HAL
open science

Comparison of different methods for extraction from Tetraclinis articulata: Yield, chemical composition and antioxidant activity

Nejia Herzi, Jalloul Bouajila, Séverine Camy, Mehrez Romdhane, Jean-Stéphane Condoret

To cite this version:

Nejia Herzi, Jalloul Bouajila, Séverine Camy, Mehrez Romdhane, Jean-Stéphane Condoret. Comparison of different methods for extraction from Tetraclinis articulata: Yield, chemical composition and antioxidant activity. Food Chemistry, 2013, vol. 141, pp. 3537-3545. 10.1016/j.foodchem.2013.06.065 . hal-00875913

HAL Id: hal-00875913

https://hal.science/hal-00875913v1

Submitted on 23 Oct 2013

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Open Archive TOULOUSE Archive Ouverte (OATAO)
OATAO is an open access repository that collects the work of Toulouse researchers and makes it freely available over the web where possible.

This is an author-deposited version published in : http://oatao.univ-toulouse.fr/
Eprints ID : 9749

To link to this article : DOI: 10.1016/j.foodchem.2013.06.065
URL : http://dx.doi.org/10.1016/j.foodchem.2013.06.065

To cite this version : Herzi, Nejia and Bouajila, Jalloul and Camy, Séverine and Romdhane, Mehrez and Condoret, Jean-Stéphane Comparison of different methods for extraction from Tetraclinis articulata: Yield, chemical composition and antioxidant activity. (2013) Food Chemistry, vol. 141 (n° 4). pp. 3537-3545. ISSN 0308-8146

Any correspondance concerning this service should be sent to the repository administrator: staff-oatao@listes-diff.inp-toulouse.fr

Analytical Methods

Comparison of different methods for extraction from *Tetraclinis articulata*: Yield, chemical composition and antioxidant activity

Nejia Herzi [a,b], Jalloul Bouajila [c,*], Séverine Camy [b], Mehrez Romdhane [a], Jean-Stéphane Condoret [b,*]

[a] *Unité de recherche MACS, ENIG, Route de Médenine, 6029 Gabès, Tunisia*
[b] *Université de Toulouse, INPT, UPS, Laboratoire de Génie Chimique, UMR CNRS 5503, 4 Allée Emile Monso, F-31030 Toulouse, France*
[c] *Université de Toulouse, Laboratoire des Interactions Moléculaires et Réactivité Chimique et Photochimique, UMR CNRS 5623, Université Paul-Sabatier, 118 Route de Narbonne, F-31062 Toulouse, France*

Keywords:
Tetraclinis articulata essential oil
Soxhlet extraction
Hydrodistillation
Supercritical carbon dioxide
Antioxidant activity

A B S T R A C T

In the present study, three techniques of extraction: hydrodistillation (HD), solvent extraction (conventional 'Soxhlet' technique) and an innovative technique, *i.e.*, the supercritical fluid extraction (SFE), were applied to ground *Tetraclinis articulata* leaves and compared for extraction duration, extraction yield, and chemical composition of the extracts as well as their antioxidant activities. The extracts were analyzed by GC–FID and GC–MS. The antioxidant activity was measured using two methods: $ABTS^{\bullet+}$ and $DPPH^{\bullet}$. The yield obtained using HD, SFE, hexane and ethanol Soxhlet extractions were found to be 0.6, 1.6, 40.4 and 21.2–27.4 g/kg respectively. An original result of this study is that the best antioxidant activity was obtained with an SFE extract (41 mg/L). The SFE method offers some noteworthy advantages over traditional alternatives, such as shorter extraction times, low environmental impact, and a clean, non-thermally-degraded final product. Also, a good correlation between the phenolic contents and the antioxidant activity was observed with extracts obtained by SFE at 9 MPa.

1. Introduction

With the incentive of following Green Chemistry principles and increasing interest of consumers in functional foods, research into more effective and cleaner extraction of natural products using 'natural' processes is on-going, especially in relation to aromatic and medicinal plants (AMP), the essential oils (EOs) of which have been reported to offer other biological activities. The need for these other biological activities from natural products has been reinforced recently by the emergence of many micro-organisms that have proved to be resistant to conventional medicines. Globally, EOs can be and have been employed in the pharmaceutical, food, cosmetic and perfume industries and also to control post-harvest diseases (Rashid, Rather, Shah, & Bhat, 2013; Sacchetti et al., 2005; Sanchez-Gonzalez, Vargas, Gonzalez-Martınez, Chiralt, & Chafer, 2011). Approximately 3000 EOs are known, 300 of them having commercial importance (Khajeh, Yamini, & Shariati, 2010; Shee, Raja, Sethi, Kunhambu, & Arunachalam, 2010). However, many plants still remain unstudied. For instance, very few works (Barrero et al., 2003; Ben Hadj Ahmed et al., 2011) have been devoted to *Tetraclinis articulata.*

This plant belongs to the family Cupressaceae and is native plant of the South-Western Mediterranean, mainly North Africa. It is an important element of the Maghreb vegetation (Morocco, Algeria and Tunisia).

T. articulata is a high quality health food, which is commonly used in Tunisian traditional medicine, because of identified pharmacological effects, including antioxidant activity that may explain the benefits in treating circulatory disorders perceived from the use of the herb over the centuries (Bourkhiss, Hnach, Paolini, Costa, Farah & Satrani, 2010). So, the macerated leaves were used by oral administration such as the herbal teas. Moreover, *T. articulata* was used as anti-diabetic plants which allow reducing the use of traditional medicines used in the treatment of diabetes (Patel, Kumar, Laloo, & Hemalatha, 2012).

The EO of such plants is traditionally extracted by hydrodistillation (HD). However, this technique presents many disadvantages such as long extraction times, which result in the degradation of unsaturated compounds (due to the high temperature, which is around 100 °C), low yield and losses of volatile compounds (Costa et al., 2012; Schaneberg & Khan, 2002; Yamini, Khajeh, Ghasemi, Mirza, & Javidnia, 2008).

Alternative methods have been proposed for extracting natural products, and more particularly volatile fractions, from plants by using supercritical fluid extraction (SFE) and extensive reviews

* Corresponding author. Address: Université de Toulouse, INPT, UPS, Laboratoire de Génie Chimique, 4 Allée Emile Monso, F-31030 Toulouse, France. Tel.: +33 5 34323713 (J.-S. Condoret). Address: Université de Toulouse, IMRCP UMR 5623. Tel.: +33 562256885 (J. Bouajila).

E-mail addresses: jalloul.bouajila@univ-tlse3.fr (J. Bouajila), jeanstephane.condoret@ensiacet.fr (J.-S. Condoret).

http://dx.doi.org/10.1016/j.foodchem.2013.06.065

and articles exist on the topic (Costa et al., 2012; Grosso, Ferraro, Figueiredo, Barroso, & Palavra, 2008; Herrero, Mendiola, Cifuentes, & Ibanez, 2010; Khajeh, Yamini, Sefidkon, & Bahramifar, 2004; Reverchon, 1997). SFE is based on the use of supercritical carbon dioxide as the solvent of extraction. SFE of volatile fractions is operated at moderate pressure (9 MPa) and relatively low temperatures (40–60 °C). An important characteristic of this technique is the potential to modify the solvent, and therefore selectivity of the process, by changing solvent density using pressure and temperature variation (Reverchon, 1997; Vergas, Mendes, Azevedo, Pessoa, & Uller, 2010). A crucial advantage of this technique over HD or Soxhlet extraction is that it involves a relatively short exposure time to a solvent, which is at low temperature. In addition, high yields, lower energy requirements and high purity of the extract are obtained (Guan, Li, Yan, Tang & Quan, 2007; Khajeh, Yamini, Sefidkon, & Bahramifar, 2004).

The use of carbon dioxide as the supercritical fluid is explained mainly by the properties of CO_2, which is a safe, non-combustible, inexpensive, odorless, colorless, tasteless, and readily available solvent. Its low viscosity enables it to penetrate the matrix to reach the material to be extracted, and its low latent heat of vaporization and high volatility mean that it can be removed easily without leaving solvent residue. By varying the temperature and pressure of the CO_2 during extraction, different components can be selectively extracted (Morales, Berry, McIntyre, & Aparicio, 1998; Reverchon, Ambruosi, & Senatore, 1994). Finally, the prominent advantage of CO_2 for the processing of natural products undoubtedly lies in its non-toxicity, which makes it acceptable for the industry and ensures 'clean' characteristics of the extracted products.

In the present study, three techniques of extraction HD, solvent extraction and SFE were compared. This work provides a brief overview on the principal application of clean processing for isolating natural products from *T. articulata* although special attention is given to the extraction of antioxidant compounds because of their important role in food preservation and health promotion. Note that it is well known that antioxidant activity detected by *in vitro* experiments may be non-effective *in vivo*. This aspect was not considered here and would need further investigation.

The main objective of this study was to compare these methods by identifying and quantifying the chemical composition of each extract and also by determining their antioxidant activity.

2. Materials and methods

2.1. Plant material

T. articulata is a traditional medicinal plant used in Tunisia, Morocco and Algeria. Leaves from cultivated plants of *T. articulata* were collected by hand during October 2010 (morning) from the same location Korbos, located in the region of Cap Bon at 60 km from Tunis (latitudes 36.82/36° 49′ 44″ and longitudes 10.59/10° 35′ 36″), Tunisia. Harvested material was dried in the air, protected against direct sunlight, until a constant weight was achieved. The average particle size of *T. articulata* obtained after sieving of grinding leaves (using laboratory knife grinder) was 0.8 mm. The moisture content of the air-dried plant material, determined by Karl Fischer volumetric titration, was 9% w/w.

2.2. Chemicals

All chemicals were of analytical reagent grade. All reagents were purchased from Sigma-Aldrich-Fluka (Saint-Quentin, France). Commercial carbon dioxide (99.99% purity) was purchased from Air Liquide (Bordeaux, France) for the extraction of volatile components by SFE extraction.

