cell proliferation by 50%).

RESULTS

In this work, SFE technology was applied to five different natural matrices, namely *L. angustifolia*, *L. stoechas*, *M. spicata*, *M. piperita* var. *citrata* and *M. pulegium*, in order to extract different bioactive compounds with promising anticancer activity.

Prior to extractions, the plants were air-dried and milled. Then, the plant material was sieved using suitable sieves and a vertical vibratory sieve shaker, in order to determine the mean particle diameter. The moisture content of the dried plants was also determined and the extractions were preformed. These results are summarized in Table 1.

Table 1: Natural matrices initial characterization: mean particle size and moisture content.

Sample		Mean Particle Diameter (μm)	Moisture Content (%)
Lavandula spp.	*L. angustifolia*	496	7.00 ± 0.71
	L. stoechas	509	4.50 ± 0.71
Mentha spp.	*M. spicata*	515	10.25 ± 0.35
	M. piperita var. *citrata*	520	9.50 ± 0.00
	M. pulegium	485	7.00 ± 0.71

Aiming at obtaining extracts rich in terpenes and fatty acids, supercritical fluid extraction technique was employed. All extractions were performed at 323K and 25 MPa, and the methodology comprised one step with supercritical CO_2 for 60 minutes, which can efficiently isolate perillyl alcohol, a monoterpene known for its chemopreventive and cytotoxic activities against a wide variety of cancer cell lines. [11] Moreover, at low CO_2 densities (temperatures between 313 and 323K and pressures between 8 and 9 MPa), terpenes are much more soluble than fatty acids. However, at high CO_2 densities (temperatures between 313 and 323K and pressures between 10 and 20 MPa), terpenes are completely miscible in supercritical CO_2, and the solubility of fatty acids increases. [19] Consequently, it was expected that, under the selected conditions, fatty acids could be extracted as well, since there are several studies that used similar supercritical CO_2 densities, and successfully isolated these compounds. [12]

The resulting extracts were analyzed by TLC for the presence of terpenes (Figure 1). Results showed that extracts of the same genus exhibited similar terpenes profile.

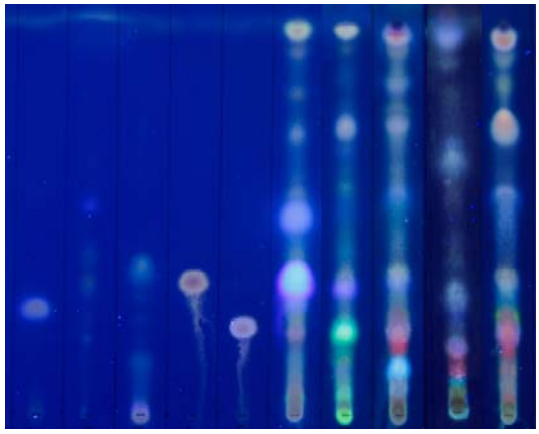

Figure 1: TLC analysis of the natural extracts, obtained by SFE, to reveal terpenes and steroids. Legend: 1 – perillyl alcohol standard; 2 – limonene standard; 3 – linalool standard; 4 – lupeol standard; 5 – cholesterol standard; 6 – *L. angustifolia* extract ; 7 – *L. stoechas* extract; 8 – *M. spicata* extract; 9 – *M. piperita* var. *citrata* extract; 10 – *M. pulegium* extract.

The study of the chemical content of the natural extracts, as well as its quantification, were performed by GC-MS. The major compounds are schematically shown in pie charts and the results are expressed as relative peak areas (Figures 2 and 3). The charts on the left represent the major peaks (which were saturated), whereas the charts on the right represent the minor compounds, whose relative peak areas were calculated excluding the percentages of the saturated compounds.

The antioxidant effectiveness of *Lavandula* and *Mentha* extracts were assessed using two complementary chemical assays, namely ORAC and HOSC, were performed. The results are presented in Figure 4, and demonstrated that *M. piperita* var. *citrata* and *L. angustifolia* extracts have the highest antioxidant capacity. These data suggest that the extracts have similar ORAC and HOSC values, except for the extracts of *L. angustifolia* and *M. piperita* var. *citrata*, which presented higher HOSC values.

