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Chemical Composition and Antioxidant Activity of *Laurus Nobilis*L. Extracts Obtained By Different Extraction Techniques

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Abstract: *Laurus nobilis L. is a source of monoterpenesand otherantioxidant compounds, as tocopherol.The aim of the study was to identify and relate the chemical compounds of the laurel leaves extracts, obtained by different techniques (hydrodistillation (HD), Soxhlet (SOX), ultrasound-assisted (UAE) and Supercritical CO_2 (Sc-CO_2) with their antioxidant characteristics. The SOX and UAE extractions obtained higher yield. The yields of the Sc-CO_2 extraction were similar or greater than the HD extraction. Oxygenated monoterpenes were found in the HD, SOX and UAE extracts. A significant amount of α-tocopherol was extracted by supercritical fluid. Laurel leaves extracts are sources of antioxidant compounds.*

Keywords:*1,8-cineole, α-tocopherol, monoterpenes, antioxidant.*

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I. Introduction

Laurus nobilis L. is a perennial tree native from the Mediterranean region. It is grown commercially for its aromatic leaves in Turkey, Algeria, Morocco, Portugal, Spain, Italy, France,Mexico and in south and southeast Brazil. As an important aromatic plant, fresh or dried laurel leaves are commonly used as food flavoring and the herbal tea and its essential oil (EO) are used in cosmetics and for medicinal purposes[1,2].

Essential oil from *L. nobilis* is a highly valued product due to beneficial functions such as antibacterial[3–8], antifungal[9] and antioxidant activities[10–15]. As the pharmacological properties of the laurel EO have been reported for antiproliferative [16] and anti-inflammatory activities [17].

Studies by Sellami et al. [18] detected the presence of forty-seven compounds in the laurel leaves essential oil, especially oxygenated monoterpenes. The main components (1,8-cineole, methyl eugenol, terpinen-4-ol, linalool and eugenol) suffer due to the extraction method or the drying method applied. Monoterpenes are important constituents of essential oils, but are highly volatile and brittle at high temperature [19].

In the literature, were found works of the laurel leaves extraction, comparing the yield and the chemical composition of the extracts obtained by supercritical fluid extraction (Sc-CO_2) and distillation[20–23], in **Table 1**. The yield for hydrodistillation (HD) extraction varying from 0.9% to 2.6%[20,23], while for the Sc-CO_2, the yield varying from 0.6 % to 1.37 %[21,22].The chemical composition of the EO fromlaurel leaves has been studied by different researchers and1,8-cineolewas found to be the major component. The quantity of 1,8-cineole varying from 22.84 % to 49.7 %[20,23] for the extracts obtained by HD and 2.53 % to 43.0 %[20,22] for Sc-CO_2 extract, according to **Table 1**.

Other compounds were present in appreciable amounts such aslinalool, D-limonene, transhidratosabinene, α-terpinol acetate, ethyl-and-methyl eugenol, sabinene and eugenol[20–23]. The EOcomposition is influenced by different factors, including the area of culture, ecotype, harvest season, light intensity, climate conditions and organ age[24,25].

The quality and the composition of the extracts is also strongly dependent on the extraction procedure and type of solvent employed, which must be carefully selected to provide an adequate balance to enhance yield and selectivity [26,27]. Traditionally, from oil laurel leaves is extracted by steam distillation, but in the last few years, new extraction techniques have been studied to reduce the volume of solvents or the time required to extract those types of compounds.

The supercritical fluid extraction (Sc-CO_2) technology meets some desirable propertiesfor the production ofnatural products and/or functional ingredients. Due to the consumer's demands and the increasing legal restrictions for delivering healthy foods to consumers, the method could be the alternative to the solvents extraction of heat labile components. Carbon dioxide (CO_2) is the most commonly used solvent to extract volatiles and essential oils due to its low polarity, mild critical conditions, non-flammability, low cost and

easiness to be removed from the extract. Thus, high quality products, free from solvents could be obtained. The extraction with Sc-CO_2may retain the essential oil components without any change or degradation; in addition, this solvent may also remove other groups of compounds[28,29].

Promising results of the total phenols content of the laurel leaves was reported in extracts obtained by maceration [12], sonication [14], infusion [30] and ultrasound [13,15], with ethanol [12,13], methanol [14,15,30] and water [30] solvents. The antioxidant activity of the laurel leaves extracts has been proven by several methods [10–12,14]. As well as the content of tocopherols present in laurel leaves [10,11,31].

α-Tocopherol is a group belonging to vitamin E, predominant in olive, wheat germ, and sunflower oils [32]. The antioxidant activity of vitamin E is due to its ability to donate phenolic hydrogen to lipid-free radicals and to retard autocatalytic lipid peroxidation process. Several studies suggest that vitamin E may contribute to lower the risks of specific chronic and degenerative diseases such as Alzheimer, age-related muscle degeneration, some types of cancer, cataracts and ischemic heart disease [33].

Tocopherol contents of Tunisian *Laurus nobilis* vegetative organs were obtained by probe sonicationand microscale saponificationtechniques [10]. The extract obtained from the laurel leaves by probe sonication presented 49.69 ± 2.4 mg/100 g fresh weight, and microscale saponification extract of 139.34± 8.8 mg/100 g fresh weight, both α-tocopherol[11]. Dias et al. [11]found total amount of tocopherols of 655.7 ± 22.62 to 780.12 ± 2.36 mg/100 g for cultivated and wild laurel leaves, respectively, by HPLC method. However, we did not find in the literature studies of the phenolic compounds, antioxidant activity and tocopherols content of the laurel leaves extract obtained by supercritical fluid.