2.3. Extraction methods

2.3.1. Hydrodistillation

HD was operated in a conventional Clevenger-type apparatus. This set-up was composed of a 2-L boiler, a condenser and a measuring tube with a stopcock. A return tube for the aqueous part of the distillate allowed the cohobation technique to be used. Dried leaves of *T. articulata* (100 g) were subjected to HD with 1 L of water for 180 min. The volatile distillate was collected over anhydrous sodium sulfate and refrigerated at 4 °C prior to analysis.

2.3.2. Supercritical fluid extraction

A SFE200 extraction pilot unit with three cyclonic separators from SEPAREX (France) was used. The 115 mL stainless steel extraction tank was loaded with 50 g of dried *T. articulata* leaves. The system was operated at a constant temperature of 40 °C and a pressure of 9 MPa for recovery of the volatile fraction. For all experiments, conditions in the recovery section were set at 5 MPa/30 °C in the first separator, 5 MPa/25 °C in the second one and 0.1 MPa/20 °C in the last one.

2.3.3. Soxhlet extraction

Ground *T. articulata* leaves (100 g) were extracted with 400 mL of ethanol or hexane (99% purity) during 6–8 h in a conventional Soxhlet apparatus (500 mL boiler). At the end of extraction, the liquid extract was filtered and evaporated to complete dryness in a vacuum at 35 °C using a rota-vapor apparatus. Finally, the dried extract was stored at 4 °C for further studies. All experiments were performed in triplicate. These two solvents were chosen because of their opposite polarities allowing extracting different kinds of compounds (non polar to polar).

2.4. Chemical components analysis: GC–FID and GC–MS

Quantitative and qualitative analysis of the extracts were carried out by gas chromatography–flame ionization detection (GC–FID) and gas chromatography–mass spectrometry (GC–MS). Gas chromatography analyses were performed on a Varian Star 3400 Cx chromatograph (Les Ullis, France) fitted with a DB-5MS fused silica capillary column (5% phenylmethylpolysyloxane, 30 m × 0.25 mm, film thickness 0.25 μm). Chromatographic conditions were a temperature rise from 60 °C to 260 °C with a gradient of 5 °C/min and then 15 min at 260 °C. A second gradient of 40 °C/min was applied to reach 340 °C. Total analysis time was 57 min. For analysis purposes, each extract was dissolved in petroleum ether. Samples (1 μL) were injected in the split mode at a ratio of 1:10. Helium (purity 99.999%) was used as the carrier gas at 1 mL/min. The injector was operated at 200 °C. The mass spectrometer (Varian Saturn GC–MS–MS 4D) was adjusted for an emission current of 10 μA and electron multiplier voltage between 1400 and 1500 V. The temperature of the trap was 150 °C and that of the transfer line was 170 °C. Mass scanning was from 40 to 650 atomic mass units.

Compounds were identified by comparison of their Retention indices (RI), obtained on a nonpolar DB-5MS column relative to C5–C24 n-alkanes, with those provided in the literature, by comparison of their mass spectra with those recorded in NIST 08 (National Institute of Standards and Technology) and reported in published articles, and by co-injection of available reference compounds. The samples were analyzed in duplicate. The percentage composition of the extract was calculated by the normalization method from the GC peak areas, assuming identical mass response factors for all compounds. Results were calculated as mean values after two injections of extract, without using correction factors. The identification is only made for the volatile compounds.

Especially, for Soxhlet and SFE extracts, some non volatile compounds cannot be identified.

2.5. Determination of total phenolic content

The phenolic content of each extract was determined by the Folin–Ciocalteu method (Singleton, Orthofer, & Lamuela-Raventós, 1999). A diluted solution of each extract (0.5 mL) was mixed with Folin–Ciocalteu reagent (0.2 mol/L, 2.5 mL). This mixture was kept at room temperature for 5 min and then sodium carbonate solution (75 g/L in water, 2 mL) was added. After 1 h of incubation, the absorbance was measured at 765 nm against water blank. A standard calibration curve was plotted using gallic acid (0–300 mg/L). The results were expressed as mg of Gallic Acid Equivalent (GAE)/kg of dry plant material.

2.6. Antioxidant activity

Two analytical methods was studied: $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ (Thaipong, Boonprakob, Crosby, Zevallos, & Byrne, 2006) described below.

2.6.1. $DPPH^{\cdot}$ free radical scavenging activity

Antioxidant scavenging activity was determined using the 1,1-diphenyl-2-picrylhydrazyl free radical ($DPPH^{\bullet}$) as described by Blois (1958) with some modifications; various dilutions of the test materials (ascorbic acid or extracts, 1.5 mL) were mixed with a 0.2 mM methanolic $DPPH^{\bullet}$ solution (1.5 mL). After an incubation period of 30 min at 25 °C, the absorbance at 520 nm was recorded as $A_{(sample)}$. A control experiment was also carried out by applying the same procedure to a solution without the test material and the absorbance recorded ($A_{(blank)}$). The free radical scavenging activity of each solution was then calculated as percentage inhibition according to the following Eq.:

$$\% \quad \text{inhibition} = 100 \times [(A_{(blank)} - A_{(sample))}/A_{(blank)}]$$

Extract antioxidant activity was expressed as IC_{50}, defined as the concentration of the test material required to cause a 50% decrease in initial $DPPH^{\bullet}$ concentration. Values were estimated using linear regression. Ascorbic acid was used as a reference.

2.6.2. $ABTS^{\cdot+}$ radical-scavenging

The radical scavenging capacity of the samples for the $ABTS^{\bullet+}$ (2,2′-azinobis-3-ethylbenzo-thiazoline-6-sulfonate) was determined as described by Re, Pellegrini, Proteggente, Pannala, Yang and Rice-Evans (1999). $ABTS^{\bullet+}$ was generated by mixing a 7 mM solution of $ABTS^{\bullet+}$ at pH 7.4 (5 mM NaH_2PO_4, 5 mM Na_2HPO_4 and 154 mMNaCl) with 2.5 mM of potassium persulfate (final concentration) and stored in the dark at room temperature for 16 h before use. The mixture was diluted with water to give an absorbance of 0.70 ± 0.02 units at 734 nm using spectrophotometry. For each sample, a diluted methanol solution of the sample (100 μL) was allowed to react with fresh $ABTS^{\bullet+}$ solution (900 μL), and the absorbance was measured 6 min after initial mixing. Ascorbic acid was used as a reference and the free radical scavenging capacity was expressed by IC_{50} (mg/L) values, which denote the concentration required to scavenge 50% of $ABTS^{\bullet+}$. The free radical scavenging capacity IC_{50} was determined using the same equation as used previously for the $DPPH^{\bullet}$ method.

2.7. Statistical analysis

All data were expressed as means ± standard deviations of triplicate measurements. The confidence limits were set at $P < 0.05$. Correlations were sought using the correlation and regression functions available in Excel software (Microsoft® Office). Standard deviations (SD) did not exceed 5% for the majority of values obtained. Data analysis procedure (ANOVA) was performed in order to assess the data.

3. Results and discussion

3.1. Operation of the different techniques of extraction

In order to estimate the potential for extracting different natural substances from *T. articulata* leaves, HD, Soxhlet extraction and SFE (at 9 MPa and 40 °C) were performed. For each extraction method, the global yield was defined as Y=g of oil extracted/kg of plant material loaded into the extractor. The yield was measured for each run and averaged for triplicate experiments.

Extraction methods were firstly compared in terms of extraction yields and, in particular, the influence of the main operating parameters. The best operating conditions, in terms of obtained global yield, were defined for each of the three extraction processes. In a second part, the chemical composition and antioxidant activity of the extracts were compared.

The brownish-yellow SFE extracts and dark green Soxhlet extracts were semi-solid under ambient conditions (20 °C and 0.1 MPa) while the oil obtained by HD was a transparent to pale yellow liquid with a specific odor, different from that of the untreated plant material. Conversely, the SFE extracts had an aroma very similar to that of the untreated plant material.

3.1.1. Hydrodistillation

The choice of the best operating conditions was done by varying the extraction time (0–300 min), the ratio of the mass of water to the mass of plant material (R = 8, 10, 12 and 14), and the condensation flow-rate at the condenser (0.01, 0.02, 0.03, and 0.04 mL/s). The condensation flow-rate (mL/s) is directly related to the vapor flux leaving the boiler and therefore related to the heating power at the boiler, which was set to a given value manually. Best choices of operating conditions were investigated to obtain the highest yield of EO.

Fig. 1 presents the time variation of the global extraction yield (Y) obtained by HD. The kinetics of extraction presented in Fig. 1-a exhibits a very conventional shape, indicating a first step of rapid extraction and then a slow-down due to the decrease of the solute concentration in the solid. From this curve, it can be seen that extraction is mostly complete after 180 min. Fig. 1-b and -c show the influence of the water/plant-material ratio and the influence of the condensation flow-rate for extraction duration of 180 min. Both curves present an optimum. Fig. 1-b indicates that a minimum volume of water (R = 10) is necessary to obtain an efficient solid–liquid boiling system (or a solute unsaturated liquid phase) and also that too much water (R > 12) is detrimental for the yield, probably because it induces loss of water-soluble compounds in the overlarge volume of water. This negative influence of excessive values of the water/plant-material ratio was also observed by Sovová and Aleksovski (2006), and this was explained by their modeling approach.

