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Der Pharmacia Lettre, 2017, 9 [6]:49-62
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ISSN 0975-5071
USA CODEN: DPLEB4

THE MECHANISM OF ANTIBACTERIAL ACTION AND THE ANTI-OXIDATIVE ACTIVITIES OF THE ESSENTIAL OIL OF CARUM MONTANUM (COSS. ET DUR.) BENTH. ET HOOK. AERIAL PARTS FROM ALGERIA

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ABSTRACT

In order to discover the virtues of untapped local plants, the essential oil (EO) of a local species (Carum montanum) has been evaluated for its antibacterial and anti-oxidative qualities. The EO was obtained by hydrodistillation by a Clevenger apparatus. This EO was screened for antibacterial activity against 11 ATCC Bacterial strains by agar diffusion method. MICs of the EO against the susceptible strains were determined by both macro and micro-dillution. The mechanism of action of the EO was determined on the susceptible strains by the time kill assay and the lysis experience. Anti-oxidative properties were studied by both free DPPH radical scavenging and reducing power techniques. B. cereus was the most sensitive strain with MIC of 55.5 µg/ml followed by K. pneumoniae (111 µg/ml). A remarkable decrease in the survival rate as well as in the absorbance at 260 nm was recorded, which suggest that the cytoplasm membrane is one of the targets of the EO. Anti-oxidative effect was concentration dependent and IC50 values was 1.09 ± 0.37 µg/ml by DPPH method and a reducing power dose-dependent. In conclusion, C. montanum EO showed potent antimicrobial and anti-oxidative activities and have acted on the cytoplasm membrane. These activities could be exploited mostly in food preservation.

***Key Words:** C. montanum, antimicrobial activity, time kill assay, anti-oxidative activity*

INTRODUCTION

The Apiaceae/Umbelliferae is a large and taxonomically complex family of flowering plants. They are relevant in a number of very different scientific fields, and have many uses. They are, also, of great interest to taxonomists, florists, ecologists, phytosociologists, phytochemists, horticulturists, as well as professionals involved in medicinal plants, aromatic, food industry and gastronomy. Common caraway is one of the oldest herbs known [1] and the genus Carum was established since 1753[2]. It is naturally found in Asia, central Europe, North Africa [1], Siberia and Turkey [3]. The genus Carum is an important genus of the Apiaceae family, and contains about 20-30 species. The best-known species of this genus is Carum carvi [4]. This herb was first used by the ancient Arabs and Pliny recommended it for hysterical complaints and pale complexions [3]. Caraway seeds are used as a flavoring of bread, cheese, sauerkraut, candies, meat products and sauces and as a source of carvone for cosmetics, toothpaste, chewing gum and pharmaceutical preparations. The seeds have been used in alternative medicine as a laxative, in colic treatment and as a breath freshener. The seeds have been found to have antipasmodic, carminative, emmenagogue, expectorant, galactagogue, stimulant, stomachic and tonic properties [3]. C. montanum (syn. Selinopsis montana Coss. et Dur.) grows wild in calcareous mountainous regions, such as the Constantine Tell and Saharian Atlas, as well as in the Kabyle and Numidian areas. It is characterized by smooth stems (10-15 cm), white flowers and an oblong fruit (2-2.6 mm) [5]. Carum carvi, the generic type of this genus, has been widely studied regarding its chemical constituents and biological activities but there seems to be no report on C. montanum. Contrary to C. carvi, this species has neither medical nor culinary applications, but is commonly grazed by the livestock [6]. In developing countries, as Algeria, people tend to use medicinal plants for their affordable prices as well as for their safety. In order to screen new bioactive chemicals from local wild plants, we have investigated antimicrobial and anti-oxidative properties of the essential oil of this species for possible use in therapeutics.

MATERIALS AND METHODS

Plant material

The aerial parts of C. montanum were collected from the mountain Megress (located about twenty kilometers northwest of the capital of Sétif) at an altitude of 1500 m above sea level. The plant was identified by Pr H. Laouer (Laboratory of Valorization of Natural Biological Resources, University of Sétif, Algeria) then they were freed of impurities and dried in the shade at room temperature.