Previous studies have compared the yield of different extraction techniques, performed the identification of chemical compounds, phenolic compounds, antioxidant activity and tocopherols content of laurel leaves extracts. However, we did not find the correlation of this information with the monoterpenes content and antioxidant characteristics of the extracts obtained by supercritical fluid (CO_2). Therefore, the aim of the study was to relate the chemical compounds of the laurel leaves extracts, obtained by different techniques, with their antioxidant characteristics, highlighting the extracts obtained by Sc-CO_2.

II. Materials And Methods

2.1 Raw material and sample preparation

Fresh leaves of *Laurusnobilis* L.were purchased in the local commerce of the city of Florianópolis/SC/Brazil.The raw material was dried in a vacuum oven at 40 °C for 12 h (EL 003, Odontobrás, Brazil)the moisture content from fresh and dry samples evaluated by the 925.09 methodology of AOAC[34]. The dried leaves were ground in a knife mill (De Leo, Porto Alegre/RS − Brazil). The raw materials were stored at 4 °C until their use.

2.2Essential oil extraction

2.2.1 Hydrodistillation (HD)

The hydrodistillation method consisted in placing 30 g of ground fresh or dryof laurel leaves insideof a Clevenger type apparatus with 1000 mL of distilled waterduring 3 h. The extract was separated from the water by density difference and dried with sodium sulfate (Na_2SO_4).

2.2.2Soxhlet (SOX)

Soxhlet extraction of dried laurel leaves using hexane as solvent was performed according to the 920.39 C method of AOAC [34]. The procedure consisted of 150 mL of solvent recycling over 5 g of dried sample, in a Soxhlet apparatus for 6 h at the boiling temperature of the solvent used. The solventsof the resulting extracts were evaporated under reduced pressure in a rotary evaporator (Fisatom, Brazil).

2.2.3 Ultrasound-assisted extraction (UAE)

Ultrasound-assisted extraction was carried according to the method described by Vinatoru[35]. The procedure was conducted using5 g of dry of laurel leaves and 150 mL of hexane placed inside a covered glass balloon, for 2 h. The equipment used was an ultrasonic bath, which operates at a frequency of 40 kHz and potency of 60 W. The system was filtered and the solvent was removed in a rotary evaporator under reduced pressure.

2.2.4Supercritical Fluid Extraction with CO_2(Sc-CO_2)

The experimental unit and the procedure for the high-pressure process were previously described by Michielin et al. [36]. The supercritical extraction used pure CO_2 (99.9%) delivered at a pressure up to 6 MPa (White Martins, Brazil). Briefly, the extraction procedure consisted of placing dried and milled material inside a stainless-steel column (329 mm length × 20.42 mm inner diameter, and internal volume of 100 mL) to form the

fixed particle bed, followed by the control of process variables (temperature, pressure, and solvent flow rate). The extract was collected from the extraction unit in amber flasks and weighted.

Preliminary tests were performed, varying the pressure (8 and 10 MPa) at the temperature 40 °C, setting the solvent flow rate of 8 ± 2 g min^{-1} CO_2, resulting in very low extraction yields, quantities not sufficient for further analysis. Therefore, higher conditions of temperature (45 °C) and pressure (15 MPa) were used, when comparing those found in the literature for laurel leaves extraction by supercritical fluids.

The duration of the Sc-$CO_2$for laurel leaves was determined analyzing the overall extraction curve (OEC). This assay was carried on using supercritical CO_2 at 15 MPa, 45 °C, 12 g of raw material and solvent flow rate of 8 ± 2 $gmin^{-1}$ CO_2, where the extract samples were collected at pre-established time intervals. Taking the OEC into account, Sc-CO_2 time for the following steps was chosen in order to recover all the extractable material and was conducted until the diffusion-controlled period was completely established. The process time was defined at 2.5 h for the application of the Sc-CO_2assays for all experimental conditions of temperature and pressure tested. The subsequent Sc-CO_2assays were performed with 12 g dry and ground laurel leaves,the solvent flow rate of 8 ± 2 $gmin^{-1}CO_2$at temperatures of 45 and 55 °C and pressures of 15, 20 and 25 MPa.

The experiments (HD, SOX, UAE and Sc-CO_2) were performed in duplicate. All extracts (HD, SOX, UAE and Sc-CO_2) were stored in sealed amber glass bottles at −18 °C.

2.2.5 Extraction yield (X_0)

The extraction yield (X_0) was calculated by percentage (%) of the mass of extract ($m_{extract}$) relative to the total mass of raw material ($m_{rawmaterial}$), according equation: $X_0 = \frac{m(extract\ )}{m(rawmaterial\ )} * 100.$

2.3 Qualitative and quantitative analysis of the most volatile fraction of the extract

Identification and relative quantification of the chemical compounds present in the extracts was performed in a chromatograph-mass spectrometer (GC/MS, model 7890 A, mass detector 5975C, Agilent Technologies, USA), attached to a HP-5MS column (30 m × 0.25 mm internal diameter × 0.25 mm film thickness, Agilent Technologies, USA). The 1,8-cineole quantification from laurel leaves extracts, was performed bygas chromatography coupled to the flame ionization detector (GC-FID), in headspace flasks, according to methodologies ofCaredda et al. [23] and Ivanovic et al. [22]with modifications.

The GC column temperature program was from 60to280°C, at 3 °Cper minute, followed by 30 min hold under isothermal conditions. The injector was maintained at 250 °C.Helium was the drag gas with a flow rate of 1.0 $mLmin^{-1}$; the sample (1 µL) was injected using a split ratio of 1:20.The conditions of the mass spectrum detector (MS 5975C) were as follows:the ionization energy 70 eV and quadrupole mass analyzer operating with scanning in the range of 35 to 550 u for the SCAN mode.Identification of the compounds extracted from the samples was performed by comparison with the library spectral data (NIST 11) and with data from the reviewed literature. Quantification of 1,8-cineole, the major compound of laurel leaves, was performed by GC-FID, using the same GC-MS operating conditions.