The antioxidant activity in plants is commonly attributed to phenolic compounds, which are broadly distributed in the plant kingdom and are their most abundant secondary metabolites. However, it is possible that extracts rich in nonphenolic compounds also have antioxidant potential. Moreover, it was demonstrated that some terpenes have significant antioxidant effects. [20]

Thus, it is possible that the antioxidant activity demonstrated by *M. piperita* var. *citrata* and *L. angustifolia* extracts is due to their content in linalyl acetate, which is one of their major constituents. However, it is also possible that some other compounds enhance the activity and that there are synergistic interactions between some extract constituents.

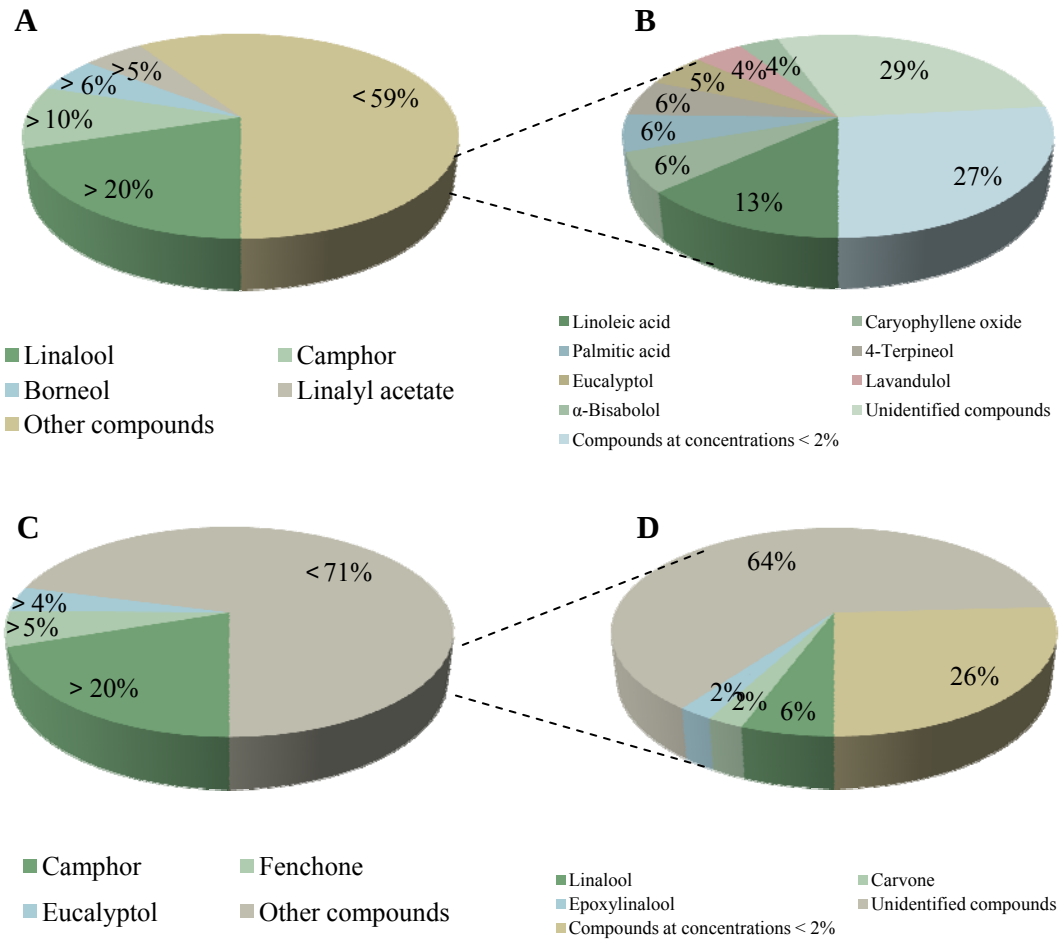

Figure 2: Major compounds found in SFE extracts of *Lavandula* plants. Legend: A – Major compounds of *L. angustifolia* extract (saturated peaks); B – Other compounds of *L. angustifolia* extract; C – Major compounds of *L. stoechas* extract (saturated peaks); D – Other compounds of *L. stoechas* extract.

