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Particle Size Effects on Antioxydant and Hepatoprotective Potential of Essential Oil from *Eucalyptus camaldulensis* Leaves against Carbon Tetrachloride-Induced Hepatotoxicity in Rats

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Abstract

Particle size fractionation by sieving is used to optimize antioxydant potential of natural substances. The aim of the present study was to evaluate particle size effects on antioxidant and hepatoprotective potential of *Eucalyptus camaldulensis* essential oils (EO) on CCl_4-induced hepatic damage in Wistar rats. Animals were daily orally treated with the EOs extracted by hydrodistillation from powder sieved at four particle sizes (≥355, 200 - 355, 100 - 200, ≤100 µm) and those of the unsieved powder at dose of 50 mg/Kg for 7 days. Compounds that are evaluated for these activities are hydrocarbons and oxygenated terpenes that were identified and quantified by GC/MS. Activities of enzymes markers of hepatocellular damage in serum and antioxidant enzymes in the liver homogenates were measured. In this research, EOs significantly prevented the increase in serum ALT and AST ($p < 0.05$), total cholesterol, triglyceride and LDL-cholesterol level in acute liver damage induced by CCl_4 and significant increase level of plasma HDL-cholesterol. Also, significantly ($p < 0.05$) decreased the extent of malondialdehyde (MDA) formation and elevated the activities of superoxide dismutase (SOD) and catalase (CAT) liver in comparison to negative control group. The best antioxidant and hepatoprotective activities were those of EOs from two fine powder fractions

(≤100 μm and 100 - 200 μm) was correlated to their high concentration in oxygenated terpenes (70.9% and 46.4%, respectively), when compared to the large particles (200 - 355 μm and ≥355 μm, with 33.3% and 41.8%, respectively) and unsieved powder (37.4%).

Keywords

Eucalyptus camaldulensis, Powder Particle Size, Essential Oils, Hepatoprotection, Oxidative Stress

1. Introduction

Hepatic diseases represent a major public health problem, thus resulting in increased morbidity and mortality all over the world, with available treatment options being very costly. They damage the hepatic tissue or to the liver functions, which can be caused by different factors, such as viruses or bacteria, chronic alcoholism, autoimmune diseases, or by the external action of different hepatotoxic chemicals [1] [2] [3]. Chemicals such as carbon tetrachloride, thioacetamide and galactosamine are mostly used in experimental models to study injury of hepatocytes both *in vivo* and *in vitro* conditions. Particularly, the presence of CCl_4 results in the generation of tricholoromethyl radicals which induce toxicity in rat liver [4], it also increases lipid peroxidation in the hepatic cells and eventually damage the liver [5]. Thus, most of the hepatotoxic chemicals damage liver cells mostly by causing lipid peroxidation which generates a variety of relatively stable decomposition products, mainly α and β-unsaturated aldehydes such as malondialdehyde (MDA), 4-hydroxy-2-nonenal, 2-propenal and isoprostanes and other oxidative damages resulting in generation of highly toxic reactive oxygen species [6] [7].

Due to the global rise of liver diseases, tremendous scientific advancement in the field of medicinal plants and finding effective and safe natural hepatoprotective agents is one of the future directions [8]. The phytoconstituants with hepatoprotective activity such as phenyl compounds, coumarins, essential oils, monoterpenoids, steroids, alkaloids and other nitrogenous compounds has been reported [9] [10] [11] [12]. Thus, as functional food components and sources of peculiar phytochemicals, the increased usage of aromatic or medicinal plants rich in essential oils, has raised due to their numerous valuable biological activities such as their potential preventive properties against hepatic or liver diseases [13] [14].

E. camaldulensis (The River Red Gum, Myrtaceae family) leaves are well known for the medicinal properties due to their contained in EO, which emerges as functional ingredients widely used in modern cosmetics but also in a number of functional food (nutraceuticals, biopolymers), food additives (flavourings, antioxidant…), medicines (pharmaceuticals, therapeutic products), and nutritional supplements [15] [16]. EO obtained from *Eucalyptus*, beyond holding antioxi-

dant activity, reveal other properties such as hepatoprotective activity [17] [18]. Essential oil of *E. camaldulensis* administered by gavage in rats demonstrated toxic effects above 2500 mg/kg [19]. Terpenes are the main constituents of EO of various plants and are basically a complex mixture of terpenic hydrocarbons, especially monoterpenes and sesquiterpenes, and oxygenated derivatives like aldehydes (citronellal, sinensal), ketones (menthone, p-vetivone), alcohols (geraniol, α-bisabolol), phenols (thymol) and esters (γ-tepinyl acetate, cedryl acetate) [20]. EO contain are also non terpenic compounds known as phenylpropanoids which give a specific flavor and odor when they are present [21].

Generally, extraction of EO from Eucalyptus is mainly based on hydrodistillation. A way to enhance extraction could be to grind plants. It is also an effective method to grind the plants into fine particulates powders which could cause marked differences in chemical composition and to enhance extraction of active compounds [22] [23] [24] [25]. Indeed, the size reduction to an appropriate level can modify or enhance the physico-chemical and functional characteristics of food materials. Our recent work *E. camaldulensis* leaves were demonstrated that the size of the particles powders has an influence on the levels of the chemical compounds in the EO [26]. It was found that the levels of oxygenated terpene compounds (terpineol, borneol, exo-fenchol, campholenol and L-trans-pinocarveol) was the highest in the EOs from powders fractions of ≤100 µm (70.9%) and 100 - 200 µm (46.4%), when compared to that of the large particles of ≥355 µm (33.3%), 200 - 355 µm (41.8%) and unsieved powder (37.4%).