2.4 Determination of Total Phenolic Content (TPC)

Total phenolic content (TPC) of the laurel leaves extractswas determined by Folin–Ciocalteu method[37], with some modifications. Briefly, 10 µL aliquot of extract solution (concentration 10 $mgmL^{-1}$) and 600 µL water were mixed to 50 µL undiluted Folin–Ciocalteu reagent (Sigma-Aldrich, USA). After 1 min, 150 µL of 20% (*w/v*) Na_2CO_3 were added and the volume completed up to 1 mL of water. The samples were incubated for 2 h at 25 °C in the dark. The absorbance was measured at 760 nm in a spectrophotometer. The standard curve with series of gallic acid solutions was used for calibration. The phenolic content was expressed as mg of gallic acid (GAE) per *g* of extract. Measurements were done in triplicate.

2.5 Antioxidant activity

The free radical scavenging of laurel leaves extractswas evaluated using 1,1-diphenyl-2-picrylhydrazil (DPPH) method performed accordingto the methodology described byMensor et al. [38]. Briefly, different extract concentrations were tested (5 different concentrations for each extract) by mixing 25 µL of extract solution to 975 µL of DPPH diluted solution to complete the final reaction medium (1 mL). After 30 min in the absence of light and at room temperature, the absorbance values were measured at 517 nm. The percentage antioxidant activity was obtained considering the mean value of triplicate assays. The DPPH results were expressed as the effective concentration at 50% (EC_{50}), i.e., the concentration of the solution required to give a 50% decrease in the absorbance of the test solution compared to a blank solution and expressed in μgmL^{-1}. The EC_{50} values were calculated from the linear regression of the percentage antioxidant activity curves obtained for all extract concentrations. Results are presented by average ± standard deviation of triplicate assays.

2.6 Statistical analysis

Extraction yield, TPC, and antioxidant activity results were statistically evaluated by a one-way analysis of variance (ANOVA), using Microsoft Excel (2013). The significant differences ($p < 0.05$) were analyzed using a Tukeytest.

III. Results and Discussion

3.1 Sc-CO_2extraction kinetics

The Sc-CO_2 extraction kinetics curve was performed with the objective of determining the period required to perform the maximum extraction,that is, the time necessary to reach the diffusional period of the extraction.**Figure1**, shows the yield extraction (X_0) against extraction time for the Sc-CO_2 performed at 15 MPa and 45 °C, operational condition tested. The shape of the extraction curve indicates that at different stages of the extraction, 'constant extraction rate' (CER)period, 'falling extraction rate' (FER) period, and diffusional period (DC), characterized by the dominance of a specific or combined mass transfer mechanisms [39], determined by linear regression of the extraction curve at each stage. As can be observedthe constant extraction rate*(CER)*ends at 62.9 min and the falling extraction rate*(FER)*occurs between 62.9 and 112 min. The time for laurel leaves extraction was fixed at the diffusion-controlled period, at 150 min (2.5 h).

3.2 EO extraction yield(X_0)

As can be observed at **Table 2**, SOX and UAE, with hexane, presented the highest yield, 7.5 and 6.0 %, respectively. The common high yield of the SOX method is due tohigh temperature (boiling temperature of hexane 68 °C), process time (6 h), solvent recycle, and solvent-solute interactions that contribute to enhancing the extraction. However, as a disadvantage, not indicated for extraction of volatile compounds, such as essential oils, due to the use of high temperature, higher than those used in UAE and Sc-CO_2, and solvent residue could be retained in the final product due to incomplete removal [40].

UAE presented a highestyield extraction than HD and Sc-CO_2. This behavior was probably due to the ultrasonic waves that improve extraction efficiency. Moreover, the UAE reduces extraction temperature, when compared to SOX, being beneficial to botanical materials which are sensitive to temperature[35]. However, the extracts also could present solvent residue retained due to use of hexane, as for SOX.

Hydrodistillation is the simplest technique for EO isolation[41]. Furthermore, it is the method established by the International Organization for Standardization of Technical Committee (ISO/TC 54), being considered the method that allows the better control of the quality of essential oils [42]. However, it was the method that presented the lowest extraction yields.

The HD extraction yield was highest to dried laurel leaves (1.5 %) than to fresh sample (0.2 %), with moisture content of 43.0 ± 0.2 % (w/w) and 4.4 ± 0.1 % (w/w), respectively. The decrease in moisture content favors the increase of the concentration of the essential oil in the surface of the leaves of laurel [43], facilitating the process of extraction, besides guaranteeing the repeatability of the procedure.

The result found by this work for HD extraction yield is highest than that found by Caredda et al.[23] (0.9 %), similar to those found byIvanovic et al. [22] (1.43 %) and Simsen& Lobo [44] (1.33 %), and lower than those verified by Ozek, Bozan & Baser[20] (2.6 %). Therefore, results that corroborate with those found in the literature.

Supercritical CO_2 have been considered as an alternative for essential oil extraction, with the advantage of being selective, non-thermal and green. The Sc-CO_2 yields extraction varied from 1.5 to 2.9%, being highest orsimilar to yieldof dried leaves HD extract ($p < 0.05$).

Analyzing only the yields obtained by Sc-CO_2extraction, the minor yield (1.5 ± 0.1 %) and only that has significant difference, ($p < 0.05$), was obtained for lower density of CO_2, in **Table 2**. The power of solvation of carbon dioxide (CO_2)is dependent on its density, which increase with increasing pressure, at constant temperature, and decrease withincreasing temperature, at constant pressure [28].

This behavior can be observed in Figure 2**,** with the increase of the extraction yieldwhen the pressure increased from 15 to 20 MPa,under isothermal condition of 55 °C. However, yields did not show significant difference, ($p < 0.05$), with increase pressure from 20 to 25 MPa, at constant temperature.