EO's extraction

To obtain the essential oil (EO), the air-dried parts of the plant were cut into thin parts and were subject to a hydrodistillation for three hours using a Clevenger-type apparatus. The oil was stored in refrigerator (4°C), until use.

Antibacterial activity

Agar diffusion method

A preliminary antibacterial activity of the EO was determined by the agar diffusion method using the six mm diameter discs. Briefly, The Mueller-Hinton Agar (MHA) was seeded by swab with a young culture of 0.5 Mc Farland density [7]. Then discs impregnated with a volume of 10 µl of different dilutions are deposited on the seeded Petri dish which is pre-incubated for 1/2 h at room temperature, allowing the complete diffusion of the EO and then incubated at 37°C for 24 h. The antibacterial activity was determined by measuring the inhibition zone diameters (mm). Gentamicin (GM), with a charge of 10µg, was used as a positive control.

Determination of MICs

Agar dilution method

A solution of sterilized Tween80 in distilled water (10%) was added to an amount of EO so that the ratio EO/Tween was 80/20 (v/v). The mixture was stirred for 2 to 3 minutes to disperse in the EO stock solution (S). Next, twofold series dilutions were made to obtain the range of dilutions. In test tubes each containing 18 ml of sterilized agar medium and kept molten at 50 °C in a water bath, was added, aseptically, 50µl of the solution S or various dilutions. After solidification of the medium, inoculating the agar, containing the EO or not (negative control) was performed on the surface. Seeding surface was made of a bacterial suspension with a density of 105 UFC/ml [8,9,10].

Broth micro-dilution method

This method involves the use of small volumes of broth dispensed into sterile plastic micro-dilution trays. A twofold dilution of the EO volumetrically in broth was made. Then, it was dispensed into the wells so that each well contained 0.1 ml. A standardized inoculum of 5 × 105 UFC/ml was inoculated in each well. The inoculated micro-dilution trays were incubated at 35 ± 2°C for 24 h [10].

Broth macro-dilution method

A standardized inoculum at 0.5 McFarland was prepared, then diluted in a broth such that the final density was 5 × 105 UFC / ml. 1 ml of the adjusted inoculum was added to each tube containing 1ml of extract in dilution series and mixed. A positive control was prepared containing only inoculated broth. The MIC is the minimum concentration of extract that completely inhibits the growth of microorganisms; it is presented by the first tube showing a disorder and is detected by the naked eye [10].

Mechanisme of the antibacterial activity

Time kill assay

This method allows the characterization of the antibacterial EO activity over time. It assesses the decrease of bacteria, which are subject to a given EO concentration over several hours. A standardized suspension of 108 UFC/ml was diluted on 1/20. A total of 1 ml of this inoculum was introduced into 9 ml of Muller-Hinton broth-Tween 80 (0.01%, v/v) in the absence (growth control) or in the presence of a concentration corresponding to the MIC of the EO in the liquid medium. The suspension obtained contained approximately 5 × 105 UFC/ml and was maintained under stirring at 37 °C. A total of 100 ml of the suspension were removed at different time (0, 2, 4, 6, 8 and 24 h) to carry out a counting on methionine hydroxy analog agar after incubation at 37 °C for 24 h. The quantification of the number of bacterial colonies was limited to the value of 102 UFC/ml. Results were interpreted by a bactericidal curve representing time intervals on the abscissa axis and the number of survivors on the ordinate axis [11, 12].

Bacterial lysis

This method determines if there is a bacteriolytic action of EO by measuring the absorbance at 620 nm [12]. Indeed, nonlysed bacteria absorb in 620 nm, so if there is a bacteriolysis, absorbance at 620 nm over time will decrease. A young bacterial suspension was standardized at 3.1010 UFC/ml (OD620 ~ 0.3), placed in a sterile tube in the absence (negative control) or in the presence of EO at two concentrations, one corresponding to the MIC and the other two times the MIC. Suspensions obtained were subjected to agitation. On time 0 s, 30 s, 30 min, 60 min, 90 min and 120 min, they were diluted to 1/100 and

absorbencies were measured at 620 nm. The results were expressed as the relative optical density (OD620) in each time interval.