The main purpose of this research was to determine particle sizes effects and further evaluate the modulatory of antioxidant and hepatoprotective potential of EO from *E. camaldulensis* against CCl_4-induced hepatotoxicity in rats.

2. Materials and Methods

2.1. Collection of *E. camaldulensis* Leaves and Preparation Powders

The fresh leaves of *E. camaldulensis* were harvested from Dang locality of Ngaoundere Region and identified [26]. Plant was transported at the laboratory of Biophysics, Food Biochemistry and Nutrition of Ngaoundere University, Cameroon. After washing and cleaning to remove all the dirt, the fresh leaves were dried in the shade of a hangar with air circulation at room temperature which varied from 14˚C to 38˚C. The leaves are spread on a clean sheet in thin layers and turned over frequently for seven days. It was powdered after which an essential oil extraction was made.

The dried leaves were ground in the Moulinex robot blender mill supplied with a sieve drilled with 1 mm trapezoid holes according to the analytical method used by Nguimbou *et al.* [27]. Obtained powder sample was sieved using mechanical sieve shaker (ENDECOTTS, MINNOR 1332-06) equipped with a set of suitably selected series of three sieves of various apertures of the following sizes: 100 µm, 200 µm and 355 µm; and selected on the basis of the previous par-

ticle size analysis by Mastersizer 3000 (Malvern Instruments, Orsay, France). For that, 100 g of powder sample was weighed and put on the first sieve and sieve shaker vibration amplitude was set at 0.5 mm for 10 min. The quantity retained on each sieve was collected, when stored in polyethylene bags at room temperature (25˚C ± 2˚C) until they were used for essential oils extraction.

2.2. Isolation and Qualitative and Quantitative Analysis of Essential Oil

The EOs were extracted from each powder fraction (≤100 µm, 100 - 200 µm, 200 - 355 µm, ≥355 µm and Unsieved powder) of *E. camaldulensis* leaves using an adapted device of Clevenger's hydrodistillation method operating in ambient conditions for 5 hours. Apparatus consist of one round bottom flask of 1000 mL which is connected with another two way round flask which holds raw material. The top flask is connected with condenser through the connecter. The separating funnel is used for the separation of essential oil and water. The EO were collected by decantation was filtered through a column of anhydrous sodium sulfate. Obtained EO were introduced into dark bottles and stored at 4˚C for subsequent analysis.

The identification and quantification of chemical constituents of essential oils were performed on a Varian CP-3380 type chromatograph equipped with a flame ionization detector and a capillary column (30 m × 0.25 mm) with a stationary apolar phase of methylsilicone type (DB5, film thickness 0.25 µm) and a quadrupole type detector (ionization energy 70 eV).

Analytical Conditions

The oven was programmed from 50˚C - 200˚C with a temperature gradient of 5˚C/min. The injector and detector temperatures were set at 200˚C and 200˚C, respectively. Nitrogen was used as the carrier gas with a flow rate of 1 mL/min. A sample of 1% solution of the essential oil in ethanol (1 µL) was injected in split mode (split ratio, 1:30). The retention indices of the constituents were determined relative to the retention times of a series of n-alkanes and their relative percentages calculated by electronic integration without taking into account their response factors. The coupling gas chromatography-mass spectrometry was carried out using an apparatus of the brand Hewlett-Packard HP 5970 A, equipped with an apolar capillary column (30 m × 0.25 mm) in fused silica of type HP-1 (film thickness 0.25 µ) and a quadrupole type detector (ionization energy 70 eV). The temperature of the injector was 220˚C and that of the interface area was 210˚C. The oven temperature was programmed from 70˚C to 200˚C with a gradient of 10˚C·min^{-1}. The carrier gas is helium with a flow rate of 0.6 mL·min^{-1}. The acquisition was made in scan mode (35 - 300 amu) at 2.96 scan·sec^{-1}. The components were identified on the basis of their retention indices and their mass spectra by comparison with data from NIST (National Institute of Standards and Technology) [28] [29]. The percentages of the compounds were calculated from the GC peak areas, using the normalization method.

2.3. Animal Treatment

Three months old Wistar rats (*Rattus norvegicus*) male weighting 250 - 300 g were used for the experiment. The inbreed colonies of rats were raised at the animal house of Laboratory of Biophysics, Food Biochemistry and Nutrition (LABBAN) of Ngaoundéré University, Cameroon. The rats were maintained at room temperature (25˚C ± 4˚C) in standard cages and kept under natural day and night cycle. The animals will feed on standard laboratory animal diet [30] and water *ad libitum*. And all experimental protocols were approved by the University Animals Ethical Committee and carried out with approval from the Cameroonian National Ethics Committee Ref. No FWIRD00001954.