The increase of temperature, at constant pressure, provides two opposite effects: 1. reduction of solvent (CO_2) power due to density decrease, observed with the increase of temperature from 45 to 55 °C, at pressure of 15 MPa; 2. the increase of the temperature favors the increase of the solutes vapor pressure, facilitating its transfer to the supercritical phase[28].

Besides the effects of density, temperature and pressure on the yield of extraction Sc-CO_2, the chemical constituents of the plant are complex mixture including a number of groups with different properties and distribution in the matrix.

Figure 2suggests a region of crossover. At 15 MPa, a negative effect of temperature is noticed on yield, which is caused by the reduction of solvent density. Otherwise, at 20 and 25 MPa,there is a tendency for predominance of solutes vapor pressure in the solubility.

The results for the Sc-CO_2 extraction yields found by this work are higher than those found in the literature (0.6%[21], 0.82% (fractional extraction)[23], 1.13 %[20], 1. 34%[20] and 1.37%[22]), in **Table 1**. Probably the highest conditions of temperature and pressure used in the Sc-CO_2 extraction process contributed to increase the yield.

The difference in the yields of the extracts obtained by this work compared with other works found in the literature, also can be explained by the natural difference of the raw material, since the essential oils are part of the plant metabolism and, therefore, they are in constant fluctuation, besides the external factors, like moment of the development, growth or schedule and plant harvest day, in addition, the moisture of the raw material [45].

3.3Chemical profile of the laurel leavesextracts

In orderto evaluate the effect of extraction methods in the chemical profile, an analytical procedure, gas chromatography-mass spectrometry (GC-MS)was applied to all extracts. **Table 3** presents the compounds identified from the various laurel leaves extracts.Twenty-three (23) different chemical compounds were identified inthe laurel leaves extractions.

Oxygenated monoterpenes were the major class in HD, SOX, and UAE extraction methods, i.e, 1,8-cineole and D-limonene,besides other monoterpenes as eugenol, methyleugenol, acetyleugenol, 3-carene and aromatic hydrocarbons (α-pinene),in **Table 3**.On the other hand, the Sc-CO_2 extracts had a highest concentration of α-tocopherol, 3-tetradecyn-1-ol, and D-limonene, compounds that act in synergy with other compounds, such as hydrocarbons, in the antioxidant and antibacterial activity [46].

SOX extract presented only, approximately, 21%of oxygenated monoterpenes and 3.5% relative area for α-tocopherol, despite the high yield, characterizing as a low selectivity method for chemical compounds that have bioactive characteristics. Therefore, the SOX method is a poor extract in monoterpenes. The UAE extract obtained 61%, approximately, of oxygenated monoterpenes and 8.0% relative area for α-tocopherol, being superior to the SOX extract, but without the predominance of a specific chemical compound.

The HD extract is rich in oxygenated monoterpenes, with 86% (approximately)of the compounds identified and the predominance of 1,8-cineole, but with the disadvantage of having the lowest yield when compared to other extraction methods. According to the literature, the EO composition can vary according to climate, plant age, soil composition and the plant organ from which the oil was extracted [47,48]. Although the major compounds in EO can vary, several studies have shown that the major compounds of laurel leaves EO are oxygenated monoterpenes, corroborating with the results of this work [20,22,23].

Besides 1,8-cineole, other monoterpenes were found in HD, SOX, and UAE extraction methods. In **Table 3**, D-limonene, methyl eugenol, eugenol, 3-carene and acetyleugenol, these compounds, in synergy, act as antioxidant and antibacterial agents. As can be observed at **Table 3**, the D-limonenemonoterpene was identified in all extracts. This compound is the main precursor of carvone, a terpene ketone with odoriferous and herbal properties widely used by the food industry on food flavoring, cakes, confectionery, and liquor factory [49].

Other authors found the monoterpenes D-limonene, methyl eugenol, eugenol, 3-carene in extracts obtained by HD extraction[20,22,23]. D-limonene was found in extracts obtained by Sc-CO_2 in the work of Ozek, Bozan & Baser[20], Caredda et al.[23], Ivanovic et al.[22] and De Corato et al.[21], collaborating with the results found by this work.

However, α-tocopherol also has been reported in extracts obtained from laurel leaves, by Soxhlet, microscale saponification and probe sonication extractions[10,11]. α-Tocopherols a compound of greater interest to the pharmaceutical, cosmetic and food industries because it has highest antioxidant capacity[11,12].

The Sc-CO_2 process conditions (T and P) favored the removal of α-tocopherol, polar compound of high molecular mass (502.88 g mol^{-1}). The obtainment of this compound is not possible by the HD method due to its limitation in extracting volatile compounds of low molecular mass. The difference of the Sc-CO_2 extracts from the SOX and UAE extracts, as the relative area of the α-tocopherol, is the predominant presence of this compound, demonstrating its high selective capacity. We did not find in the literature, data on the extraction of α-tocopherol from laurel leaves by Sc-CO_2.

On **Table 3**, we found high relative area perception of α-tocopherol in the Sc-CO_2, varying from 30.0 to 51.5%.It was observed that the increase in temperature, at constant pressure, increase the percentage relative area of α-tocopherol by 20 and 25 MPa, indicating that the extraction of this component was favored by the increase in the vapor pressure, whereas the increase of pressure, at constant temperature, was only favorable at 55 °C. The best result for extraction of α-tocopherol was at 20 MPa and 55 °C.

Ouchikh et al. studied the tocopherols contents in *L. nobilis* vegetative organs, by microscale saponification and probe sonication extractions. They noted that the highest concentration of tocopherol homologues was found in laurel leaves (139.34 ± 8.8 mg/100 g fresh weight) for α-tocopherol, by microscale

saponification extraction, and a lower concentration (9.69 ± 2.4 mg/100 g fresh weight), by probe sonication extraction.