Anti-oxidative activity

DPPH radical scavenging assay

The DPPH radical absorbs at 517 nm and the anti-oxidative activity can be determined by recording the decrease of the absorbance of the extracts. 50µl of each different EO dilution were mixed with 1250 µl of a methanol solution of DPPH (0.004 %). The absorbance was measured after 30 min of incubation in the dark. Synthetic antioxidant, BHT was used as positive control. Thus, the calibration curves representing the percentage of inhibition versus concentrations were performed using Graph-pad prism programs. The ability to scavenge DPPH radical is calculated as follows;

I% = [(Abs517 control–Abs517 sample) / Abs517control] x 100. The IC50 values were estimated by a linear regression. The values are presented at least as the mean of triplicate measures [13].

Reducing power assay

It is a technique that measures the reduction of Fe3+ (ferric iron) to Fe2+ (ferrous iron) in the presence of the extract tested. The presence of reducers in plant extracts causes the reduction of Fe3+ in a complex of ferricyanid to form ferrous iron Fe2+. Therefore, Fe2+ can be assessed by measuring the increase of the density of the green color in the reaction medium at 700 nm [14]. In a test tube containing 1 ml of EO dilution, was added 2.5 ml of phosphate buffer (0.2M, pH 6.6) and 2.5 ml of potassium hexacyanoferrate [K3Fe (CN)6] (10g/l). The whole is heated to 50 °C in water bath for 20 minutes. A volume of 2.5 ml of trichloroacetic acid (TCA) (100 g/l) was then added to stop the reaction. Finally, 2.5 ml of the supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of ferric chloride [FeCl3] (1g/l). A blank sample is prepared in the same conditions. The absorbance was read at 700 nm. BHT is used for the positive control. An increase in absorbance corresponds to an increase of reducing power of the extract tested [15, 16, 17]. The values are presented as the means of triplicate measures.

Statistical analysis

All experiments were done in triplicate and results were reported as mean ± SD. The results of various tests were analyzed by the Student t test for single comparisons and ANOVA followed by Tukey and Dunnett's test for multiple comparisons and for the determination of significance level. The analysis was done through “Graphpad Prism” version 5.0 software.

RESULTS AND DISCUSSION

Antimicrobial activity

Disc diffusion method

Carum EO, which is very rich in phenylpropanoids (Table 1) with 71.1%, exhibited a moderate non-selective activity against the tested strains (Table 2).

Table 1. Chemical composition of the essential oil of C. montanum

Chemical compound Percentage	Percentage

Phenylpropanoids	71.9
Oxygenated sesquiterpenes	0.2
Sesquiterpene hydrocarbons	15.4
Hydrocarbon monoterpenes	5.0
Other compounds identified	1.9
Other unidentified compounds	5.6

Table 02: Inhibition diameters in mm of C. montanum extracts

Microbial strain	**10 µl EO in**	**GM**
Pseudomonas aeruginosa (ATCC 27853)	-	8
Escherichia coli (ATCC 25922)	08 ± 0.00	22
Salmonella typhimurium (ATCC 13311)	11 ± 0.00	20
Acinetobacter baumanii (ATCC 19606)	11 ± 0.00	20
Staphylococcus aureus (ATCC 25923)	13 ±0.00	31
Klebsiella pneumoniae (ATCC 700603)	20 ±0.00	25
Bacillus cereus (ATCC 10876)	16 ± 0.00	19
Enterococcus faecalis (ATCC 49452)	12 ± 0.00	20
Lysteria monocytogenes (ATCC 15313)	-	19
Citrobacter freundii (ATCC 8090)	-	18

Proteus mirabilis (ATCC 35659)	8 ± 2.8	26
GM :gentamicin, -: no activity.		