For that, thirty five Wistar rats were randomly organised in group of 5 rats per cage; and treated according to the protocol described by [31]. In total, 7 rat groups were formed that are control group, 1 negative control group, 1 positive control group, and 5 experimental groups (receiving the EO from different particle sizes (≤100 µm, 100 - 200 µm, 200 - 355 µm, ≥355 µm and unsieved powder) of *E. camaldulensis* leaves. Treatment procedure is follows:

***Group* 1** (negative control): Received saline solution at 1 mL/Kg bw for 7 days once daily followed by CCl_4 alongside olive oil at 1 mL/Kg bw, intraperitoneally On the 7th day and in addition to normal animal diet and water *ad libitum*.

***Group* 2** (normal control): Received saline solution at 1 mL/Kg bw, intraperitoneally for 7 days once daily and in addition to normal animal diet and water *ad libitum*.

***Groups* 3 - 7** (experimental rat groups): Received EO of *E. camaldulensis* from different powders particle sizes (≤100 µm, 100 - 200 µm, 200 - 355 µm, ≥355 µm and unsieved powder) at 50 mg/Kg bw *per os* for 7 days once daily followed by CCl_4 alongside olive oil at 1 mL/Kg bw intraperitoneally on the 7th day and in addition to normal animal diet and water *ad libitum*.

All the animals were treated as shown above for a period of 7 days. Before foods being given to rats, each EO sample was previously suspended in a saline water by stirring using a magnetic stirrer at 3500 rpm for 30 minutes until to obtain a white emulsion. Saline water (negative control and normal control rat groups) and suspend EO in saline solution (experimental rat groups) were administrated every day for seven days. On the 7th day, 1 h after the last dose of EO samples, rats were treated intraperitoneally with CCl_4 alongside olive oil (0.5:0.5, v/v) which will serve as it mediator and for conveniences [32]. During this period of treatment, the rats were maintained under normal diet and water. Twenty-four hours after the dose of CCl_4 injection, all animals were anesthetized by light ethyl ether inhalation. After cardiac puncture in the neck region of the animals, 1 - 2 mL of blood samples were quickly collected from the carotid arteries on three animals from each group which were selected randomly. Collected blood in heparin tubes was centrifuged at 3500 rpm for 15 min under cool temperature of 4˚C for serum separation. The separated serum samples were subsequently kept frozen at −4˚C until they were used for biochemical analyses. After

collection of blood samples, the rats in different groups were also sacrificed by dissection and their livers were removed carefully; and washed immediately with ice cold saline to remove as much blood as possible. Liver homogenate was prepared from 1 g of liver tissues which homogenized in 5 mL of phosphate buffer (pH 7.4) using a china mortar. Liver homogenate was subjected to centrifugation (3500 rpm for 15 min) to separate serum, and was kept frozen at −4˚C until used to determination of oxidative stress markers.

2.4. Serum Biochemical Parameters Determination

The parameters of the lipid status of each rat were determined by measuring the levels of triglyceride (TG), total cholesterol (CT), HDL cholesterol and LDL cholesterol as well as the measurement of the activity of the enzymes which mark hepatocellular lesions (alanine aminotransferase, ALT and aspartate aminotransferase, AST) using commercially available kits based on the wellestablished spectrophotometric methods, according to the manuals supplied. Triglycerides and cholesterol were quantitated by enzymatic colorimetric methods. Glycerol phosphate oxidaseperoxidase (GPO-POD) and cholesterol oxidaseperoxidase (CHOD-POD) methods were applied for determination of triglycerides and cholesterol, respectively. AST and ALT activities were performed according to standard IFCC methods.

2.5. Determination of Oxidative Status Markers in the Liver

Lipid peroxidation (malondialdehyde content), catalase (CAT) activity and superoxide dismutase (SOD) activity were determined in the organ homogenates and blood plasma as recently reported [25].

2.5.1. Lipid Peroxidation

100 µL of homogenate liver, 400 µL of TBA reagent, and 80 µL of HCl were successively introduced into a test tube. Mixture was vortexed and incubated in a boiling water bath for 15 min. After cooling in a cold water bath for 30 min, mixture was centrifuged at 603 g for 15 min. Absorbance of collected supernatant was read at 530 nm using UV-visible spectrophotometer. Results was expressed as malondialdehyde (MDA) content in µmol/mg protein using molar extinction coefficient of MDA ($\varepsilon = 1.56$, 105 $mol^{-1} \cdot cm^{-1}$).

2.5.2. Estimation of Antioxidant Enzymes

SOD activity: 2.5 mL of 0.1 M carbonate buffer solution at pH 10.2 was added to 0.2 mL of liver homogenate or blood plasma. Then, 0.3 mL of adrenalin solution prepared at 5 µg/mL in water was added to the mixture to trigger the reaction and whole was vortexed. Absorbance was measured every 30 s until 150 s in order to follow an increase of absorbance at 480 nm. 0.3 ml of distilled water was used in a reference tube. Calculated SOD activity was expressed as units per milligram of protein.

CAT activity: One milliliter of 0.1 M phosphate buffer at pH 7.4 and 0.4 mL

of 0.2 M H_2O_2 were added to 100 µL of liver homogenate or blood plasma contained in tube. The reaction was stopped at 30, 60 and 90 s by adding 2 mL of dichromate/acetic acid mixture (5:95, v/v). Absorbance was measured at 620 nm, and CAT activity expressed in units per milligram of protein using molar extinction coefficient of CAT ($\varepsilon = 0.036$ $mmol^{-1} \cdot cm^{-1}$).