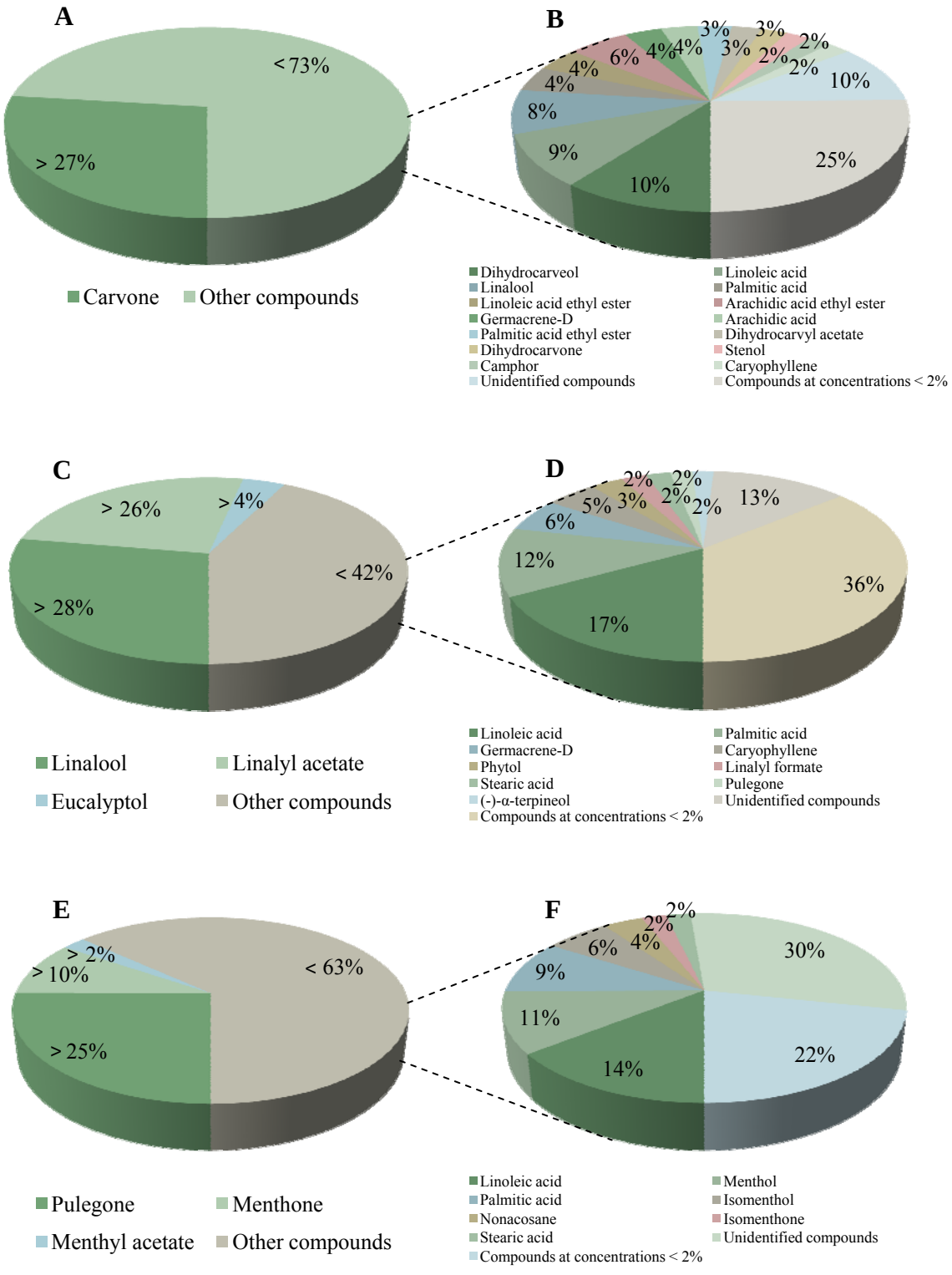

Figure 3: Major compounds found in SFE extracts of *Mentha* plants. Legend: A – Major compounds of *M. spicata* extract (saturated peaks); B – Other compounds of *M. spicata* extract; C – Major compounds of *M. piperita* var. *citrata* extract (saturated peaks); D – Other compounds of *M. piperita* var. *citrata* extract; E – Major compounds of *M. pulegium* extract (saturated peaks); F – Other compounds of *M. pulegium* extract.