The detailed analysis of the EO of *C. montanum* was previously reported [6]. it was mainly composed of phénylpropanoides (71.9%) and sesquiterpene hydrocarbon (15.4%). The predominance of a single compound in our EO (Nothoapiole) is not surprising but rather rare. According to **Bakkali et al., (2008)**, the main constituants of essential oils are terpenes, terpenoids and the other of aromatic and aliphatic constituents, all characterized by low molecular weight [18]. In a case similar to ours, EOs of the wild carrot (*D. carota*) is composed of 45.0% phenylpropanoids [19]. Although **Raut and Karuppayil (2014)** confirm the presence of phenylpropanoids more frequently in Apiaceae family [20]. According to **Garnero (1996)**, it is not unusual to recognize certain EOs that are very rich in a given constituent. This is the case of the Brazilian rosewood EO, rich in linalo, EO of badian, green anise and fennel rich in trans-anethole, EO *Eucalyptus globulus* are rich in 1,8-cineol [21]. Thus, *C. copticum* EO is constituted, in major part, by a monoterpenoid which is γ-terpineol (55.75%) [22]. The oil of the root bark of *Securidaca longepedunculata* containss more than 90% of methyl salicylate [23].

The non-selectivity of the antibacterial activity is due to the huge variety in composition of EOs which does not define any particular spectrum at each oil or even to classify them by adopting the conventional activity test Gram+ and Gram [24]. The susceptibility of different bacteria toward Carum's EO has been reported by several authors; C. carvi [25, 1, 26] and C. copticum [27]. The species B. cereus was most susceptible, which was the same with the EO of C. carvi [25], but S. aureus sensitive to this oil was moderately sensitive to C. carvi EO, from 17 different regions [26]. The α-pinene, the limonene, the β-pinene, the thymol, the γ-terpinene, the myrcene, the phellandrene, the geranial, the dillapiole and the cymene are present in our oil and they are considered as potential antimicrobials [28]. Generally, phenylpropanoids, may be the cause of the antibacterial activity because they have been isolated previously from a species known to exert a protective action against phyto-pathogens (bacteria and fungi) [29]. Noting that the activities observed by the EO were all bacteriostatic.

Determination of MICs

The MICs of the EO were evaluated only on the strains which gave inhibition diameters equal to or greater than 15mm. The inhibition in agar occurred in the highest concentration tested (35.80 µg/ml). *B. cereus* was the most sensitive strain with MIC of 55.5 µg/ml by broth micro-dilution method. Then came *K. pneumoniae* (111 µg/ml). Indeed, the MIC value cannot be determined exactly because of the spaced concentrations. However, we can say that the MIC is in the range where the minimum value is greater than the highest concentration where there was the bacterial development and lower than or equal to the lowest concentration where there was a complete absence of bacterial growth. The observed differences in MICs values can't be considered a significant difference in activity due to changes usually known and observed in these techniques, even using standardized methods [35]. For example, by comparing the micro-dilution technique with that of the E-test, there was different values of MICs, it was concluded that this phenomenon can be explained by the high frequency of spontaneous mutations and

loss of activity of antimicrobial agents unstable during incubation, which can lead to concentrations inhibitors that could encourage the emergence of resistant strains **[**30]. According to the **CLSI (2012)**, the MIC from the micro-dilution for Gram- bacilli are the same or lower than those from the macro-dilution as in the case of *K. pneumonia***[**10]. By testing the sensitivity of several strains overlooked antibiotics, it was found that the method of micro-dilution seems to be a simple and reliable method for the determination of MICs and it can offer an interesting alternative to the agar dilution method **[**31]. In fact, the choice of method to be used is made according to the advantages and inconveniences of each. The micro-dilution is economic in material as well as in extracts but delicate, macro-dilution is easy in the interpretation but requires quantities of the extract. The macro-agar dilution is fast and economical and can test several strains at once and contamination can be directly recognized but requires large amounts of extracts.

Mechanism of action of the EO

Time kill assay

It is found that the activity of the EO against the tested bacteria persists over time (**Figure 1**). This activity begins slowly, and then there is a rapid decrease leading to low concentrations, but without the total absence of viable forms of the bacteria.