2.6. Statistical Analysis

Obtained data were recorded in Excel file, and analysis was carried out in triplicate. The experimental results were expressed as mean ± standard error mean deviation. Comparison between groups were performed statistically using One-way analysis of variance (ANOVA), followed by Duncan's multiple range test performed by Statgraphics to determine significant differences among the samples or intergroup variation, and $p < 0.05$ was considered as significant difference. Principal components analysis (PCA) was performed using XLSTAT 2016 to highlight correlation between studied essential oils samples and terpene contents.

3. Results

3.1. EO Chemical Composition

The contents of hydrocarbons and oxygenated terpenes in essential oils from different powder fractions of *Eucalyptus camaldulensis* Leaves were analysed by GC/MS and the results are presented in Table 1. The level of terpenic compounds in the EO from *E. Camaldulensis* leaves powders depends on the particle size. There was a significant difference ($p < 0.05$) between the terpenic compounds of the investigated EO from *E. Camaldulensis* leaves powders: EO from unsieved powder had maximum terpenic hydrocarbons (62.6%) followed by EO from powders with particle size of ≥355 µm (66.7%), 200 - 355 µm (58.2%) and 100 - 200 µm (53.6%), while EO from finer powder of ≤100 µm had the least amount of terpenic hydrocarbons. Contrarily, a significant ($p < 0.05$) decrease was observed in the levels of oxygenated terpenes with the particle size. The levels of oxygenated terpenes have increased significantly 1.90 and 1.24 times in EO from powders with particle size of ≤100 µm and 100 - 200 µm, respectively and as compared to that of EO from unsieved powder (37.4%).

Table 1. Contents of two terpenes compounds (%) present in essential oils from different powder fractions of *Eucalyptus camaldulensis* Leaves.

Terpenic components (%)	Essential oils samples from powder fractions:				
	≤100 µm	100 - 200 µm	200 - 355 µm	≥355 µm	Unsieved Powder
Terpenic hydrocarbons	29.1	53.6	58.2	66.7	62.6
Terpenic oxygenated	70.9	46.4	41.8	33.3	37.4

3.2. Effects of EOs on CCl_4-Induced Serum Biochemical Parameters

Table 2 shows the effect of treating CCl_4 induced hepatotoxicity in rats with essential oils from different powder fractions of *Eucalyptus camaldulensis* for 7days on serum lipid profile (Triglyceride, TG; total cholesterol, TC; high density lipoprotein, HDL; low density lipoprotein, LDL). After 7 days of experiment levels of TC, LDL, TG and HDL-C in négative control group were 170.6 ± 2.82 - 97.41 ± 3.37 - 95.71 ± 4.07 and 41.3 ± 3.02 mg/dl respectively. After the same period (7 days) with 50 mg/kg EO unsieved powder of *E. camaldulensis* treatment, TC, LDL and TG decreases to 130.70 ± 10.32 - 60.96 ± 4.76 - 70.70 ± 1.22 mg/dl, while HDL-C increase to 55.6 ± 1.6 mg/dl compared to the negative control group. EO fraction ≤100 µm was the most effective to restoring the balance of CT, LDL, TG (126.28 ± 6.96 - 63.09 ± 3.77 - 58.67 ± 8 .76 mg/dl) and stimulated HDL-C (62.86 ± 3.44 mg/dl).

The hepatotoxic agent, CCl_4, caused significant liver damage as indicated by an increase in the level of liver chemistry biomarkers such as; AST and ALT (Table 2). The results show a significant increase 13,13 and 23,33 times in serum levels (684.60 ± 18.81 and 2972 ± 133.59 U/I) of ALT and AST in the negative control group compared to the normal control group (52.13 ± 5.39; 127.36 ± 6.07 U/I). After administration of the EOs at a dose of 50 mg/Kg, a slight decrease in the activity of transaminases was observed in all the groups treated with EO compared to the group treated only with CCl_4. The strongest inhibition is achieved by the EO of the fractions ≤100 µm (213.74 ± 14.74; 964.83 ± 9.95 U/I) and 100 - 200 µm (311.51 ± 12.9; 1324.98 ± 90.97 U/I) compared to the other fractions. These results are similar to those obtained by Raskovic *et al.* [31]

Table 2. Serum lipid profile of carbon tetrachloride-induced hepatotoxicity rats administered with EO from powder fractions of *Eucalyptus camaldulensis* Leaves.