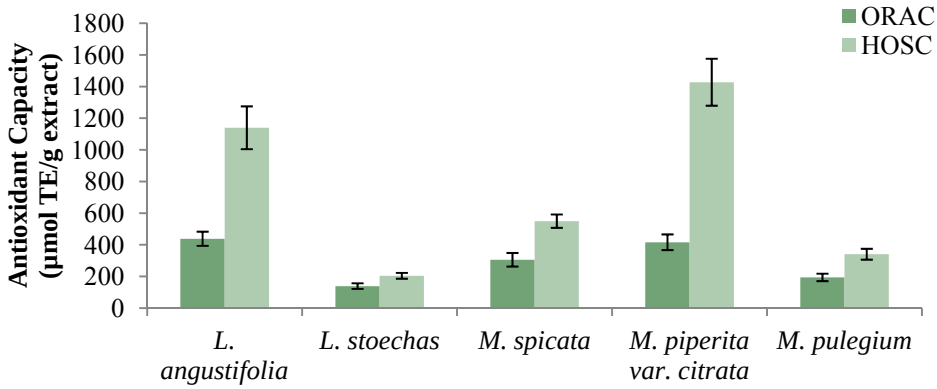

Figure 4: Antioxidant capacity of *Lavandula* and *Mentha* extracts.

In order to examine the anticancer potential of the extracts, antiproliferative assays were conducted in human colorectal adenocarcinoma cells (HT-29). The cells were exposed to the natural extracts for 24h and the percentage of viable cells was evaluated using MTT assay. Results showed that all extracts inhibited colorectal cancer cell growth. In Figure 5 are presented the EC50 values of all fractions. Among all samples, the *Lavandula* plants extracts were more effective than the extracts of *Mentha* plants. In other words, a lesser amount of *Lavandula* extracts is needed to inhibit half of the cell viability. Moreover, it is important to highlight that, at this concentration, the natural extracts did not exhibit cytotoxicity in Caco-2 cells (data not shown), which are a good model of the human intestinal barrier.

Among all extracts, *L. angustifolia* exhibited the highest antiproliferative effect (EC50 value of 0,112±0,011 mg extract/mL) followed by *M. spicata* and *L. stoechas* extracts. It is important to note that the extract obtained from *L. stoechas*, which had showed the lowest antioxidant capacity, was one of the most effective in inhibiting HT-29 cells growth. These results suggest that there are some compounds that are present in the extract that contribute to this effect, but do not have antioxidant properties.

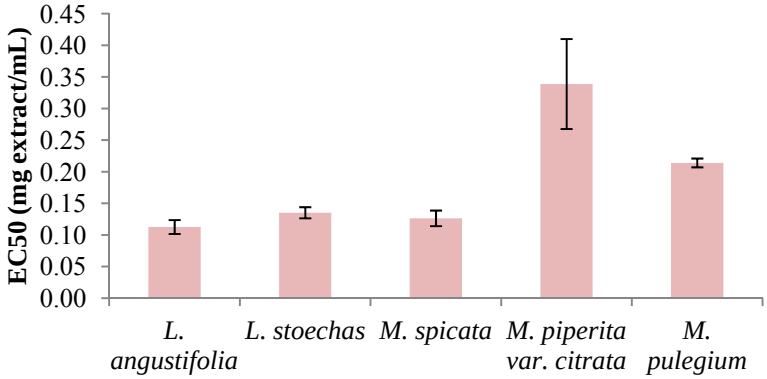

Figure 5: Antiproliferative effect on HT-29 cell line after 24 h of treatment with *Lavandula* and *Mentha* extracts – EC50 values.

The anticancer activity was correlated with the phytochemical composition of the natural extracts, since linalool, camphor, fenchone, carvone and linoleic acid, among others, have demonstrated cytotoxic activity against several tumor cell lines. [21, 22, 23] However, it is also possible that the exhibited activity was due to a synergistic effect between several compounds that were present in extracts.

CONCLUSION

Supercritical CO_2 proved to be a potent solvent to develop phytochemical-rich fractions, from *Lavandula* and *Mentha* plants, with promising anticancer activity.

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