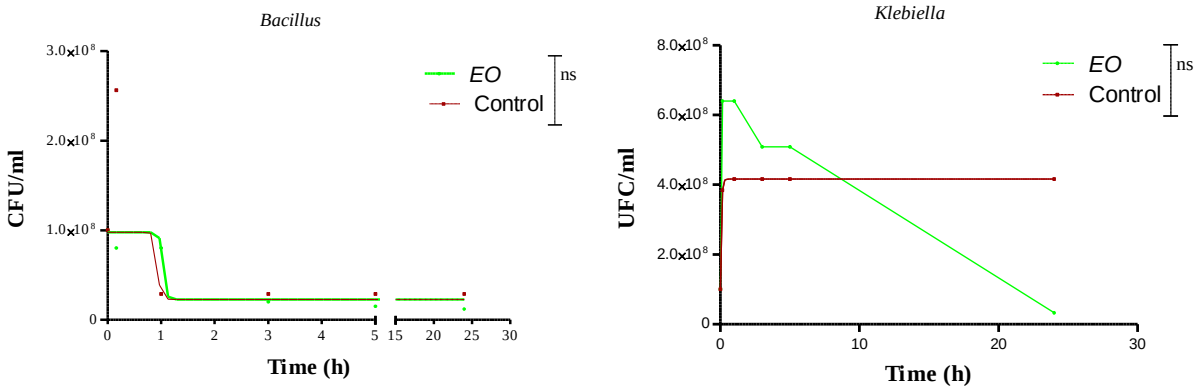

Figure 1: Time kill curves of bacterial strains exposed to *C montanum* EO.

The persistence of viable forms of the bacteria can be explained by the fact that the EO is bacteriostatic and not bactericidal. Despite the continuous agitation of the reaction medium, there is always the problem of miscibility, which shares the medium giving rise to the bacterial development away from the micelles of the EO. Indeed, the activity of the EO differs according to the growth phase; the action is lower during the stationary phase when the bacterium is in a state of rest, in the phase of decline the activity increases. These results showed that the treatment time and concentration of the EO had a great influence on the antibacterial effect.

Bacterial lysis

This experiment informs us if EOs act on the cell membrane thus inducing lysis. This is one of the frequent mechanisms caused by antibacterial agents. The results obtained are represented by curves (**Figure 2**) showing the relative percentage of the absorbance of the bacterial suspensions as a function of time. The decrease in absorbance is explained by a rate of cell lysis because only the living cells absorb at the wavelength of 620 nm, which is not the case for lysed cells. The absorbance measurements are carried out for two hours at different intervals. The relative absorbencies corresponding to the control strains represent an increase of more than 400% indicating the ordinary growth of the bacteria in the exponential phase. Then, a drop in absorbance appears in the stationary phase. Each strain has a different absorbance after one and a half hour of incubation. When the bacteria were exposed to concentrations corresponding to MICs, relative absorbance values decreased by 100% at lower but different values; 66.7% (*B. cereus*), 12.5% (*K. pneumoniae*). Only in the second case there was a continuous decrease until the end of the experiment. The incubation of the strains with concentrations corresponding to four times the MIC did not give significant differences ($P \leq 0.01$) in absorbencies.