In Fig. 1-c, it can be seen that a low vapor flux (*i.e.*, condensation flow-rate <0.02 mL/s) gave a low extraction yield because it slowed the kinetics. When there is no internal mass transfer limitation, the recovery rate in the separator is directly proportional to the vapor flow. So, more time is necessary to obtain the same yield at low vapor fluxes, and the extraction was probably not complete after 180 min. A further increase in the vapor flow rate would ultimately yield a plateau. Nevertheless, in our case, increased vapor flow (0.045 mL/s) lowered the global yield. A probable explanation is

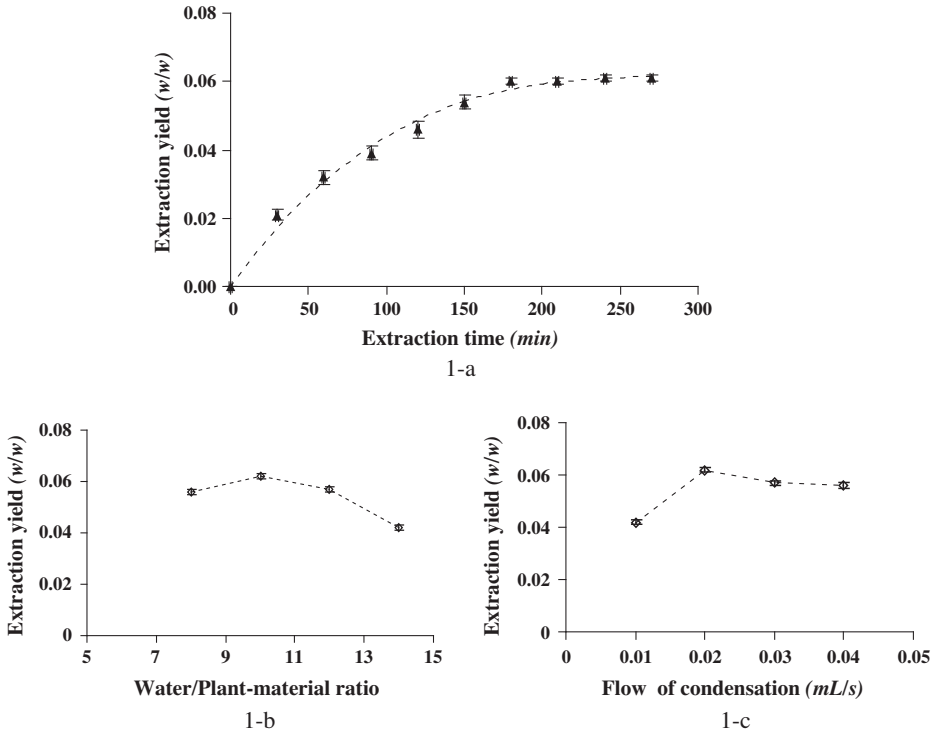

Fig. 1. Extraction yield of hydrodistillation for 100 g of raw material. (a) Kinetics of extraction with a condensation flow-rate of 0.02 mL/s and a Water/Plant-material ratio of 10, (b) Influence of the Water/Plant-material ratio for an extraction time of 3 h and a condensation flow-rate of 0.02 mL/s, and (c) Influence of the condensation flow-rate for an extraction time of 3 h and a Water/Plant-material ratio of 10.

that the condenser capacity was too small for this volume of steam, which led to loss of oil in the non-condensed vapor phase at the top of the condenser.

Finally, the best choice of operating extraction conditions for the *T. articulata* HD process were found to be 3 h duration, 0.02 mL/s condensation flow-rate and a water/plant-material ratio *R* equal to 10.

3.1.2. Supercritical CO_2 extraction (SFE)

For the SFE process, the extraction pressure and temperature, CO_2 flow-rate, particle size and extraction duration are generally considered as the most important factors that influence extraction efficiency. The influence of each parameters:temperature (at three levels of 40, 50 and 60 °C), pressure (using four levels of 9, 15, 28 and 100 MPa), flow rate of CO_2 (five flow rate 5, 10, 15, 20 and 30 g/min) and particle size (four values of $0.5 < dp < 1.2$; $1.2 < dp < 1.8$; $1.8 < dp < 2.5$; and $dp > 2.5$ mm) on the global extraction yield is shown in Fig. 2.

Fig. 2-a presents an example of kinetics of the extraction process. The shape of the curve suggests that extraction is mainly limited by the solubility of oil in CO_2 or by solid–fluid equilibrium. As expected, on Fig. 2-b, an increase in extraction pressure had a strong positive influence on the global yield of extraction. Pressure has a direct effect on the density of CO_2, increasing its solvent power. However, high extraction pressure favors the extraction of heavy compounds such as lipids or waxes. As volatile fractions of plant material are soluble in supercritical CO_2 at 9 MPa, selectivity for these compounds was not favored by high extraction pressure. Such conditions are mentioned several times in the literature and were confirmed by a preliminary study of the plant material (Gomes, Mata, & Rodrigues, 2007; Sovova, Komers, Kucera, & Jez, 1994).

Fig. 2-c shows that an increase in temperature, inducing a decrease in CO_2 density, leads to a decrease in the global extraction yield. This may be related to the retrograde solubility effect described for example by da Francisco and Sivik (2002) where increased vapor pressure is not sufficient to balance the loss of solvent power due to the decreased CO_2 density when temperature is increased.

Fig. 2-d indicates that, for 60 min of extraction, a CO_2 flow rate of 20 g/min is necessary to achieve complete extraction. The initial quasi-linear influence of the CO_2 flow-rate confirms the hypothesis that this SFE process is solubility or solid–fluid equilibrium limited. The plateau obtained for high flow-rates corresponds to complete extraction. From these results, the optimal conditions for *T. articulata* extraction were found to be 9 MPa, 40 °C and 20 g/min of CO_2 for 60 min. Extraction was apparently complete at 30 min but an additional 30 min was included to propose a complete kinetic study and to ensure complete extraction. Indeed, SFE requires actually shorter extraction times (30 min) compared to HD (3 h) and Soxhlet extraction (6 h).

The influence of particle size was also studied and is shown on Fig. 2-e, where optimal yield was obtained for $1.2 < dp < 1.8$ and $1.8 < dp < 2.5$ mm-diameter particles. Smaller particles gave lower yields. This was most probably related to a loss of solutes during the grinding step, as previously evidenced in a study by Khajeh, Yamini, Bahramifar, Sefidkon, and Pirmoradei (2005). During grinding, compounds including oils may be degraded either by local overheating or by being left on the grinder blades. A decrease in yield was also observed with larger particles (>0.5 mm), probably as a result of internal diffusional limitations inducing lower extraction kinetics and, therefore, lower yields over a given duration.

3.1.3. Soxhlet extraction

Hexane and ethanol, which have very different polarity properties, were tested as solvents for the Soxhlet extraction of *T. articulata*. Hexane extraction yield was found to be the highest (40 g/kg) against 26.8 g/kg for ethanol (see Table 1). In this study, a Solvent/

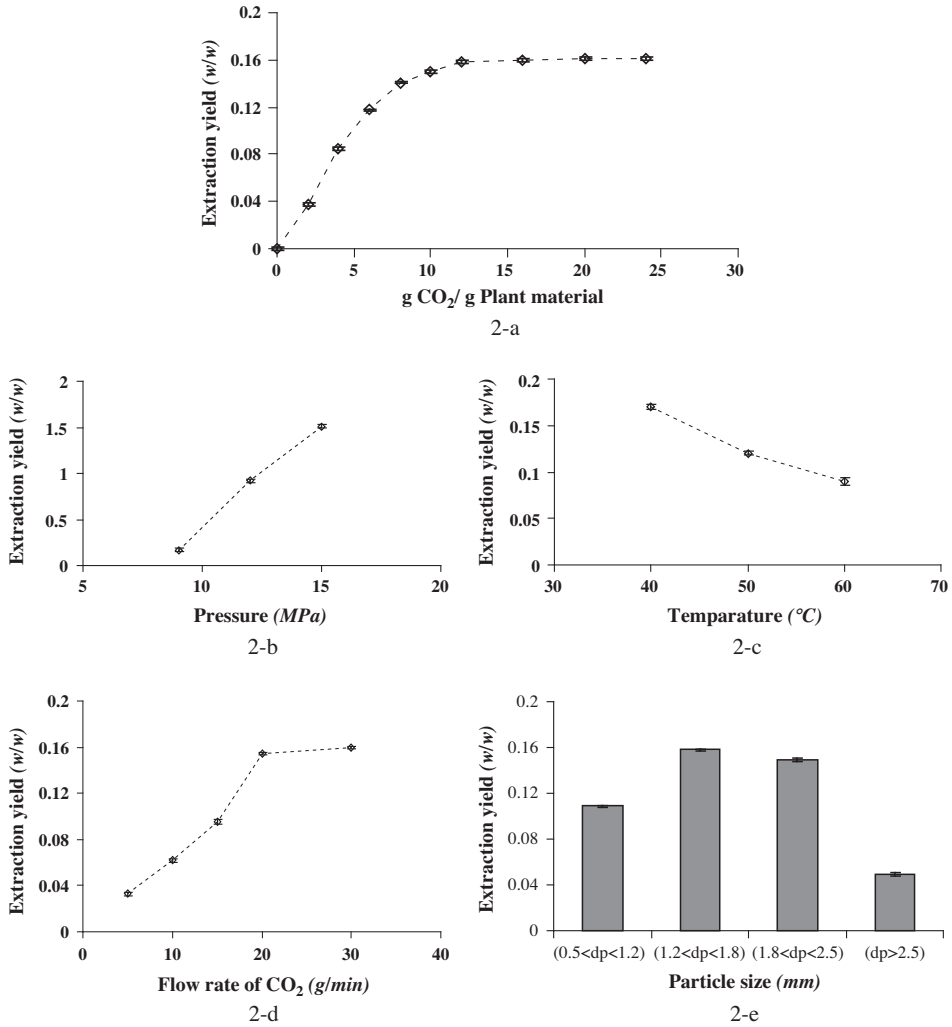

Fig. 2. Extraction yield of SFE for 50 g of raw material of *T. articulata* at Q = 20 g/min, P = 9 MPa and T = 40 °C, (a) Kinetics of SFE extraction process at t = 60 min, (b) Influence of the pressure on the extraction yield at t = 30 min, (c) Influence of the temperature on the extraction yield at t = 30 min, (d) Influence of CO_2 flow-rate on the extraction yield at t = 30 min, (e) Influence of the particle size on the SFE extraction yield at t = 30 min.

Plant-material ratio of 4 was used. Different extraction durations were tested for ethanol. The results presented in Table 1 shows the effect of the duration on the quantity and quality of products. The best performance and quality of product were obtained for 6 h of extraction with ethanol.