Rat groups	TG (mg/dl)	TC (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	AST (U/l)	ALT (U/l)
Negative control	$95.71^{e} \pm 4.07$	$170.65^{e} \pm 2.82$	$41.30^{a} \pm 3.02$	$97.41^{e} \pm 3.37$	$2972.87^{g} \pm 133.59$	$684.60^{h} \pm 18.81$
Normal control	$83.78^{d} \pm 2.47$	$152.81^{f} \pm 3.55$	$47.35^{bc} \pm 4.34$	$80.7^{de} \pm 3.01$	$127.36^{a} \pm 6.07$	$52.13^{a} \pm 5.39$
≤100 µm	$58.67^{ab} \pm 8.76$	$126.28^{a} \pm 6.96$	$62.86^{f} \pm 3.44$	$63.69^{a} \pm 3.77$	$964.83^{b} \pm 9.15$	$213.74^{d} \pm 14.74$
100 - 200 µm	$58.15^{ab} \pm 2.16$	$133.53^{bc} \pm 9.68$	$53.23^{cd} \pm 4.49$	$68.67^{b} \pm 2.48$	$1324.98^{c} \pm 90.97$	$311.51^{e} \pm 12.9$
200 - 355 µm	$74.10^{c} \pm 3.07$	$136.98^{cd} \pm 4.37$	$44.61^{ab} \pm 2.26$	$77.55^{c} \pm 1.60$	$2419.33^{f} \pm 22.96$	$400.23^{g} \pm 8.06$
≥355 µm	$53.83^{a} \pm 8.47$	$134.6^{bc} \pm 6.97$	$48.58^{bc} \pm 5.39$	$75.26^{c} \pm 4.13$	$1638.03^{e} \pm 45.0$	$386.86^{f} \pm 7.97$
Unsieved powder	$70.7^{c} \pm 1.22$	$130.7^{ab} \pm 10.32$	$55.6^{de} \pm 1.60$	$60.96^{a} \pm 4.76$	$1474.81^{d} \pm 66.18$	$180.51^{b} \pm 6.59$

Each column represents a mean ± SEM of 3 animals. Means followed by the same superscripted letter (a, b, c, d, e, f, g) were not significantly different ($p < 0.05$). Triglyceride, TG; total cholesterol, TC; high density lipoprotein, HDL; low density lipoprotein, LDL; alanine aminotransferase, ALT; aspartate aminotransferase, AST; standard error mean, SEM.

who demonstrated a significant increase in transaminase activity in rats treated with CCl_4 injection after 24 hours.

3.3. Effects of EO on CCl_4-Induced Oxidative Stress

Figures 1(a)-(c) shows the effect of treating CCl_4 induced hepatotoxicity in rats

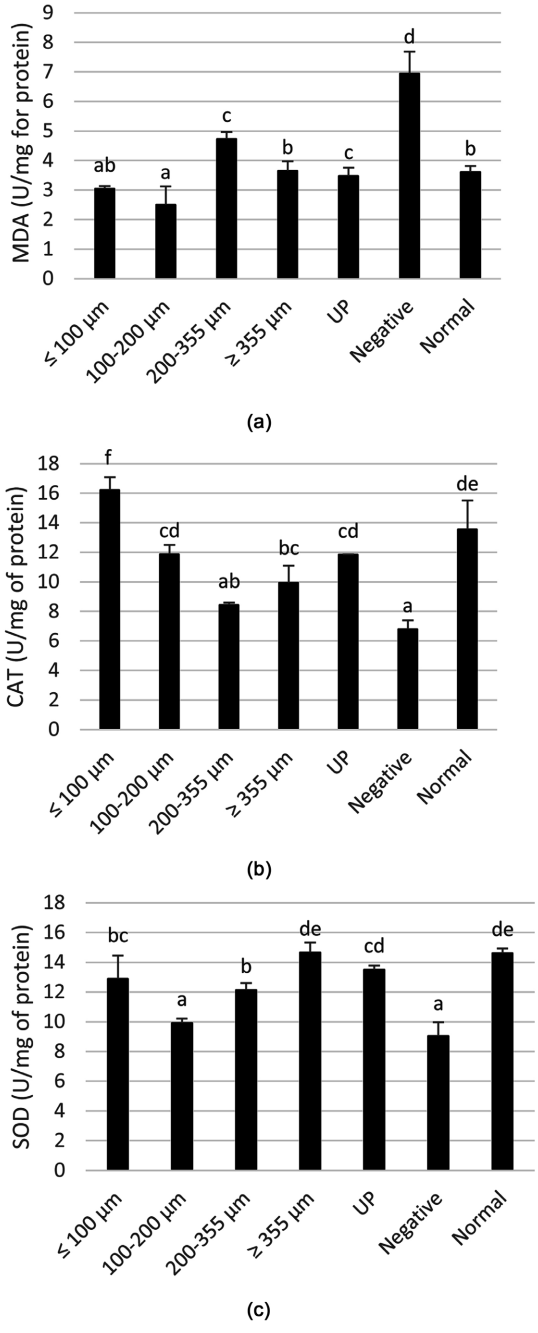

Figure 1. Effect of treating CCl_4 induced hepatotoxicity in rats with essential oils from different powder fractions of *Eucalyptus camaldulensis* liver tissue homogenate antioxidant enzymes and lipid peroxide. Each band represents a mean ± SEM of 3 animals. Means followed by the same letter were not significantly different ($p < 0.05$). Malondialdehyde, MDA; Catalase, CAT; Superoxyde dismutase, SOD; Unsieved powder, UP.