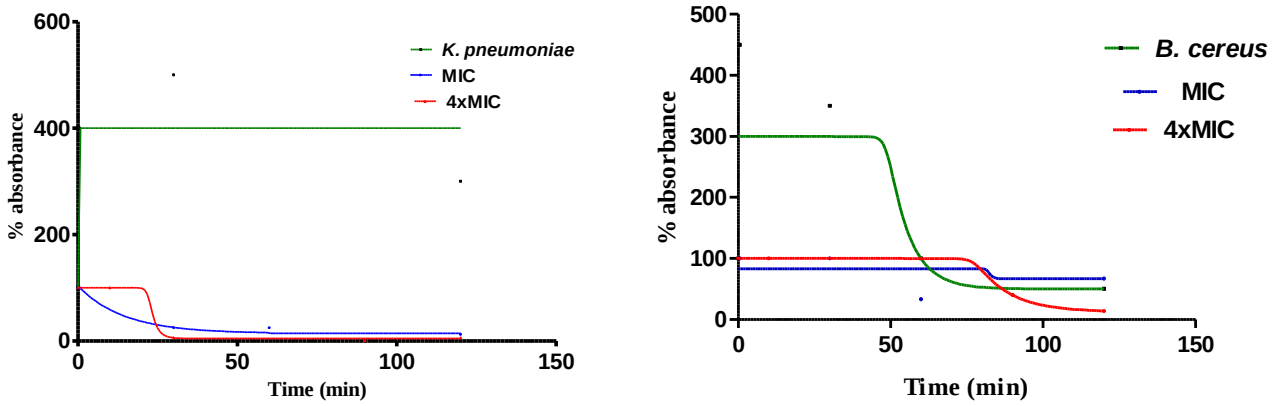

Figure 2: Curves of bacterial lysis of the bacteria exposed to the EO of *C. montanum*.

Hydrocarbon monoterpenes or sesquiterpenes and their oxygenated derivatives exhibit potential antimicrobial activity [32]. Interactions with the hydrophobic structures of the bacteria play a key role in the antimicrobial effect of these molecules [12]. Bacteria are less sensitive in the stationary phase than in the exponential phase. Since antimicrobial agents that act on the synthesis process often have small effects in the stationary phase, these results suggest that the primary EO target is not the synthesis of macromolecules [12]. The penetration of active plant compounds into the cytoplasmic membrane is likely to have a profound effect on the physical property of the phospholipid bilayer. This modification could interfere with transmembrane transport processes resulting in changes in the secretion of proteins associated with bacterial virulence in the surrounding environment [33]. Some antimicrobial agents cause large changes in the plasma membrane, causing complete cell lysis. Autolytic enzymes induce lysis. Although activation of auto-lytic enzymes may be responsible for this effect, lysis may also be due to

weakening of the cell wall and subsequent rupture of the cytoplasmic membrane due to osmotic pressure (Rather than a specific action on the membrane) [12].

Antioxidant activity

Scavenging ability on DPPH radicals

It is clear that the scavenging activity of the EO is dose-dependent (**Figure 3**). The IC_{50} value 1.09 ± 0.37 µg/ml showed that this EO was more active by comparing it with that of the BHT (87.26 ± 0.001 µg/ml), that is the same with *C. bulbocastanum* [34]. Despite these values, the difference remained statistically not significant at $p \leq 0.05$**.** Many studies on the antioxidant activities of EOs of a wide variety of aromatic plants show that these properties are related to their chemical composition, it is difficult to attribute these activities to one compound but that is the result of a synergistic effect between the various compounds. Some EOs has the potential to preserve food. Oregano EO, rich in Thymol and Carvacrol, had significant antioxidant effects on the oxidation process [36]**.** The activity of the EO of *C. bulbocastanum* was attributed to the Nothoapiole [34] which is the main component in *C. montanum* EO [6]. This value is very good compared to that obtained by the phenolic extract of *C. carvi* (IC_{50}: 2.7 mg/ml) [37] and the ethanol extract of *C. nigrum* (IC_{50}: 14 mg/ml), where antioxidant activity was also dose-dependent [38, 1]**.** However, the ME of *C. Carvi* from the Himalayas presented a higher activity than that of our extract with an IC_{50} of 6.9 µg/ml [44]**.** The antioxidant effect can be due to the presence of hydroxyl groups in their chemical structures **[**36].