Table 1 summarizes the results of the three extraction procedures. The hexane Soxhlet extraction provided an extraction yield of *T. articulata* equal to 40 g/kg, which is the highest yield. SFE extraction at 9 MPa resulted in a yield of 1.6 g/kg, while the HD process gave the lowest yield, of 0.6 g/kg.

The extracts obtained with HD and SFE at 9 MPa are assumed to contain only volatile fractions of the plant. So, the extraction yield of volatile oil is increased by about 62% with SFE as compared to HD. Soxhlet extractions with ethanol or hexane lead to extracts containing volatile compounds together with high-molecular-mass compounds, which means low selectivity with respect to volatile compounds (Hawthorne, Grabanski, Martin, & Miller, 2000). Another drawback of the Soxhlet procedure is the extraction duration (6 h) and the presence of organic solvent traces in the extracts.

3.2. Effect of extraction technique on the chemical composition of the extracts

The results of the chemical identification of all of the extracts, percentages and extracted amounts, are summarized in Table 1. A total of 58 compounds were identified in HD extracts, 72 compounds in SFE extracts and 78 compounds in Soxhlet extracts, which accounted for 93.71%, 98.62% and 98.5% of the total oil composition respectively (Kelen & Tepe, 2008; Mara, Leal, Carvalho, & Meireles, 2003). For the sake of simplicity, the discussion has been restricted to the major components identified for the different methods. These major components are presented by 32 components present at fraction greater than 2%.

The major components identified in HD extracts were α-pinene (24.9%), linalool acetate (21.44%) and caryophyllene oxide (4.24%). The major components in SFE extracts were α-pinene (9.39–31.32%), linalool acetate (15–18.18%), alloaromadendrene (7.55–11%) and γ -caryophyllene (4.16–6%). The major components identified in Soxhlet extracts were α-terpinene (13–16.65%), trans-isolimonene (4–8.19%), abietic acid (0–5.68%) and linalool acetate (5.52–7%). In the Soxhlet extracts, other major high molecular-mass components were detected but not identified by GC–MS (8–11%).

Remarkable differences concerning the nature of components and the amounts extracted were observed with respect to the type of extraction process.

Although compositions of the oils obtained by SFE and HD were similar, they were quantitatively different. For instance, the best SFE operating conditions (9 MPa, 40 °C, 20 g/min for CO_2 flow-rate) gave more than 3-fold greater α-pinene recovery (501 mg/kg

Table 1
Chemical composition (GC–MS) and global yield of *T. articulata* extracts obtained by HD, Soxhlet, and SFE.

			Type of process											
			SFE						Soxhlet				HD	
		Flow rate (g/min)	20		20		5						0.02 mL/s	
		Temperature (°C)	40		60		40		–	–	–	–	–	
		Solvent	CO_2		CO_2		CO_2		EtOH	EtOH	EtOH	Hexane	Water	
		Extraction time (min)	30		30		30		240	360	480	480	180	
		Global yield (g/kg)	1.6		1.6		0.6		21.2	26.8	27.4	40.4	0.61	
NI	RI	Compounds	N°1		N°2		N°3		N°4	N°5	N°6	N°7	N°8	
			%	(mg/kg)	%	(mg/kg)	%	(mg/kg)	%	%	%	%	%	(mg/kg)
1	919	α-pinene	31.32 ± 1.21^{a}	501 ± 22	21.92 ± 1.43^{c}	351 ± 18	9.35 ± 0.42^{d}	56 ± 1	2.68 ± 0.10^{g}	3.14 ± 0.14^{f}	2.63 ± 0.12^{g}	6.79 ± 0.22^{e}	24.90 ± 1.08^{b}	152 ± 6
2	967	β-thujene	2.64 ± 0.12^{a}	42 ± 1	1.56 ± 0.09^{c}	25 ± 1	0.91 ± 0.04^{d}	5 ± 0.2	0.45 ± 0.02^{f}	0.53 ± 0.02^{f}	0.43 ± 0.01^{f}	0.68 ± 0.02^{e}	1.99 ± 0.09^{b}	12 ± 0.4
3	983	Trans-isolimonene							5.85 ± 0.23^{c}	8.19 ± 0.42^{a}	7.28 ± 0.21^{b}	4.93 ± 0.21^{d}		
4	1004	Pseudolimonene	0.26 ± 0.01^{c}	4 ± 0.1	0.16 ± 0.01^{d}	3 ± 0.1	0.16 ± 0.00^{d}	1 ± 0.08				3.22 ± 0.11^{a}	0.37 ± 0.01^{b}	2 ± 0.1
5	1008	3-Carene	3.39 ± 0.15^{a}	54 ± 1	2.30 ± 0.12^{b}	37 ± 1	1.50 ± 0.07^{c}	9 ± 0.4				3.32 ± 0.10^{a}	3.44 ± 0.14^{a}	21 ± 1
6	1012	α-Terpinene							13.36 ± 0.66^{b}	16.65 ± 0.72^{a}	13.87 ± 0.52^{b}	4.21 ± 0.21^{c}	0.12 ± 0.01^{d}	1 ± 0.04
7	1128	α-Campholenal	3.44 ± 0.17^{a}	55 ± 2	2.67 ± 0.12^{b}	43 ± 1	3.43 ± 0.12^{a}	21 ± 0.5	1.51 ± 0.06^{d}	1.81 ± 0.07^{c}	0.95 ± 0.42^{e}	1.90 ± 0.08^{c}	3.84 ± 0.16^{a}	23 ± 1
8	1148	Camphene hydrate	1.25 ± 0.05^{d}	20 ± 1	1.67 ± 0.7^{c}	27 ± 1	2.51 ± 0.08^{b}	15 ± 0.6	1.10 ± 0.05^{e}	1.57 ± 0.05^{c}	0.78 ± 0.02^{f}	1.33 ± 0.06^{d}	4.36 ± 0.18^{a}	27 ± 0
9	1266	Linalool acetate	18.18 ± 0.70^{b}	291 ± 12	15.78 ± 0.7^{c}	252 ± 7	17.69 ± 0.12^{b}	106 ± 5	6.15 ± 0.29^{e}	5.52 ± 0.24^{f}	2.48 ± 0.11^{g}	7.44 ± 0.23^{d}	21.44 ± 1.08^{a}	131 ± 5
10	1383	β-Bourbonene	1.74 ± 0.07^{b}	28 ± 1	1.73 ± 0.06^{b}	28 ± 1	1.90 ± 0.06^{a}	11 ± 0.6	1.22 ± 0.06^{c}	0.75 ± 0.02^{d}	0.67 ± 0.01^{d}	1.14 ± 0.04^{c}	0.44 ± 0.01^{e}	3 ± 0.11