with essential oils from different powder fractions of *Eucalyptus camaldulensis* liver tissue homogenate antioxidant enzymes and lipid peroxide. The results show a significant increase in the hepatic level (6.94 ± 0.74 U/mg of protein) of MDA in the negative group 1.92 times compared to the normal control group (3.60 ± 0.21 U/mg of protein). Furthermore, it is observed that the administration of EOs to the animals corrected the level of MDA relative to the negative control group. The fractions ≤100 µm and 100 - 200 µm (3.04 ± 0.09 and 2.50 ± 0.62. U/mg of protein) were the most effective. However, the levels of Catalase (6.78 ± 0.60. U/mg of protein) and SOD (9.04 ± 0.94. U/mg of protein) are relatively low in the negative control group compared to the normal control group 13.55 ± 1.95 and 14.61 ± 0.31 U/mg of protein respectively. In animals treated with EOs the activities of CAT and SOD increased slightly. Of all the samples, the fraction ≤100 µm shows the highest catalase and SOD activity (16.20 ± 0.89 and 12.88 ± 1.57. U/mg of protein) followed by the fraction 100 - 200 µm (11.86 ± 0.65 and 14.65 ± 0.68. U/mg of protein).

3.4. Principal Component Analysis (PCA)

The principle of this analysis is based on the correlation between the variables for which the virtual axes generated (F1 and F2) are linearly correlated.

Figure 2 shows the fractions in the F1 × F2 axis system. We can see in this figure correlation circles which illustrate the correlations between the variables analyzed and the particle size fractions. The PCR makes it possible to distinguish 2 groups of fractions constituted for group 1 of the Fractions ≤100 µm and 100 - 200 µm and the unieved powder. For group 2 of the fractions 200 - 355 µm, ≥355

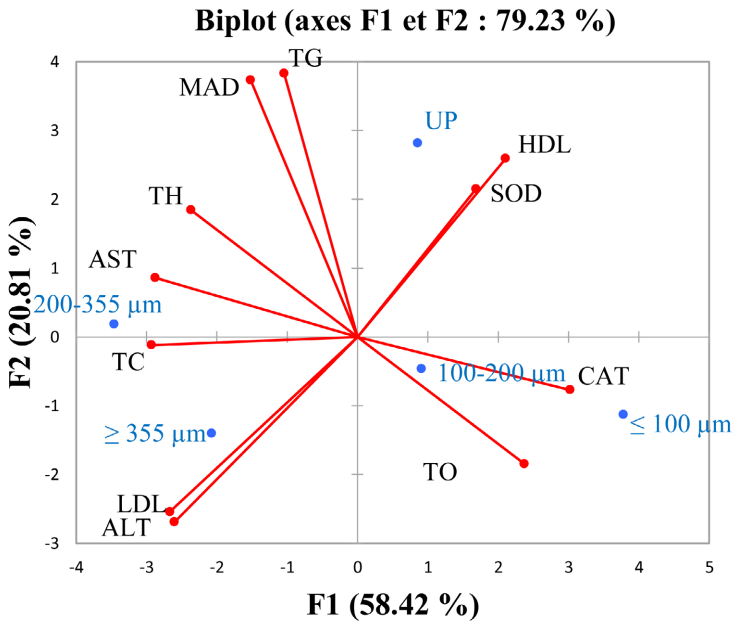

Figure 2. Distribution of particle size fractions and variables on the F1 × F2 axis system. TO: terpenic oxygenated; TH: terpenic hydrocarbon; UP: Unsieved powder.

μm. This figure shows that the levels of oxygenated terpenes are higher in the group 1 fractions; which would give them the highest catalase activity and SOD. On the other hand, these contents are low in the group 2 fractions. Hence the weak antioxidant enzymatic activities and the lowest MDA reduction rate.

4. Discussion

In this research, we determined the chemical composition of the studied EO and its potential to ameliorate liver injury induced by pro-oxidant agent CCl_4 According to this result, it must be recognized that production of plant powder followed by EO extraction are accompanied by physicochemical alteration induced by oxygen or heat (brought to plant during drying or grinding process and hydrodistillation) and can result in autooxidation of EO components. Indeed, temperature, light as well as oxygen availability are considered to exert the most decisive impact on essential oil stability [33] [34] [35]. As terpenic components tend to be both volatile and thermolabile and may be easily oxidized or hydrolyzed depending on their respective structure [36], it is well accepted that the chemical composition of essential oils is moreover dependent on the conditions during processing and storage of the plant material, upon distillation as well as in the course of subsequent handling of the oil itself [37] [38]. The contact of EO components with oxygen can occur during plant grinding followed by sieving operations which would induce the release of EO with increase of the powders surface contact with environment and allow oxidation induced by light and oxygen. Then, oil oxidation accelerates with the concentration of dissolved oxygen, which in turn depends largely on oxygen partial pressure in the headspace. Really, a reduction in the powder particles size increases the contact surface with air-oxygen which promotes the oxygenation and hydroxylation reactions of the carbon double carbon bond favoring the transformation of hydrocarbon terpenes into oxygenated terpenes [39] and with atmospheric oxidants (hydroxyl radical OH, Nitrate NO_3, Ozone O_3) react with terpenes promoting the self-oxidation process [40]. For example, d-limonene could react with the OH radical to form *α*-terpineol. In the course of this spontaneous, air-induced oxidative process, unsaturated molecules react in a free radical chain mechanism with aerial oxygen into a range of primary and secondary oxidation products. As a result, the EO self-oxidation process is more important in small powder particles compared to large powder particles. In conformity with our previous studies [26], this results clearly points out that the reduction in the levels of terpenic hydrocarbons, particularly *α*-pinene and limonène in the EO of ≤100 μm and 100 - 200 μm powder fractions. On the other hand, it was reported that the particle temperature in the grinder surrounding can rise up to 90˚C, attesting the deleterious impact of grinding process on extracted EO. Terpenoids, especially mere terpenes and aldehydes, are commonly known to be thermolabile and susceptible to rearrangement processes at elevated temperatures. Terpenic conversion reactions upon heating have been reported both for isolated compounds