The largest isomer wasα-tocopherol, and its amount was extremely high in laurel leaves, whereas the other tocopherol isomers were not detected. Therefore, the results obtained by this work demonstrate that the Sc-CO_2 extraction was able to remove the α-tocopherol present in the laurel leaves.

The α-tocopherol is a fat-soluble antioxidant naturally present in foods of plant origin, especially in those with dark green coloration, as laurel leaves. This compound was identified in the laurel leaves by others authors [10,11,50–52].

The 1,8-cineole only was identified in Sc-CO_2 extracts at 20 MPa. The literature reported the extraction of 1,8-cineole by supercritical CO_2 at pressures of 80 to 110 bar and temperatures of 40 and 50 °C. It is likely that 1,8-cineole was removed during supercritical extraction, because essential oils, in general, are very soluble in compressed carbon dioxide and their solubility increases a result of the rapid rise of the density of the carbon dioxide [53,54]. Therefore, partial loss of 1,8-cineole may have occurred because of its solubility in supercritical CO_2, together with the fact that the use of higher conditions of temperature and pressure in supercritical extraction, the 1,8-cineole that has been drawn out of the extractor, came out with the supercritical CO_2.

The 1,8-cineole and α-tocopherol were obtained predominantly in the extracts for ambient pressure and high-pressure methods, so an evaluation of the 1,8-cineole concentration and the amount of α-tocopherol was performed separately.**Figure 3** shows the 1,8-cineoleconcentrationand amount of α-tocopherol of relative area percentagepresents in laurel leaves extracts from SOX, UAE, HD and Sc-CO_2 extractions.

The SOX and UAE extracts presented the 1,8-cineole concentration of 7.00 mg mL^{-1} and 8.30 mg mL^{-1}, respectively. Considering the amount of raw material (laurel leaves) used in the extraction, the yield the of 1,8-cineole concentration was approximately of 0.05 % (m/m), both for SOX and UAE extractions. Therefore, there is no difference between these methods when considering the 1,8-cineole concentration. However, UAE extract has greater amount of monoterpenes (61.0%) and α-tocopherol (8.0%) presenting superior characteristics compared to the SOX extract.

As it can be observed in **Figure 3**, thehigher 1,8-cineole concentration was obtained at HD extraction. The 1,8-cineole concentration of dried laurel leaves (62.70 $mgmL^{-1}$)was higher than fresh sample (54.00 $mgmL^{-1}$), confirming the importance of drying for a higher yield. These results correspond to a yield of 0.09% and 0.07% (m/m), respectively, considering the amount of raw material (laurel leaves) used in the extraction, reaffirming the low yield of the extraction method.

All extracts from Sc-CO_2presented the higher amount of α-tocopherol, in **Figure 3**,values between 30% and 51.5%, in the relative area, indicating the predominance of this component and highest selectivity of supercritical extraction. The results indicate that the amount of α-tocopherol from all Sc-CO_2 extracts is much higher than those found for UAE extracts. Therefore, the supercritical fluid extraction method can be a promising alternative for the removal of α-tocopherol from the laurel leaves.

We concluded that SOX extract is composed of several chemical compounds, not selective and poor in oxygenatedmonoterpenes. UAE extract has a significant amount of oxygenatedmonoterpenes, but without predominance of some chemical component characteristic of the laurel essential oil. HD extract is rich inoxygenated monoterpenes, mainly of the major compound of laurel leaves, 1,8-cineole. Sc-CO_2 extractions are rich in α-tocopherol and D-limonene, bioactive compounds associated with antioxidant and antibacterial activities of laurel essential oil. The GC/MS results show that the different extraction techniques interfered in the characteristics and quantities of the chemical compounds obtained from the laurel leaves.

3.4 Total phenolic compounds andantioxidant activity

Table 4 shows the determination of total phenols content (TPC) and total antioxidantactivity (DPPH method) of laurel leaves extracts. The TPC analysis was performed due to the presence of α-tocopherol in extractions, because the Folin–Ciocalteu reagent, a mixture of phosphotungstic ($H_3PW_{12}O_{40}$) and phosphomolybdic ($H_3PMo_{12}O_{40}$) acids, is reduced to blue oxides of tungstene (W_8O_{23}) and molybdene (Mo_8O_{23}) during phenoloxidation, indicating the total phenols content [12].

The EC_{50} values from DPPH assay represent the required concentration for an antioxidant to scavenge 50% of initial DPPH free radical concentration, using to estimate of antioxidant activity[11]. The TPC and EC_{50}results of the extracts were compared to the synthetic productbutylated hydroxytoluene(BHT), used as standard.

The UAE extract presented the best result for TPC (47 mg GAE (g extract)$^{-1}$), and the HD extract shows the best result for EC_{50} (38 ± 1 μg mL^{-1}), since they are the closest to the results obtained for BHT, synthetic antioxidant commonly known. The other extracts presented similar TPC results, with no significant difference ($p<0.05$) between ambient pressure and high-pressure techniques. The UAE and SOX extracts revealed highestantioxidant activity (lower values of EC_{50}) than the of the Sc-CO_2 extracts.

Although phenolic compounds are the main natural antioxidants, they are not the only class of substances that contribute to antioxidant performance of natural products [55], which explains the EC_{50} good result for the HD extract, since this extract does not possess α-tocopherol.

Studies have shown that the polarity of the solvent influences the extraction of phenolic compounds[56,57]. Ethanolic and water solvents reveled promising results in the extraction of phenolic compounds[10,13,58]. Our TPC result for the UAE extractwas lower than those found byMuñiz-Márquez et al.[13] and Skerget et al.[15], both extracted by UAE of the laurel leaves. Theydetermined of phenolic compounds of 17.32 ± 1.52 mg GAE g^{-1} of plant (35% ethanol solvent), and 99.7 g GAEkg^{-1} (pure methanol solvent), respectively.Probably this difference is due to the polarity of the solvent, we extracted with hexane, a non-polar solvent.