11	1400	γ-Caryophyllene	4.16 ± 0.19^{c}	67 ± 2	5.43 ± 0.23^{b}	87 ± 4	5.81 ± 0.25^{a}	35 ± 0.8	1.51 ± 0.06^{f}	1.68 ± 0.06^{e}	1.35 ± 0.06^{g}	1.71 ± 0.06^{e}	2.53 ± 0.09^{d}	15 ± 0.6
12	1461	Alloaromadendrene	7.55 ± 0.31^{c}	121 ± 6	11.80 ± 0.41^{a}	189 ± 6	10.86 ± 0.98^{b}	65 ± 2		2.04 ± 0.10^{d}	1.38 ± 0.05^{e}	2.03 ± 0.10^{d}	1.14 ± 0.08^{f}	7 ± 0.2
13	1494	α-Zingiberene	2.14 ± 0.10^{e}	34 ± 1	3.52 ± 0.17^{c}	56 ± 2	4.75 ± 0.14^{a}	29 ± 0.9	3.96 ± 0.22^{b}	2.93 ± 0.14^{d}	3.13 ± 0.12^{d}	4.03 ± 0.19^{b}	0.53 ± 0.02^{f}	3 ± 0.1
14	1501	α-Amorphene	1.39 ± 0.05^{d}	22 ± 1	1.98 ± 0.07^{b}	32 ± 1	2.76 ± 0.08^{a}	17 ± 0.8					1.64 ± 0.06^{c}	10 ± 0.4
15	1557	Caryophyllene alcohol	1.06 ± 0.04^{b}	17 ± 0.6	3.23 ± 0.12^{a}	52 ± 1	2.43 ± 0.06^{b}	15 ± 0.4						
16	1568	Caryophyllene oxide	1.82 ± 0.06^{g}	29 ± 1	2.88 ± 0.11^{d}	46 ± 2	4.04 ± 0.18^{b}	24 ± 1.2	4.21 ± 0.19^{a}	2.44 ± 0.08^{e}	2.20^{f} ± 0.08	3.54 ± 0.13^{c}	4.24 ± 0.11^{a}	26 ± 1
17	1594	Cedrenol	0.48 ± 0.02^{e}	8 ± 0.3	0.96 ± 0.03^{d}	15 ± 0.4	1.12 ± 0.05^{c}	7 ± 0.3	1.56 ± 0.07^{b}	1.20 ± 0.03^{c}	0.93 ± 0.03^{d}	1.16 ± 0.04^{c}	2.00 ± 0.08^{a}	12 ± 0.3
18	1613	1-Epi-cubenol	0.52 ± 0.01^{f}	8 ± 0.4	1.23 ± 0.04^{c}	20 ± 0.6	1.67 ± 0.04^{b}	10 ± 0.2	1.17 ± 0.04^{c}	0.95 ± 0.04^{d}	0.84 ± 0.02^{e}	0.92 ± 0.02^{d}	2.83 ± 0.12^{a}	17 ± 0.5
19	1626	γ-Eudesmol	0.58 ± 0.02^{g}	9 ± 0.2	1.01 ± 0.03^{f}	16 ± 0.6	1.35 ± 0.06^{d}	8 ± 0.1	1.73 ± 0.07^{b}	1.53 ± 0.06^{c}	1.17 ± 0.05^{e}	1.12 ± 0.02	2.56 ± 0.09^{a}	16 ± 0.4
20	1642	Spathulenol	0.43 ± 0.01^{f}	7 ± 0.2	0.77 ± 0.02^{e}	12 ± 0.5	1.05 ± 0.03^{d}	6 ± 0.1	1.50 ± 0.06^{b}	1.52 ± 0.05^{b}	1.36 ± 0.06^{c}	1.47 ± 0.04^{b}	2.37 ± 0.11^{a}	14 ± 0.3
21	1678	Cis-α-santalol	0.44 ± 0.01^{g}	7 ± 0.3	1.07 ± 0.02^{f}	17 ± 0.6	1.36 ± 0.04^{e}	8 ± 0.2	2.41 ± 0.09^{b}	2.80 ± 0.13^{a}	2.24 ± 0.12^{c}	2.19 ± 0.11^{c}	1.74 ± 0.06^{d}	11 ± 0.1
22	1778	Platambin					0.31 ± 0.01^{e}	2 ± 0.1	1.96 ± 0.08^{b}	2.82 ± 0.14^{a}	1.90 ± 0.08^{c}	0.82 ± 0.02^{d}		
23	1962	Palmitic acid							2.69 ± 0.08^{a}	1.32 ± 0.04^{d}	2.20 ± 0.11^{b}	1.52 ± 0.04^{c}		
24	2095	Heneicosane	0.40 ± 0.02^{e}	6 ± 0.2	0.40 ± 0.02^{e}	6 ± 0.2	0.59 ± 0.02^{d}	4 ± 0.2	2.26 ± 0.08^{b}	2.01 ± 0.09^{c}	2.20 ± 0.09^{b}	2.53 ± 0.11^{a}	0.09 ± 0.00^{f}	1 ± 0.1
25	2135	1R-pimaral	0.13 ± 0.00^{d}	2 ± 0.1	0.16 ± 0.01^{d}	3 ± 0.0	0.27 ± 0.01^{c}	2 ± 0.1	2.14 ± 0.09^{a}		1.83 ± 0.06^{b}		0.27 ± 0.01^{c}	2 ± 0.1
26	2216	15-Ripperten-3 α-ol	0.41 ± 0.01^{h}	7 ± 0.3	0.94 ± 0.03^{f}	15 ± 0.6	1.62 ± 0.06^{e}	10 ± 0.4	2.20 ± 0.11^{c}	1.76 ± 0.07^{d}	2.85 ± 0.13^{a}	2.73 ± 0.12^{b}	0.52 ± 0.02^{g}	3 ± 0.1
27	2368	Androst-5-en-17-ol, 4,4 dimethyl							4.43 ± 0.21^{b}	1.73 ± 0.08^{d}	6.54 ± 0.29^{a}	3.73 ± 0.17^{c}		
28	2383	Podocarp-7-en-3-one, 13-β-methyl-13-vinyl							1.60 ± 0.09^{d}	2.53 ± 0.11^{c}	5.79 ± 0.21^{a}	4.56 ± 0.20^{b}		
29	2473	ni							2.56 ± 0.11^{b}	2.99 ± 0.13^{a}	2.86 ± 0.11^{a}	2.28 ± 0.11^{c}		
30	2922	Abietic acid							4.41 ± 0.20^{b}	5.68 ± 0.27^{a}	5.68 ± 0.26^{a}	0.51 ± 0.02^{c}		
31	2925	ni							4.89 ± 0.21^{c}	3.86 ± 0.17^{d}	6.26 ± 0.27^{a}	5.46 ± 0.25^{b}		
32	2930	Podocarp-8(14)-en-15al, 13 β-methyl-13-vinyl									4.25 ± 0.18^{a}			
33	906	Santolinatriene	0.85 ± 0.02^{a}	14 ± 0.6	0.67 ± 0.02^{b}	11 ± 0.3	0.23 ± 0.01^{d}	1 ± 0.1	0.08 ± 0.01^{f}	0.10 ± 0.00^{f}	0.09 ± 0.00^{f}	0.17 ± 0.01^{e}	0.39 ± 0.01^{c}	2 ± 0.1
34	931	Artemesiatriene	0.80 ± 0.02^{a}	13 ± 0.5	0.47 ± 0.01^{c}	8 ± 0.3	0.25 ± 0.01^{d}	2 ± 0.1	0.11 ± 0.00^{f}	0.11 ± 0.01^{f}	0.09 ± 0.01^{f}	0.19 ± 0.01^{e}	0.60 ± 0.02^{b}	4 ± 0.3
35	940	Cumene	0.33 ± 0.01^{a}	5 ± 0.1	0.14 ± 0.00^{c}	2 ± 0.1	0.24 ± 0.01^{b}	1 ± 0.0						
36	953	α-Fenchene	0.22 ± 0.00^{a}	4 ± 0.1	0.14 ± 0.01^{b}	2 ± 0.0	0.08 ± 0.00^{c}					0.06 ± 0.00^{c}	0.14 ± 0.00^{b}	1 ± 0.1
37	958	Camphene	0.49 ± 0.02^{a}	8 ± 0.2	0.37 ± 0.01^{b}	6 ± 0.2	0.24 ± 0.01^{c}	1 ± 0.1	0.10 ± 0.01^{e}	0.13 ± 0.01^{e}	0.10 ± 0.00^{e}	0.17 ± 0.00^{d}	0.45 ± 0.02^{a}	3 ± 0.2
38	973	1-ethyl-4-methylbenzene	0.43 ± 0.01^{a}	7 ± 0.2	0.22 ± 0.00^{b}	4 ± 0.2	0.29 ± 0.01^{b}	2 ± 0.1						
39	990	Myrcene	0.17 ± 0.01^{b}	3 ± 0.0	0.06 ± 0.00^{c}	1 ± 0.0	0.12 ± 0.01^{b}	1 ± 0.1					0.48 ± 0.02^{a}	3 ± 0.1
40	1037	Limonene	0.16 ± 0.01^{a}	3 ± 0.1	0.09 ± 0.00^{c}	1 ± 0.0	0.10 ± 0.0^{c}	1 ± 0.0	0.11 ± 0.01^{c}	0.15 ± 0.01^{a}	0.13 ± 0.00^{b}	0.10 ± 0.00^{c}	0.18 ± 0.01^{a}	1 ± 0.0
41	1047	3-methyl-1-decene	0.13 ± 0.00^{b}	2 ± 0.0	0.12 ± 0.01^{b}	2 ± 0.1	0.16 ± 0.0^{a}	1 ± 0.0	0.05 ± 0.00^{c}	0.07 ± 0.00^{c}		0.07 ± 0.00^{c}		
42	1068	Isoterpinolene	0.26 ± 0.01^{c}	4 ± 0.02	0.14 ± 0.01^{d}	2 ± 0.0	0.16 ± 0.0^{d}	1 ± 0.0	0.26 ± 0.01^{c}	0.35 ± 0.01^{b}	0.28 ± 0.01^{c}	0.25 ± 0.01^{c}	0.46 ± 0.01^{a}	3 ± 0.1