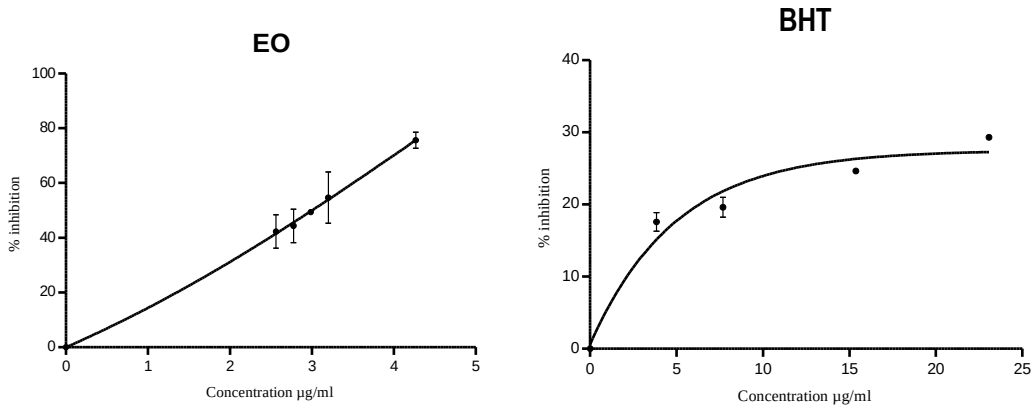

Figure 3: DPPH scavenging effect of *C. montanum* EO and this of BHT Each value represents the mean ± SD (n= 3)

Reducing power

The reducing power of the EO is concentration dependent **(Figure 4)**, in the range of 49.47 µg/ml to 84,57µg ml, it increased only slightly. However, the power of BHT is much higher but statistically not significant at $p \leq 0.05$. The reducing power of the

EO is concentration dependent. Other species tested of *Carum* have also presented interesting reducing powers; The EO of *C. nigrum*, *C. bulbocastanum* showed a very high reducing power than BHT [34, 13]. This activity can be attributed to some substances known to be anti-oxidative such as γ-terpinene and myrcene [28]**.** Mostly, the anti-oxidative potential of the EOs may be due to the presence of various types of compounds. In addition, the anti-oxidative activity could be a result of synergistic effects of two or more compounds and most compounds natural anti-oxidative work in synergy with each other to produce a wide range of anti-oxidative properties that create a defense effect system against free radicals [34]. These results corroborate with those of **Thippeswamy et al. (2013)** [37] where the phenolic extract of *C. carvi* showed a great power than the BHT. However, **Xavier et al. (2011)** [38] found that the extract of *C. carvi* from the Himalayas was the less active among several species tested, compared to ascorbic acid, used as reference. The components with a reducing power are electron donors and can reduce the oxidized intermediates of the process of lipid peroxidation reaction, so they can act as primary or secondary antioxidants [17].

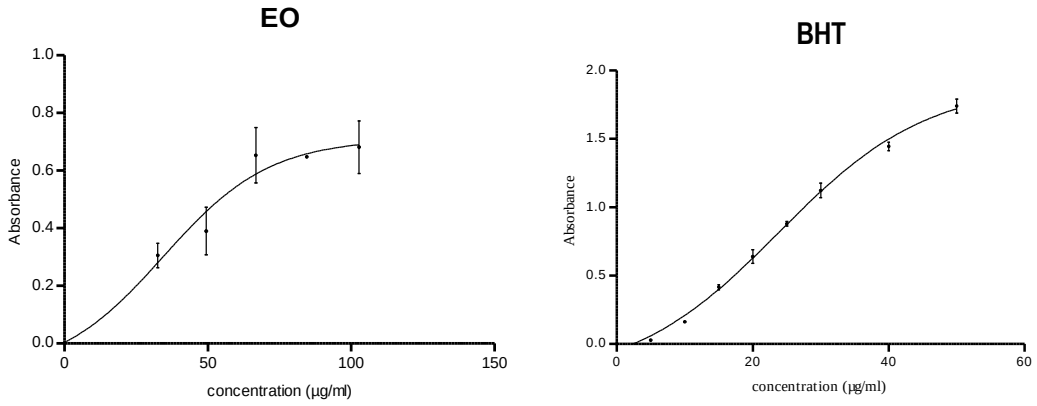

Figure 4: Reducing power of *C. montanum* EO and this of BHT

Each value represents the mean ± SD (n= 3).

CONCLUSION

In conclusion, we can say that the essential oil of *C. montanum* exhibited very interesting antimicrobial activities with MICs which differ depending on the technique used. These activities can be used in food preservation after being tested for an eventual cytotoxicity.

Acknowledgment: This work was supported by a grant from the Algerian government**.**

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