[41] as well as for essential oils [42]. Upon temperature increase, dominant alterations in essential oils from cardamom, clove bud, lavender, pine, and rosemary were revealed in decreasing amounts of terpenic hydrocarbons such as β-caryophyllene, β-myrcene, β-pinene, sabinene, or γ-terpinene and an overall rise of p-cymene [35]. Additionally, ultraviolet light and visible light are considered to accelerate autoxidation processes by triggering the hydrogen abstraction that results in the formation of alkyl radicals [43]. Especially monoterpenes have been shown to degrade rapidly under the influence of light [44]. It was true for lemon oil in which decreasing amounts of geranial, terpinolene, and γ-terpinene together with a rise in p-cymene have been observed [45].

Ketones, epoxides, peroxides as well as acids are often allylic hydrogen carrier polymers [34]. Compounds rich in allylic hydrogen atoms make up most probable targets for autoxidation considering that hydrogen atom abstraction is giving rise to resonance-stabilized radicals highly favored due to lower activation energy [46]. Polyunsaturated terpenic hydrocarbons that exhibit the structural preconditions to form several radicals stabilized by conjugated double-bonds or isomerization to tertiary radicals are therefore particularly prone to oxidative deterioration [47]. Generally, treatment with CCl_4 resulted in significant increase in all lipids parameters (TC, LDL and TG) except of HDL-C. These results are similar to those obtained by El-Hadary *et al.* [48] who found a decrease in serum lipids CT, TG, LDL, VLDL and an increase in HDL after treatment of animals with EO of *Syzygium aromaticum* at doses of 100 and 200 mg/kg. Lipids play an important role in the incidence of liver disease. This study reveals that CCl_4 treatment induces a disruption of lipid metabolism (CT, TG, LDL and HDL). The increase in CT, LDL and TG is due to radicals generated from CCl_4 which damage the ER, leading to reduced protein synthesis and lipid accumulation in the liver [49] [50]. In addition, Pan *et al.* [51] described that exposure to CCl_4 leads to covalent modification of MTP (microsomal triglyceride transfer protein) and its degradation by proteasomes. This leads to an increase in the accumulation of triglycerides and cholesterol in the tissues causing steatosis.

The hepatoprotective effects of EO were investigated by measuring ALT and AST activities, as enzyme markers of hepatocellular damage, in serum. CCl_4-induced hepatic injury is a commonly used experimental animal model. A previous study reported that CCl_4 administration is associated with activation by the cytochrome system to form trichloromethyl radicals ($CCl_3^{\bullet}$). It is generally thought that CCl_4 toxicity is due to a reactive intermediate that is generated by its reductive metabolism. This highly reactive intermediate is known to induce lipid peroxidation, oxidative stress, hepatic necrosis and apoptosis [52]. It is well known that CCl_4, as a highly toxic and pro-oxidant agent, produce the damage of hepatocytes to cause large increases in ALT and AST activities and elevation in bilirubin content in serum. In our study, intraperitoneal injection of CCl_4 induced a notable increase in ALT and AST activities in the negative control group and confirmed the hepatic damage. ALT and AST are important metabolic en-

zymes of the liver. These enzymes normally exist in the cytoplasm, but upon liver injury, they can enter the circulatory system due to toxicity-mediated altered permeability of the cellular membran [53].

EO was found to exert hepatoprotective effects in single dose (50 ml/mg) after seven days by diminishing AST and ALT activities in serum, as the most specific markers of liver injury, compared to negative group. Similarly, results of the study by Sotelo-Félix *et al*. [54] and Gutiérrez *et al*. [5]. Fine fractions (≤100 and 100 - 200 μm) show the best inhibitions of transaminases compared to large particles (200 - 355 μm and ≥355 μm). These results could be explained by the fact of the high concentration in terpenic oxygenated in the fine particles compared to large particles. Indeed, according to the work of Wilson and Hrutfiord [55] terpenes which have a small number of functional groups (hydroxyl and carbonyl) have a reduced activity in metabolic reactions. In addition, the molecular structure but also the presence of oxygen in the terpenic molecules give them a greater activity [56]. According to Ouraini *et al*. [57] the order of biological activity of the terpenic compound according to their funtional group is a follows: Phenol, Alcohol, Aldehyde, Oxide, Hydrocarbon, and Ester.