43	1077	Ethyl maltol							0.32 ± 0.01^{a}	0.36 ± 0.00^{a}	0.27 ± 0.01^{b}	0.29 ± 0.01^{b}	0.26 ± 0.01^{b}	2 ± 0.1
44	1079	Artemisia alcohol	0.19 ± 0.00^{b}	3 ± 0.0	0.17 ± 0.01^{b}	3 ± 0.1	0.25 ± 0.01^{a}	2 ± 0.1				0.12 ± 0.01^{c}		
45	1089	α-Terpinolene			0.08 ± 0.00^{a}	1 ± 0.0						0.12 ± 0.00^{a}		
46	1108	Cis-thujone	0.22 ± 0.01^{b}	4 ± 0.2	0.18 ± 0.01^{c}	3 ± 0.0	0.25 ± 0.01^{b}	2 ± 0.0	0.19 ± 0.00^{c}	0.10 ± 0.00^{d}	0.05 ± 0.00^{e}	0.13 ± 0.01^{d}	0.35 ± 0.01^{a}	2 ± 0.1
47	1122	Trans-2,8-menthadienol	0.17 ± 0.01^{c}	3 ± 0.1	0.15 ± 0.00^{c}	2 ± 0.1	0.26 ± 0.00^{b}	2 ± 0.1					0.38 ± 0.01^{a}	2 ± 0.0
48	1132	Isothujol					0.25 ± 0.01^{a}	2 ± 0.1					0.17 ± 0.02^{b}	1 ± 0.0
49	1157	Isoborneol							0.29 ± 0.01^{b}	0.35 ± 0.01^{a}		0.16 ± 0.01^{c}		
50	1159	β-Terpineol	0.18 ± 0.00^{c}	3 ± 0.0	0.20 ± 0.01^{c}	3 ± 0.0	0.22 ± 0.01^{c}	1 ± 0.0			0.36 ± 0.01^{b}		0.73 ± 0.02^{a}	4 ± 0.1
51	1172	3,5,5-Trimethyl-1,4-cyclohexanedione			0.14 ± 0.00^{d}	2 ± 0.1	0.26 ± 0.00^{c}	2 ± 0.0	0.28 ± 0.00^{c}	0.41 ± 0.02^{b}	0.28 ± 0.01^{c}	0.37 ± 0.01^{b}	1.41 ± 0.05^{a}	9 ± 0.3
52	1193	Myrtenal	0.23 ± 0.01^{b}	4 ± 0.1	0.20 ± 0.00^{b}	3 ± 0.0	0.41 ± 0.02^{a}	2 ± 0.0	0.12 ± 0.00^{c}	0.20 ± 0.01^{b}	0.09 ± 0.00^{c}		0.08 ± 0.00^{c}	
53	1200	Z-dihydrocarvone	0.18 ± 0.01^{c}	3 ± 0.1	0.13 ± 0.01^{d}	2 ± 0.1	0.26 ± 0.01^{b}	2 ± 0.1	0.13 ± 0.01^{d}	0.24 ± 0.00^{b}	0.11 ± 0.01^{d}	0.16 ± 0.00^{c}	0.34 ± 0.01^{a}	2 ± 0.1
54	1232	Cis-carveol	0.15 ± 0.01^{b}	2 ± 0.0	0.12 ± 0.00^{b}	2 ± 0.0			0.14 ± 0.01^{b}	0.18 ± 0.00^{a}		0.14 ± 0.01^{b}		
55	1279	p-sec-Butylphenol							0.06 ± 0.00^{b}	0.10 ± 0.01^{b}		0.07 ± 0.00^{b}	0.58 ± 0.02^{a}	4 ± 0.1
56	1318	(Z)-3-Hexenyl 2-methyl-(E)-2-butenoate	0.38 ± 0.02^{a}	6 ± 0.1	0.17 ± 0.01^{b}	3 ± 0.1	0.33 ± 0.01^{a}	2 ± 0.0		0.11 ± 0.00^{c}	0.10 ± 0.00^{c}			
57	1327	Trans-pulegone oxide	0.91 ± 0.03^{b}	15 ± 0.5	0.79 ± 0.02^{c}	13 ± 0.3	0.96 ± 0.02^{b}	6 ± 0.2	0.61 ± 0.01^{d}	0.53 ± 0.02^{e}		0.67 ± 0.02^{d}	1.53 ± 0.05^{a}	9 ± 0.1
58	1356	α-Cubebene	1.11 ± 0.04^{b}	18 ± 0.5	1.15 ± 0.03^{b}	18 ± 0.6	1.22 ± 0.04^{a}	7 ± 0.3	0.90 ± 0.02^{c}	0.73 ± 0.02^{d}	0.70 ± 0.02^{d}	0.95 ± 0.02^{c}	0.26 ± 0.00^{e}	2 ± 0.1
59	1370	Cyclosativene	0.72 ± 0.03^{a}	12 ± 0.4			0.65 ± 0.01^{a}	4 ± 0.1		0.36 ± 0.01^{b}	0.23 ± 0.01^{c}	0.37 ± 0.01^{b}	0.16 ± 0.01^{d}	1 ± 0.0
60	1409	α-Gurjunene	0.28 ± 0.01^{a}	4 ± 0.1	0.26 ± 0.01^{a}	4 ± 0.2			0.20 ± 0.01^{b}		0.11 ± 0.00^{c}			
61	1435	α-Trans-bergamotene	1.29 ± 0.04^{c}	21 ± 0.1	1.51 ± 0.04^{b}	24 ± 0.1	1.63 ± 0.04^{a}	10 ± 0.2	0.53 ± 0.01^{d}	0.51 ± 0.01^{d}	0.40 ± 0.01^{e}	0.54 ± 0.02^{d}	1.04 ± 0.03^{c}	6 ± 0.2
62	1444	α-Caryophyllene			0.08 ± 0.00^{b}	1 ± 0.0			0.06 ± 0.00^{b}	0.08 ± 0.00^{b}	0.06 ± 0.00^{b}	0.06 ± 0.00^{b}	0.13 ± 0.00^{a}	1 ± 0.0
63	1455	α-Patchoulene	0.48 ± 0.02^{d}	8 ± 0.2	0.61 ± 0.02^{c}	10 ± 0.0	0.75 ± 0.01^{b}	5 ± 0.1	0.84 ± 0.01^{a}				0.18 ± 0.01^{e}	1 ± 0.0
64	1474	γ-Muurolene							0.68 ± 0.02^{a}	0.64 ± 0.02^{a}	0.49 ± 0.02^{b}	0.47 ± 0.01^{b}	0.35 ± 0.01^{c}	2 ± 0.0
65	1479	γ-Curcumene	0.58 ± 0.02^{c}	9 ± 0.3	0.78 ± 0.02^{a}	12 ± 0.1	0.63 ± 0.02^{b}	4 ± 0.1					0.43 ± 0.02^{d}	3 ± 0.1
66	1494	α-Zingiberene	2.14 ± 0.10^{f}	34 ± 0.9	3.52 ± 0.15^{c}	56 ± 0.2	4.75 ± 0.21^{a}	29 ± 0.9	3.96 ± 0.17^{b}	2.93 ± 0.12^{e}	3.13 ± 0.12^{d}	4.03 ± 0.21^{b}	0.53 ± 0.01^{g}	3 ± 0.1
67	1501	α-Amorphene	1.39 ± 0.05^{d}	22 ± 0.7	1.98 ± 0.09^{b}	32 ± 0.1	2.76 ± 0.12^{a}	17 ± 0.6					1.64 ± 0.07^{c}	10 ± 0.4
68	1516	α-Selinene	0.32 ± 0.01^{a}	5 ± 0.2	0.35 ± 0.01^{a}	6 ± 0.1	0.31 ± 0.01^{a}	2 ± 0.1						
69	1534	Incisol	0.17 ± 0.00^{f}	3 ± 0.1	0.46 ± 0.01^{e}	7 ± 0.2	0.44 ± 0.02^{e}	3 ± 0. 0	0.73 ± 0.02^{a}	0.70 ± 0.02^{a}	0.52 ± 0.02^{d}	0.66 ± 0.02^{b}	0.59 ± 0.02^{c}	4 ± 0.1
70	1588	β-Oplopenone											0.38 ± 0.00^{a}	2 ± 0.0
71	1619	Trans-isolongifolanone					0.15 ± 0.00^{b}	1 ± 0.0					0.42 ± 0.02^{a}	3 ± 0.0
72	1660	14-Hydroxy-9-epi-(E)-caryophyllene	0.14 ± 0.00^{e}	2 ± 0.1	0.29 ± 0.01^{d}	5 ± 0.1	0.23 ± 0.01^{d}	1 ± 0.0	0.48 ± 0.01^{b}	0.50 ± 0.01^{b}	0.44 ± 0.01^{c}	0.42 ± 0.01^{c}	0.97 ± 0.04^{a}	6 ± 0.01
73	1673	Cedr-8-en-13-ol			0.10 ± 0.00^{b}	2 ± 0.1							0.23 ± 0.01^{a}	1 ± 0.0
74	1690	Juniper camphor					0.52 ± 0.01^{b}	3 ± 0.0	0.65 ± 0.01^{a}			0.13 ± 0.01^{c}		
75	1717	Trans-2-heptadecene*	0.22 ± 0.01^{g}	4 ± 0.2	0.40 ± 0.01^{f}	6 ± 0.2	0.74 ± 0.02^{e}	4 ± 0.2	1.10 ± 0.06^{d}	1.64 ± 0.02^{a}	1.39 ± 0.04^{c}	1.55 ± 0.07^{b}		
76	1729	Cis-β-santalol								1.13 ± 0.04^{a}		0.69 ± 0.02^{b}		
77	1761	1,10-Dihydronootkatone							0.88 ± 0.03^{a}				0.08 ± 0.00^{b}	
78	1789	14-Hydroxy-α-muurolene*			0.42 ± 0.02^{b}	7 ± 0.2			0.71 ± 0.02^{a}	0.76 ± 0.02^{a}	0.49 ± 0.01^{b}	0.36 ± 0.01^{c}		
79	1799	Alloaromadendrene oxide							0.35 ± 0.01^{b}	0.47 ± 0.01^{a}	0.19 ± 0.01^{c}	0.15 ± 0.00^{c}		
80	1928	16-Hexadecanolide					0.31 ± 0.01^{b}	2 ± 0.0	0.18 ± 0.01^{c}	0.41 ± 0.01^{a}	0.22 ± 0.01^{c}		0.15 ± 0.01^{d}	1 ± 0.0
81	1983	Biformene*			0.07 ± 0.00^{e}	1 ± 0.0	0.14 ± 0.00^{d}	1 ± 0.0	0.79 ± 0.02^{b}	0.43 ± 0.02^{c}	1.39 ± 0.06^{a}			
82	1991	Manoyl oxide	0.13 ± 0.00^{g}	2 ± 0.1	0.37 ± 0.01^{e}	6 ± 0.2	0.50 ± 0.02^{c}	3 ± 0.2	0.59 ± 0.01^{b}	0.36 ± 0.01^{e}	0.43 ± 0.01^{d}	0.66 ± 0.02^{a}	0.26 ± 0.01^{f}	2 ± 0.0
83	2008	Kaur-16-ene	0.14 ± 0.00^{e}	2 ± 0.1	0.38 ± 0.01^{d}	6 ± 0.2	0.48 ± 0.01^{c}	3 ± 0.1	0.53 ± 0.01^{b}	0.36 ± 0.01^{d}	0.68 ± 0.02^{a}	0.56 ± 0.01^{b}	0.10 ± 0.00^{e}	1 ± 0.0
84	2053	Abietatriene			0.20 ± 0.00^{g}	3 ± 0.1	0.40 ± 0.01^{e}	2 ± 0.1	0.84 ± 0.02^{a}	0.56 ± 0.02^{c}	0.67 ± 0.01^{b}	0.47 ± 0.02^{d}	0.32 ± 0.00^{f}	2 ± 0.1
85	2143	Cubitene	0.22 ± 0.01^{f}	4 ± 0.2	0.30 ± 0.01^{e}	5 ± 0.1	0.58 ± 0.02^{d}	3 ± 0.1	1.55 ± 0.05^{b}	1.47 ± 0.06^{c}	2.33 ± 0.09^{a}	1.65 ± 0.06^{b}		
86	2187	Cembrene A ketone	0.25 ± 0.01^{e}	4 ± 0.1	0.49 ± 0.02^{d}	8 ± 0.2	0.95 ± 0.03^{c}	6 ± 0.2	1.04 ± 0.04^{b}	1.01 ± 0.04^{b}	0.93 ± 0.03^{c}	1.27 ± 0.04^{a}	0.14 ± 0.01^{f}	1 ± 0.0
87	2223	Trans-totarol	0.12 ± 0.00^{c}	2 ± 0.1	0.19 ± 0.01^{b}	3 ± 0.1	0.32 ± 0.01^{a}	2 ± 0.1			0.29 ± 0.01^{a}			
88	2274	Larixol*							0.68 ± 0.02^{b}	0.66 ± 0.02^{b}	0.75 ± 0.02^{a}	0.63 ± 0.02^{c}		
89	2288	ni							0.33 ± 0.01^{b}	0.44 ± 0.01^{a}	0.44 ± 0.01^{a}	0.40 ± 0.01^{a}		
90	2311	Trans-14-Isopropylpodocarpa-8,11,13-trien-13-ol							0.67 ± 0.02^{b}	0.52 ± 0.01^{c}	0.74 ± 0.03^{a}	0.62 ± 0.02^{b}	0.07 ± 0.00^{d}	
		Number of compounds	56		62			61	66	65	63	68	56	56
		Monoterpene hydrocarbons	40.56	649	27.96	447	13.1	78	23	29.35	24.9	24.21	33.52	204
		Monoterpene oxygenated	6.92	111	6.28	100	8.8	53	4.15	5.08	2.34	4.68	12.36	75
		Sesquiterpenes hydrocarbons	21.76	348	29.2	467	31.27	188	9.9	9.72	8.52	11.3	8.83	54
		Sesquiterpenes oxygenated	5.64	90	12.42	199	14.67	88	18.34	16.82	12.28	13.63	18.41	112
		Others	21.35	342	20.28	324	25.55	153	43.14	37.46	50.29	44.71	25.03	152
		Total	96.23	1540	96.14	1538	93.39	560	98.53	98.43	98.33	98.53	98.15	597