The TPC results for the Sc-CO_2 extracts did not present a significant difference ($p < 0.05$) to those found in HD and SOX extracts, independent of temperature and pressure conditions on Sc-CO_2. The TPC values of the Sc-CO_2varying from 21.5 ± 5 to 29.0 ± 4 mg GAE (g extract)$^{-1}$. These values are probably assigned to α-tocopherol, which is a phenolic compound.

The extraction of polar compounds, such as phenolics, in supercritical extraction is favored when a polar cosolvent is used, since these are less soluble in supercritical CO_2[59,60]. However, although we did not use a polar cosolvent, our results are similar to those found by Ouchikh et al. [10] (20.94 ± 0.97 mg GAEg^{-1}), in extract of laurel leaves obtained by microscale saponification extraction, with dilution of the sample in solvent methanol. These results strengthen the suggestion of α-tocopherol extraction by Sc-CO_2 to be a promising alternative.

The EC_{50} values of HD (35 ± 1 μg mL^{-1}), UAE (28 ± 1 μg mL^{-1}) and SOX (60 ± 4 μg mL^{-1}) extracts showed highest antioxidant activity, probably due to the synergy of chemical compounds, with emphasis on monoterpenes and α-tocopherol.Similar results to our work were found byPapageorgiou, Mallouchos and Komaitis[14],where the EC_{50} values varying from 52.50 to 85.40 mg L^{-1}, laurel leaves extracts of aqueous methanol (70:30 v/v) by sonication. Conforti et al. [12]reported higherantioxidant activity of wild *Laurusnobilis*is due to the greater abundance of monoterpenes, particularly eugenol and methyl eugenol,and vitamin E (tocopherol) with known antioxidant activity.

The EC_{50} values of the Sc-CO_2 extractions showed a lower level of antioxidant activity compared to de BHT (14 μg mL^{-1}), varying from 145.0 ± 8.0 μg mL^{-1} to 270.5 ± 6.0 μg mL^{-1}. Furthermore, a positive effect of increasing the pressure, at constant temperature, in the antioxidant activity was verified in the data from **Table 4**.

No previous research was found with results for the antioxidant activity (DPPH) of extractions by Sc-CO_2. However, according to Dias et al. [11], EC_{50} values varying from 90 to 200 μg mL^{-1} of laurel leaves obtained by methanolic extract infusion methods,similar results to those found for Sc-CO_2 extracts.

Extracts with greater variety of chemical compounds presented promising results for the total phenols content and antioxidant activity.

IV. CONCLUSION

The results demonstrated that the extraction method affects the yield, chemical composition and antioxidant activity of laurel essential oil. The SOX extract has highestyield, but is poor in oxygenated monoterpenes and does not have specificity of chemical groups. The UAE extract has a good yield, with significant presence of oxygenated monoterpenes and good results for the total phenols content and antioxidant activity. The HD extract has low yield, however, is rich in oxygenated monoterpenes and compounds with antioxidant characteristics.Sc-CO_2 extracts have similar or highest yield than the HD extract and are rich in α-tocopherol and highly selective.

These findings also suggest that leaves of *Laurus nobilis*L. can represent a valuable source of divers bioactive compounds, both for biomedical, cosmetic or pharmaceutical applications, as well as to be used as food ingredients.

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FIGURE CAPTION

Figure 1. Kinetic assay of laurel leaves at 15 MPa, 45 °C and CO_2flow8 ± 2 g min^{-1}.

Figure 2. Curves isotherms for the global yield of laurel leaves extracts obtained by Sc-CO_2.

Figure 3. 1,8-Cineole concentration and α-tocopherol amount from laurel leaves of extracts by different extraction techniques (Hydrodistillation - HD, Soxhlet - SOX, Ultrasound-assisted extraction - UAE, Supercritical Fluid Extraction - Sc-CO_2).

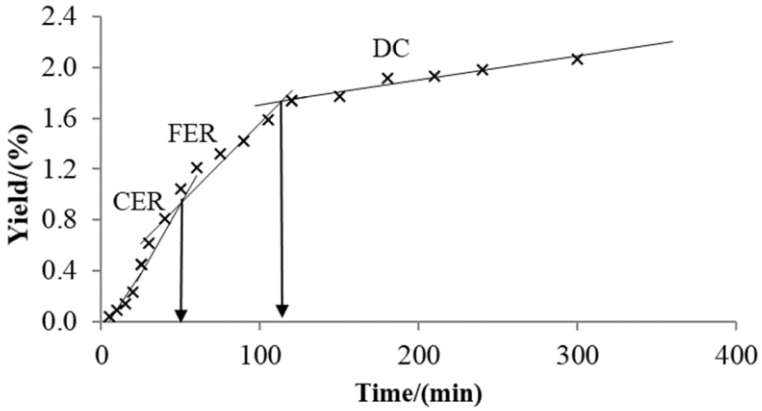

Figure 1. Kinetic assay of laurel leaves at 15 MPa, 45 °C and CO_2flow8 ± 2 g min^{-1}.