MDA is one of the well-known secondary product of lipid peroxidation. In this study, EO significantly decrease MDA level. These results are similar to those obtained by Raskovic *et al*. [30] and Hsiao *et al*. [58] who found respectively a significant inhibition of the plasma level of MDA in rats treated with CCl_4 intraperitoneal injection at dose 1 ml/kg and with the rosemary EO (10 mg/kg) on the one hand and inhibition of the activity of CAT in presence of free radicals in the rats treated with CCl_4 on the other hand. The levels of MDA suggest enhanced peroxidation leading to tissue damage and failure of the antioxidant-defense mechanisms to prevent the formation of excessive free radicals [59] [60]. However, EO at 50 mg/kg could markedly prevent the increase in MDA formation, which clearly demonstrated the ability of EO to relieve lipid peroxidation. MDA is well known to be the most abundant individual aldehyde resulting from lipid peroxidation and is commonly used as an indicator of liver tissue damage involving a series of oxidative chain reactions [61].

In this study, the CCl_4 treatment for 1 day increased the lipid peroxidation and liver enzymes, and also decreased the antioxidant enzyme levels. It has been suggested that the lipid peroxidation may be a link between tissue injury and liver fibrosis by modulating collagen gene expression [62]. It was reported that CCl_4 is suitable to induce lipid peroxidation in experimental animals within a few minutes after administration and its long-term use results in liver fibrosis and cirrhosis by lipid peroxidation pathway [63]. It is generally thought that CCl_4 toxicity is due to reactive free radical ($\dot{C}Cl_3$), which is generated by its reductive metabolism by hepatic cytochrome P_{450}. The reactive intermediate is believed to cause lipid peroxidation and breakdown of cellular membranes [64]. The best decrease in MDA levels in the groups ≤100 μm, 100 - 200 μm can be explained by the fact that in these groups, there is an increase in the concentra-

tion of oxygenated terpenes in the essential oils of said fractions. Oxygen molecules are final electron accepting substrates, important in reactive oxygen species (ROS) reduction reactions. Indeed, the presence of oxygen is a determining element of the metabolic pathways of terpenes. In these pathways, the oxygen molecules are final electron accepting substrates with production of CO_2 from the carbons derived from terpenes [65]. According to Packer *et al*. [66], the most important pathway in the elimination of trichloromethyl radicals is its reaction with molecular oxygen. From this point of view, we admit that the oxygen of terpenes reacts with the trichloromethyl radical by reducing it into more stable molecules from the redox reactions of substitution or addition, thereby neutralizing the production of free radicals resulting from metabolism, which reflects the protective effect of these EOs against the lesions of hepatocytes induced by CCl_4. In addition, the 1,8-cineole (oxygenated terpene) majority component of the EO of fine particles has an antagonistic effect on lipid peroxidation in rats exposed to nicotine [67]. Oral administration of Borneol 50 mg/kg has been shown to inhibit lipid peroxidation in rats [68]. Furthermore, body has an effective defense mechanism to prevent and neutralize the free radical induced damage by a set of endogenous antioxidant enzymes such as SOD and CAT. These enzymes constitute a mutually supportive defense team against ROS. Lipid peroxidation, a ROS-mediated mechanism, has been implicated in the pathogenesis of various liver injuries and subsequent liver fibrogenesis in experimental animals. SOD and CAT are antioxidant enzymes, they trap oxygenated species and inhibit their production to keep the oxidant/antioxidant ratio in balance to avoid the installation of oxidative stress in the body. CAT has an important role in the elimination of free radicals derived from the redox process of xenobiotic molecules in the liver, it is easily inactivated in the presence of peroxidized lipids [69].

The Pearson correlation matrix between the elements with antioxidant potential and the activity of SOD and CAT makes it possible to note that the variable oxygenated terpenes and the activity of SOD and catalase are positively correlated, in particular with regard to the activity of SOD and CAT. The strong correlation between SOD and CAT would be due to the fact that these two enzymes have synergistic mechanisms, in fact, SOD transforms the superoxide anion into H_2O_2 and CAT transforms the H_2O_2 formed into water molecules [70] [71]. This could be justified by the fact that the oxygenated terpene compounds present induced the synthesis of these antioxidant enzymes. It also appears from this table that the variables MDA and oxygenated terpenes are negatively correlated which illustrates the antagonistic effect of oxygenated terpenes on the level of MDA. Furthermore, there is a strong negative correlation between the variables CAT and AST on the one hand and between the variables CAT and ALT on the other hand. This result could be explained by the fact that: in the fractions containing the oxygenated terpenes in small percentage, the stimulation of the intracellular antioxidant enzymes by the hydrocarbon terpenes are less important. Consequently, the hepatocytes are less protected against lesions due to ROS re-

sulting from the metabolism of CCl_4, which justifies a high activity of the transaminases.

5. Conclusion

In summary, the results demonstrate that the administration of EO of *E. camaldulensis* leaves has a Hepatoprotective activity and oxidative stress reduction against lesions and toxicity caused by ROS derived from the metabolism of CCl_4. Hepatoprotective and antioxidative effect is correlated with the chemical composition and particle size of the different EOs. The ≤100 μm and 100 - 200 μm fractions significantly inhibited lipid peroxidation better than unsieved powder and protected the liver of rats from hepatocellular damage. Finer powders could be used as functional ingredients in food formulations for the management of chronic diseases. In order to better appreciate the effects of particle size fractionation on the antioxidant and hepatoprotective properties of EO, it would be wise to vary the doses to determine the most effective.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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