ni: not identified.

* Tentatively identified supported by good match of MS. Values within lines with different superscripts (a–h) were significantly different. Relative standard deviation (RSD): RSD <5% for percentage of compounds >0.2% and RSD >5% for percentage of compounds <0.2%.

compared to 152 mg/kg). Because both extractions were performed up to completion, it can be postulated that such compounds were thermally degraded in the case of HD and therefore were not recovered at the condenser. Such reasoning is supported by the fact that more caryophyllene oxide was present in HD than in SFE, indicating a probable degradation of caryophyllene into its oxidized form. These results demonstrate that shorter duration of operation at lower temperature is one of the main advantages of SFE over HD. Therefore, SFE appears to be the optimum process for obtaining *T. articulata* volatile oil of high quality with a good yield. Costa et al. (2012) obtained similar results when processing leaves from *Lavandula viridis*, where higher yields were achieved by SFE than by HD. Also, Pavela, Sajfrtová, Sovová, Bárnet and Karban (2010) obtained higher yield for SFE compared to HD when processing *Tanacetum parthenium* (*L.*).

When operating SFE, a change of the extraction temperature in the extractor has an effect on the chemical composition of the extracts and 40 °C seems to be the best condition. It is probable that, at 60 °C, the solubility of solutes decreases because of the retrograde solubility phenomenon specific to supercritical fluids.

Because it has been postulated that extraction is mainly solubility limited, experiment N°3, where the CO_2 flow-rate was reduced to 5 g/min (instead of 20 g/min), can be interpreted as being equivalent to a shorter duration experiment at 20 g/min of CO_2, *i.e.* 7.5 min duration. So it logically results in a lower yield (0.6 g/kg compared to 1.6 g/kg). Also, decreasing the flow-rate of CO_2 at constant temperature decreases the proportion of monoterpene hydrocarbons (40.56–13.1% or 40.56–27.96%, respectively) while it increases the proportion of oxygenated sesquiterpenes (5.64–14.6% or 5.64–12.32%, respectively) and sesquiterpene hydrocarbons (21.76–31.2% or 21.76–29.20%, respectively), indicating a selectively favored extraction of the latter.

All Soxhlet extractions gave significantly higher yields than HD or SFE (from 26 to 40 g/kg, as can be seen in Table 1). This procedure allows the extraction of volatile compounds but also high molecular-mass compounds, which proved to be present in large amounts. Because such heavy compounds cannot be detected by GC–MS, it was not possible to calculate the amounts extracted as for HD and SFE, so they are not given in Table 1.

3.3. Phenolic composition

Polyphenols are used for the prevention of various diseases that are mainly associated with free radicals. More generally, phenolic compounds have been recognized as antioxidant agents, which act as free radical terminators and have been known to exhibit medicinal activity as well as physiological functions (Rawat, Bhatt, & Rawal, 2011).

The total phenolic composition of the extracts was measured and the results are summarized in Table 2. The total phenolic content varied from 2.3 to 38.1 mg of Gallic Acid Equivalent (GAE)/g dry plant material. A wide range of phenolic concentrations was observed in the extracts. EO obtained by HD (24.6 ± 0.2 mg GAE/g dry plant material) and the volatile fraction obtained by SFE (4.4–2.3 mg GAE/g dry plant material) contained very much smaller amounts of phenolic compounds than did ethanol Soxhlet extract (38.1 ± 0.6 mg GAE/g dry plant material) and hexane Soxhlet extract (26.3 ± 0.4 mg GAE/g dry plant material). These results are novel because the occurrence of large quantities of phenolic compounds in *T. articulata* from Tunisia has not been reported previously.

3.4. Antioxidant activity

The antioxidant activity of extracts was assessed by two tests measuring the antiradical activity: the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ methods.

Table 2
Total phenolics and antioxidant activity of *T articulata* extracts obtained by HD, Soxhlet and SFE.

	Samples	Phenolics (g GAE/kg dry mass)[a]	$DPPH^{\bullet}$ IC_{50} (mg/L)	$ABTS^{\bullet+}$ IC_{50} (mg/L)
SFE	N°1	1.08 ± 0.06^{b}	182 ± 6^{b}	45 ± 2^{a}
	N°2	1.18 ± 0.02^{b}	146 ± 2^{a}	41 ± 1^{a}
	N°3	0.49 ± 0.00^{a}	243 ± 10^{c}	97 ± 3^{c}
SOXHLET	N°4	11.52 ± 0.16^{d}	2669 ± 125^{g}	375 ± 18^{f}
	N°5	32.90 ± 1.60^{e}	1110 ± 55^{e}	51 ± 2^{b}
	N°6	89.70 ± 3.00^{f}	1836 ± 61^{f}	293 ± 14^{d}
	N°7	140.50 ± 9.00^{g}	943 ± 50^{d}	281 ± 8^{d}
Hydrodistillation	N°8	8.89 ± 0.16^{c}	3681 ± 69^{h}	324 ± 14^{e}
Vitamin C	–	–	4.00 ± 0.14	2.01 ± 0.06

Values within columns with different superscripts (a–h) were significantly different ($p < 0.05$).

A comparison of the antioxidant activity, assessed by both $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays, is presented in Table 2.

The differences between $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ tests can be explained by the mechanism of the reactions involved. The $ABTS^{\bullet+}$ cation radical reactions involve electron transfer and take place at a much faster rate than those for $DPPH^{\bullet}$ radicals, whose degree of discoloration is attributed to the hydrogen donating ability of the tested compounds. The general trend obtained for the different tested extracts is the same by the two methods (Bendaoud, Romdhane, Souchard, Cazaux, & Bouajila, 2010).

For the $DPPH^{\bullet}$ assay, the values varied from 108 to 242 mg/L for the SFE extracts, from 943 to 2668 mg/L for the Soxhlet extracts and, finally, were equal to 3681 mg/L for HD EO. *T. articulata* EO obtained by HD (3681 ± 69 mg/L by DPPH assay) exhibited a low antioxidant activity and this result is in accordance with the one obtained by Ben Hadj Ahmed et al. (2011) (1214.13 mg/L by DPPH assay) with *T. articulata* from Algeria. For the $ABTS^{\bullet+}$ assay, the values varied from 29 to 96 mg/L for the SFE extracts, 51 to 375 mg/L for the Soxhlet extracts and, finally, 324 mg/L for HD EO. From both tests, it appears that extracts obtained from SFE at 9 MPa exhibit the highest antioxidant activity (146 mg/L for $DPPH^{\bullet}$ and 41 mg/L for $ABTS^{\bullet+}$). In contrast, EO of *T. articulata* obtained by HD exhibited quite a low antioxidant activity (3681.49 mg/L for $DPPH^{\bullet}$ and 324.45 mg/L for $ABTS^{\bullet+}$). The Soxhlet extracts exhibited intermediate activities.

For SFE extraction, the influence of temperature so as CO_2 flow-rate at a constant pressure (9 MPa) on antioxidant activity was tested. The results show that an increase in temperature (40–60 °C) increased the antioxidant activity, probably indicating a correlative increase in the solvent power of CO_2. Varying the CO_2 flow-rate (from 20 g/min to 5 g/min) decreased the antioxidant activity (45 ± 2–97 ± 3 mg/L) of the extract.

This result can be explained by the fact that, for the chosen extraction time, because the extraction process is postulated to be limited by solubility, the extraction at 20 g/min was complete but this is not true at 5 g/min. So, compounds contributing to antioxidant activity are not fully recovered.

Similarly, for the Soxhlet extraction, two solvents were tested and extraction time was varied in the case of ethanol. In this case, the results show an optimum extraction time of 6 h with a good antioxidant activity of the extracts (51 ± 2 mg/L). Also, it can be noted that the antioxidant activity of the extracts was higher with hexane than with ethanol as the solvent.

These results suggest that SFE makes it possible to recover one or more compounds with high antioxidant properties while these compounds are not extractable with HD or organic solvent extraction. A better hypothesis could be that, in the case of HD or Soxhlet extraction, these active compounds are destroyed because they are

exposed to high temperature for a long time in these two methods. This highlights one of the main interests of the SFE process as compared to traditional extraction techniques (Nerio, Olivero-Verbel, & Stashenko, 2010).

Antioxidant activity is often due to the presence of phenolic compounds but, in this case, few phenolics were found in SFE extracts and a first hypothesis could be that other compounds extracted by SFE are responsible for the antioxidant activity.

However, when the relation between total phenolic content and antioxidant activity was analyzed, the results showed a good correlation (R^2 = 0.6 for $ABTS^{\bullet+}$ and R^2 = 0.87 for $DPPH^{\bullet}$) between the antioxidant activity and the phenolic content. So, it can be postulated that, even at low concentration, phenolics present in the SFE extracts are probably very active molecules that have been extracted and preserved from thermal degradation by the SFE procedure.