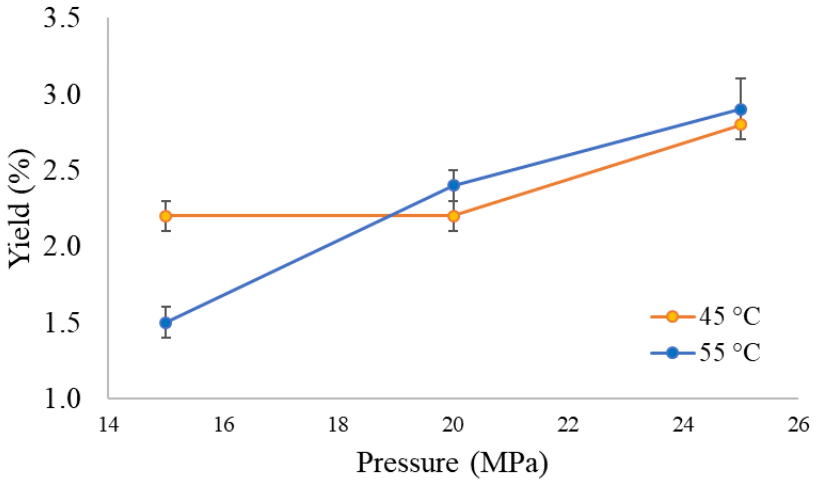

Figure 2. Curves isotherms for the global yield of laurel leaves extracts obtained by Sc-CO_2.

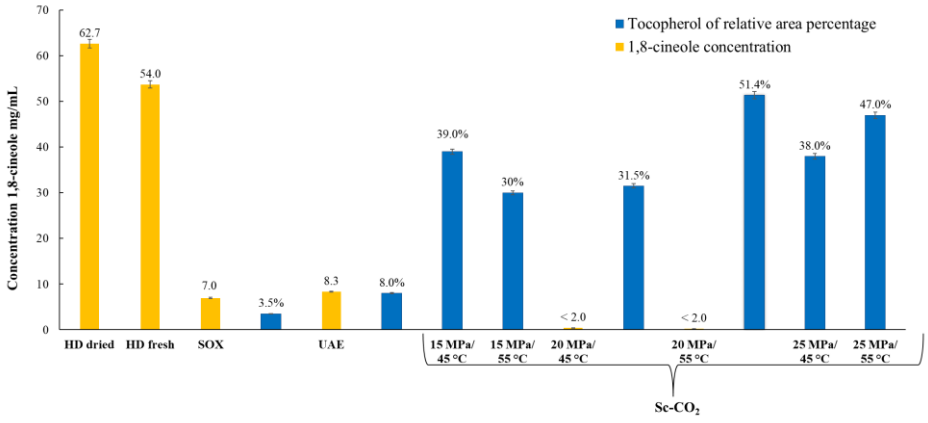

Figure 3. 1,8-Cineole concentration and α-tocopherol amountfrom laurel leaves of extractsby different extraction techniques (Hydrodistillation - HD, Soxhlet - SOX, Ultrasound-assisted extraction - UAE, Supercritical Fluid Extraction - Sc-CO_2).

TABLE CAPTION

Table 1. Extraction methods, yield and chemical composition of laurel leaves essential oil in literature.
Table 2. Extraction yield (X_0) of laurel leaves extracts obtained by different extraction techniques (Hydrodistillation - HD, Soxhlet - SOX, Ultrasound-assisted extraction - UAE, Supercritical Fluid Extraction - Sc-CO_2).
Table 3: Chemical profile (GC-MS) of laurel leaves extracts obtained by different extraction techniques (Hydrodistillation - HD, Soxhlet - SOX, Ultrasound-assisted extraction - UAE, Supercritical Fluid Extraction - Sc-CO_2).
Table 4: Total phenolic compounds (TPC) and antioxidant activity (EC_{50}) of laurel leaves extracts.

Table 1. Extraction methods, yield and chemical composition of laurel leaves essential oil in literature.

Extractionmethods	**Yield**	**Chemical compounds (GC/MS)**	**Authors**
Hydrodistillation	2.6 %	**1,8-cineole (49.7 %)** limonene (0.8 %) methyleugenol (0.4%) carvone (0.3 %) eugenol (0.3 %) linalool (0.1 %) trans-methylisoeugenol (0.1 %)	Ozek; Bozan; Baser (1998)
Steamer	1.9 %	**1,8-cineole (54.2 %)** limonene (0.8 %) methyleugenol (0.3%) eugenol (0.3 %) linalool (0.2 %) trans-methylisoeugenol (0.1 %)	
Supercritical: 80 barand 40 °C	1.34 %	**1,8-cineole (43.0 %)** limonene (0.9 %) methyleugenol (0.7%) eugenol (0.8 %) linalool (0.2 %)	
Supercrítica 100 bar/50 °C	1.13 %	**1,8-cineole (40.2 %)** limonene (0.5 %) methyleugenol (0.8%) eugenol (0.7 %) linalool (0.1 %)	
Hydrodistillation	0.9 %	**1,8-cineole (22.84%)** limonene(1.23 %) linalool (10.57 %) eugenol (1.83 %) methyleugenol (9.42 %) α-carene (0.67 %)	Caredda et al. (2002)
Supercritical: 1ª. Stage: 90 bar and 50 °C 2ª. Stage: 150 bar and 10 °C (separador)	0.82 %	**1,8-cineole (23.51%)** limonene(1.18 %) linalool (12.46 %) eugenol (2.6 %) methyleugenol (8.09 %) α-carene (0.67 %)	
Supercritical: 110 bar and 40 °C	0.6 %	**1,8-cineole (24.84 %)** linalool (14.46 %) terpineolacetate (12.36 %) methyleugenol (10.09 %) eugenol (5.60 %)	De Corato et al. (2010)
Hydrodistillation	1.43 %	**1,8-cineole (33.4 %)** linalool (16.0 %) α-terpinylacetate (13.8 %) sabinene (6.91 %) methyleugenol (5.32 %) eugenol (1.77 %) limonene-β-phellandrene (1.59 %) carene (0.24 %)	Ivanovic et al. (2010)
Supercritical: 100 bar and 40 °C	1.37 %	methyl linoleate (16.18%) α-terpinyl acetate (12.88%) linalool (9.0%) methylene eugenol (8.67%) methyl arachidonate (6.28%) eugenol (6.14%); **1,8-cineole (2.53%)**	

Table 2. Extraction yield (X_0) of laurel leaves extracts obtained by different extraction techniques (Hydrodistillation - HD, Soxhlet - SOX, Ultrasound-assisted extraction - UAE, Supercritical Fluid Extraction - Sc-CO_2).