The correlation between the amount of total polyphenols and antioxidant activity is original and is confirmed for SFE for the first time.

4. Conclusions

The extraction of EO of *T. articulata* was studied using HD, Soxhlet and SFE methods. The best results for SFE were obtained at 40 °C and 9 MPa and provided selective recovery of the volatile fraction. SFE was rapid (30 min) compared to HD (3 h) and Soxhlet (6 h), and the yield (1.6 g/kg) was improved as compared to HD (0.6 g/kg), most probably because of reduced thermal degradation of the extracted compounds. Interesting selectivity for compounds with high antioxidant activity (41 ± 1 mg/L) and correlation between phenolic content and antioxidant activity were observed for SFE extracts. Although solvent Soxhlet extractions have provided good results, their use as an industrial process is questionable, mainly from the hazards they generate, either because of their toxicity or their flammability. The use of a clean solvent like CO_2 exhibits none of these drawbacks, which are now considered as non acceptable flaws, and in addition preserves the products from long time exposure to high temperature. This is often a good incentive to overcome the drawback of higher investment costs related to the supercritical extraction technology.

References

Barrero, A. F., Moral, J. F. Q., Lucas, R., Paya, M., Akssira, M., Akaad, S., et al. (2003). Diterpenoids from *Tetraclinis articulata* that inhibit various human leukocyte functions. *Journal of Natural Products, 66*, 844–850.

Ben Hadj Ahmed, S., Sghaier, R. M., Guesmi, F., Kaabi, B., Mejri, M., Attia, H., Laouini, D., & Smaali, I. (2011). Evaluation of antileishmanial, cytotoxic and antioxidant activities of essential oils extracted from plants issued from the leishmaniasis-endemic region of Sned (Tunisia). *Natural Product Research: Formerly Natural Product Letters, 25*(12), 1195–1201.

Bendaoud, H., Romdhane, M., Souchard, J. P., Cazaux, S., & Bouajila, J. (2010). Chemical composition and anticancer and antioxidant activities of *Schinus Molle* L. and *Schinus terebinthifolius* Raddi berries essential oils. *Journal of Food Science, 75*(6), 466–472.

Blois, M. S. (1958). Antioxidant determinations by the use of a stable free radical. *Nature, 181*, 1199–1200.

Bourkhiss, M., Hnach, M., Paolini, J., Costa, J., Farah, A., & Satrani, B. (2010). Propriétés antioxidantes et anti-inflammatoires des huiles essentielles des différentes parties de *Tetraclinis articulata* (Vahl) masters du Maroc. *Bulletin de la Société Royale des Sciences de Liège, 79*, 141–154.

Costa, P., Grosso, C., Gonçalves, S., Andrade, P. B., Valentão, P., Bernardo-Gil, M. G., et al. (2012). Supercritical fluid extraction and hydrodistillation for the recovery of bioactive compounds from *Lavandula viridis* L'Hér. *Food Chemistry, 135*, 112–121.

da Francisco, J. C., & Sivik, B. (2002). Solubility of three monoterpenes, their mixtures and eucalyptus leaf oils in dense carbon dioxide. *The Journal of Supercritical Fluids, 23*, 11–19.

Gomes, P. B., Mata, V. G., & Rodrigues, A. E. (2007). Production of rose geranium oil using supercritical fluid extraction. *The Journal of Supercritical Fluids, 41*, 50–60.

Grosso, C., Ferraro, V., Figueiredo, A. C., Barroso, J. G., & Palavra, A. M. (2008). Supercritical carbon dioxide extraction of volatile oil from Italian coriander seeds. *Food Chemistry, 111*, 197–203.

Guan, W., Li, S., Yan, R., Tang, S., & Quan, C. (2007). Comparison of essential oils of clove buds extracted with supercritical carbon dioxide and other three traditional extraction methods. *Food Chemistry, 101*, 1558–1564.

Hawthorne, S. B., Grabanski, C. B., Martin, E., & Miller, D. J. (2000). Comparisons of soxhlet extraction, pressurized liquid extraction, supercritical fluid extraction and subcritical water extraction for environmental solids: recovery, selectivity and effects on sample matrix. *Journal of Chromatography A, 892*, 421–433.

Herrero, M., Mendiola, J. A., Cifuentes, A., & Ibanez, E. (2010). Supercritical fluid extraction: Recent advances and applications review. *Journal of Chromatography A, 1217*, 2495–2511.

Kelen, M., & Tepe, B. (2008). Chemical composition, antioxidant and antimicrobial properties of the essential oils of three Salvia species from Turkish flora. *Bioresource Technology, 99*, 4096–4104.

Khajeh, M., Yamini, Y., Bahramifar, N., Sefidkon, F., & Pirmoradei, M. R. (2005). Comparison of essential oils compositions of *Ferula assa-foetida* obtained by supercritical carbon dioxide extraction and hydrodistillation methods. *Food Chemistry, 91*, 639–644.

Khajeh, M., Yamini, Y., Sefidkon, F., & Bahramifar, N. (2004). Comparison of essential oil composition of *Carum copticum* obtained by supercritical carbon dioxide extraction and hydrodistillation methods. *Food Chemistry, 86*, 587–591.

Khajeh, M., Yamini, Y., & Shariati, S. (2010). Comparison of essential oils compositions of Nepetapersica obtained by supercritical carbon dioxide extraction and steam distillation methods. *Food and Bioproducts Processing, 88*, 227–232.

Mara, M. E., Leal, P. F., Carvalho, J. E., & Meireles, M. A. (2003). Comparison of yield, composition, and antioxidant activity of turmeric (*Curcuma longa* L.) extracts obtained using various techniques. *Journal of Agricultural and Food Chemistry, 51*, 6604–6611.

Morales, M. T., Berry, A. J., McIntyre, P. S., & Aparicio, R. (1998). Tentative analysis of virgin olive oil aroma by supercritical fluid extraction-high-resolution gas chromatography–mass spectrometry. *Journal of Chromatography A, 819*, 267–275.

Nerio, L. S., Olivero-Verbel, J., & Stashenko, E. (2010). Repellent activity of essential oils: A review. *Bioresource Technology, 101*, 372–378.

Patel, D. K., Kumar, R., Laloo, D., & Hemalatha, S. (2012). Natural medicines from plant source used for therapy of diabetes mellitus: An overview of its pharmacological aspects. *Asian Pacific Journal of Tropical Disease*, 239–250.

Pavela, R., Sajfrtov, M., Sovova, H., Barnet, M., & Karban, J. (2010). The insecticidal activity of *Tanacetumparthenium* (*L.*) Schultz Bip. Extracts obtained by supercritical fluid extraction and hydrodistillation. *Industrial Crops and Products, 31*, 449–454.

Rashid, S., Rather, M. A., Shah, W. A., & Bhat, B. A. (2013). Chemical composition, antimicrobial, cytotoxic and antioxidant activities of the essential oil of Artemisia indica Willd. *Food Chemistry, 138*, 693–700.

Rawat, S., Bhatt, I. D., & Rawal, R. S. (2011). Total phenolic compounds and antioxidant potential of *Hedychium spicatum* Buch. Ham. ex D. Don in West Himalaya, India. *Journal of Food Composition and Analysis, 24*, 574–579.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology Medicine, 26*, 1231–1237.

Reverchon, E. (1997). Supercritical extraction and fractionation of essential oils and related products. *The Journal of Supercritical Fluids, 10*, 1–37.

Reverchon, E., Ambruosi, A., & Senatore, F. (1994). Isolation of peppermint oil using supercritical CO_2 extraction. *Flavour and Fragrance Journal, 9*, 19–23.

Sacchetti, G., Maietti, S., Muzzoli, M., Scaglianti, M., Manfredini, S., Radice, M., et al. (2005). Comparative evaluation of 11 essential oils of different origin as functional antioxidants, antiradicals and antimicrobials in foods. *Food Chemistry, 91*, 621–632.

Sanchez-Gonzalez, L., Vargas, M., Gonzalez-Martınez, C., Chiralt, A., & Chafer, M. (2011). Use of essential oils in bioactive edible coatings. *Food Engineering Reviews, 3*, 1–16.

Schaneberg, B. T., & Khan, I. A. (2002). Comparison of extraction methods for marker compounds in the essential oil of lemon grass by GC. *Journal of Agricultural and Food Chemistry, 50*, 1345–1349.

Shee, A. K., Raja, B. R., Sethi, D., Kunhambu, A., & Arunachalam, K. D. (2010). Studies on the current antibacterial activity potential of commonly used food preservatives. *International Journal of Engineering and Technology, 2*, 264–269.

Singleton, V. L., Orthofer, R., & Lamuela-Raventós, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of folin–ciocalteu reagent. *Methods in Enzymology, 299*, 152–178.

Sovová, H., & Aleksovski, S. A. (2006). Mathematical model for hydrodistillation of essential oils. *Flavour and Fragrance Journal, 21*, 881–889.

Sovova, H., Komers, R., Kucera, J., & Jez, J. (1994). Supercritical carbon dioxide extraction of caraway essential oil. *Chemical Engineering Science, 49*, 2499–2505.

Thaipong, K., Boonprakob, U., Crosby, K., Zevallos, L. C., & Byrne, D. H. (2006). Comparison of $ABTS^{\bullet+}$, $DPPH^{\bullet}$, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. *Journal of Food Composition and Analysis, 19*, 669–675.

Vergas, C. E., Mendes, M. F., Azevedo, D. A., Pessoa, F. L. P., & Uller, A. C. (2010). Extraction of the essential oil of abajeru (*Chrysobalanus icaco*) using supercritical CO_2. *The Journal of Supercritical Fluids, 54*, 171–177.

Yamini, Y., Khajeh, M., Ghasemi, E., Mirza, M., & Javidnia, K. (2008). Comparison of essential oil compositions of Salvia mirzayanii obtained by supercritical carbon dioxide extraction and hydrodistillation methods. *Food Chemistry, 108*, 341–346.