Extractiontechniques	**ρ CO_2[1] (gcm^{-3})**	**Temperature (°C)**	**Pressure (MPa)**	**(Xo) (%)**
HD (dried)		100	ambient	1.5 ± 0.1^{d}
HD (fresh)		100	ambient	0.2 ± 0.1^{e}
SOX-hexane		60	ambient	7.5 ± 0.2^{a}
UAE-hexane		ambient	ambient	6.0 ± 0.2^{b}
Sc-CO_2	0.7477	45	15	2.2 ± 0.1^{cd}
	0.6547	55	15	1.5 ± 0.1^{d}
	0.8169	45	20	2.2 ± 0.1^{cd}
	0.7553	55	20	2.4 ± 0.1^{c}
	0.8592	45	25	2.8 ± 0.1^{c}
	0.8114	55	25	2.9 ± 0.2^{c}

[1]Angus; Armstrong; De Reuck, 1976; Different superscript letters mean groups statistically different ($p < 0.05$) in each column.

Table 3: Chemical profile (GC-MS) of laurel leaves extracts obtained by different extraction techniques (Hydrodistillation - HD, Soxhlet - SOX, Ultrasound-assisted extraction - UAE, Supercritical Fluid Extraction - Sc-CO_2).

Chemicalcompounds	**RT[1] (min)**	**Relative area (%)[2] of extracts**									
		HD dried	**HD fresh**	**SOX hx**	**UAE hx**	**Sc-$CO_2$15 MPa/45° C**	**Sc-$CO_2$15 MPa/55° C**	**Sc-$CO_2$20 MPa/45° C**	**Sc-$CO_2$20 MPa/55° C**	**Sc-$CO_2$25 MPa/45° C**	**Sc-$CO_2$25 MPa/55° C**
Oxygenated monoterpenes											
1,8-Cineole (eucalyptol)	**8.05**	**41.4**	**18.0**	**4.50**	**5.3**			**11.9**	**2.9**		
3-Carene	10.46	18.4	15.8	7.1	7.6						
D-Limonene	20.67	21.6	32.4	9.1	9.7	4.5		4.4	4.5	6.6	7.4
Eugenol	20.98		1.0		2.7						
Methyleugenol	22.98	4.5	13.1		1.4	1.3				2.6	
Acetyleugenol	27.88				34.1						
Otherschemicalcompounds											
3,5,5-Trimethylhexyl acetate	3.09					4.5					
2,5,5-Trimethyl-2-Hexene	3.25			10.2	6.2	19.7	5.4	9.1	8.3	11.4	10.3
3,5,5-Trimethyl-2-Hexene	3.36		0.1		1.7	5.6	1.5	2.6	2.2	3.2	2.6
α-Pinene	5.32	2.70	0.6								
Pyrrolidine	5.54			2.0	1.3	4.3		1.9		2.4	2.2
1-Isopropyl-4-methylbicyclo[3.1.0]hex-2-ene	6.32	8.3						4.1			
β-Pinene-(1S)-(-)	6.42	3.1	1.1					1.1			
Dodecamethylpentasiloxane	27.04			5.1							
3-Amino-2-phenazinol	33.44			5.2	2.1		1.6				
1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane	39.06			4.2			2.7				
3-Tetradecyn-1-ol	50.51			1.8	1.9	13.2		11.4		14.8	16.4
2,4-Undecadien-1-ol	50.53						15.9		25.9		
3-methyl-4-methylidenebicyclo[3.2.1]oct-2-ene	52.30							7.3			
10.12-Octadecadiynoic acid	52.31						10.4				
N-[2,6-dimethyl-4-[(trimethylsilyl)oxy]phenyl]-1,1,1-trimethyl silanamine	60.48			4.9	2.2						
Hexadecamethylheptasiloxane	63.95			8.2	2.3		8.5				

α-Tocopherol	**79.60**			**3.5**	**8.0**	**39.0**	**30.0**	**31.5**	**51.50**	**38.0**	**47.0**
Total (%)	-	100.0	82.1	65.8	86.5	92.1	76.0	85.3	95.2	79.0	85.9
Others (%)	-	-	17.9	34.2	13.5	7.9	24.0	14.7	4.8	21.0	14.1

[1]Retention time; [2]Peak area relative to internal standard peak area in total chromatogram (GC–MS).

Table 4: Total phenolic compounds (TPC) and antioxidant activity (EC_{50}) of laurel leaves extracts.

Extractiontechniques		**TPC[(1)] (mg GAE (g extract)$^{-1}$)**	**EC_{50}[(2)] (μgmL^{-1})**
$HD_{(dried)}$		22 ± 1^{c}	35 ± 1^{g}
SOX-hexane		28 ± 3^{c}	60 ± 4^{f}
UAE-hexane		47 ± 0^{b}	28 ± 1^{h}
$Sc\text{-}CO_2$	15 MPa/45 °C	25 ± 0^{c}	258 ± 2^{ab}
	15 MPa/55 °C	21 ± 5^{c}	270 ± 6^{a}
	20 MPa/45 °C	28 ± 2^{c}	145 ± 8^{e}
	20 MPa/55 °C	24 ± 9^{c}	226 ± 3^{bc}
	25 MPa/45 °C	28 ± 1^{c}	179 ± 3^{de}
	25 MPa/55 °C	29 ± 4^{c}	196 ± 17^{cd}
BHT[(3)]		69 ± 1^{a}	14 ± 1^{i}

[(1)]TPC: Total phenolic compounds; [(2)]EC_{50}: effective concentration; [(3)]BHT:butylhydroxytoluene.Different superscript letters mean groups statistically different ($p < 0.05$) in each column.

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