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Composition and biological activities of the essential oil of *Nigella sativa seeds* isolated by accelerated microwave steam distillation with cryogenic grinding

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Abstract

In this study, essential oil of Sahara *Nigella sativa* L. was extracted using a rapid extraction, the microwave steam distillation (MSD) and the cryogenic grinding (CG). Two procedures have been investigated, the MSD1 (seeds inside of oven apparatus) and MSD2 (seeds outside of oven apparatus). Forty-six compounds were identified and significant differences in quantities of the major constituents were observed, mainly were thymoquinone (CLG: 331.82-443.55 mg and CG: 272.95-413.57 mg/100 g of seeds), *p*-cymene (CLG: 181.71-244.17 mg, CG: 369.80- 374.40 mg/100 g of seeds), dehydro-sabina ketone (CLG: 24.60- 25.83 mg, GC: 44.02-50.69 mg/100g of seeds), carvacrol (CLG: 10.32-10.96 mg, CG: 3.91-12.67 mg/100 g of seeds) and longifolene (CLG: 11.90-16.43 mg, CG: 12.72-19.58 mg/100 g of seeds).Results showed that essential oils exhibit a good activity in each antioxidant system with a special attention for β-carotene bleaching test (IC50: 21 to 27 µg/ml) and reducing power (EC50: 9 to 14 µg/ml).The *N. Sativa* essential oils exhibited higher antibacterial and antifungal activities varying according to technique extraction and grinding mode used, with a high effectiveness against Gram-positive bacteria with a diameter of inhibition zones growth ranging from 9.5 to 35 mm and MIC and MBC values ranging from (0.042–0.10 mg/ml) to (0.20–0.75 mg/ml), respectively.

Keywords: *Nigella sativa,* Ranunculaceae, composition, Microwave steam-distillation, Cryogrinding, antioxidant and antibacterial activities.

1. Introduction

Herbs and spices are invaluable resources, useful in daily life as food additives, flavours, fragrances, pharmaceuticals, colours or directly in medicine.

Black seed, the seed of *Nigella sativa* L. (Ranunculaceae), has been employed for thousands of years as a spice, food preservative and curative remedy for numerous disorders [1, 2]. The historical tradition of black seed use in medicine is substantial. *N. sativa* is known to have beneficial effects on a wide range of diseases, antiasthmatic [3], antitumor [4], antiviral [5], antibacterial [6], anti-inflammatory [7], gastroprotective [8], antimalarial [2], antihypertensive [9], antidiabetic [10], anti-atherosclerotic [11], protective and antioxidant [12], nutritional [13] and anti-cholesterol [14].

Thymoquinone, the main constituent of the essential oil of *N. sativa* seeds, was capable to also exert beneficial effects on acute gastric ulcer [15]. In addition, thymoquinone and its reduced product thymohydroquinone have been reported to have an antibacterial activity and beneficial interaction with some antibiotics [16].

In this paper, the potential of the MSD1 (microwave steam distillation, seeds inside of oven apparatus) and MSD2 (microwave steam distillation, seeds outside of oven apparatus) techniques have been investigated using the classical and the cryogenic grinding, as the current techniques and commercial situation call for research into new extracts and new extraction techniques.

The fundamental principle of cryogenic grinding (cryogrinding) for herbal medicine is similar to that of grinding methods for conventional materials while using the liquid nitrogen or liquid air

as the cryogen, all of these thermo-sensitive herbal medicines can be ground below their brittle temperature. The colour and other properties of the products of cryogrinding will not be changed and the flavour and nutrition of the herbal medicines will not be lost.
In fact, the compositions are very complex, containing aromatics of high volatility, oils and fats, which are easily oxidized.
Considering the success of the culture of spices, particularly N. sativa seed in the arid areas (Adrar, Southwest of Algeria), it is useful to investigate the chemical composition and the biological activities of this seed according to the extraction technique and the grinding mode.
We have applied MSD1, MSD2, MSD1-CG and MSD2-CG techniques to extract essential oils from aerial parts (seeds) of Algerian (Adrar) N. sativa belonging to the family Ranunculaceae, which is a highly advanced and homogeneous family, largely used in food preparation and medicine. We make appropriate comparisons in term of extraction yields and rates, essential oil composition, and energy consumption. In addition, the other aim of this work was to study the antioxidant and antimicrobial effects against several pathogenic microorganisms according the techniques used.

2. Material and methods

2.1 Plant Material

Mature black cumin (*Nigella sativa* L.) seeds were cultivated and collected from Adrar (Sbaa) area (Southwest Algeria: 28° 13' 0" North, 0° 10' 0" West, elevation: 270, dry area, irrigated garden). The initial moisture of these seeds was 8%. They were stored at 4 °C until extraction. They were authenticated by the Botanic Department of the National Agronomic Institute, Algiers, Algeria. The voucher specimens are deposited at the herbarium of this Institute.
Seed material (30 g) was milled in an electric heavy-duty grinder for 20 s to 180-250 µm average size (Ika Werke standard model Germany) at a speed of 20,000 rpm, and subjected immediately to oil extraction. The cryogenic grinding (CG) was carried out by adding about 50 ml of the liquid nitrogen at -196 °C to seeds (30 g) and subjected immediately to grinding in the same conditions as the classical grinding (CLG). The average size of seeds is < to 100 µm.

2.2 MSD Apparatus and Procedure

Themicrowave extraction process was carried out in a microwave laboratory oven as described in [17, 18], at atmospheric pressure with grinded seeds. The power used is 600 W with non-focused radiations.The volume of distilled water is 100 ml.
The samples treated above was submitted to MSD using a simple column (Diameter φ=3 cm, Height h=15 cm) supporting the grinded seeds according to the figures 1-a and b coupled to a Clevenger apparatus (Conseil de l'Europe *Pharmacopée Européenne*. 1996) [19] and extracted with 300 ml water for 5 min (until no more essential oil was obtained). The plant material is in the first method inside of the microwave apparatus MSD 1 (Fig.1-a) and in the second method is placed out of the oven (MSD 2) (Fig. 1-b) where the oil is not submitted to the microwave radiations. The essential oil was collected, dried over anhydrous sodium sulphate and stored at −4 °C until used.

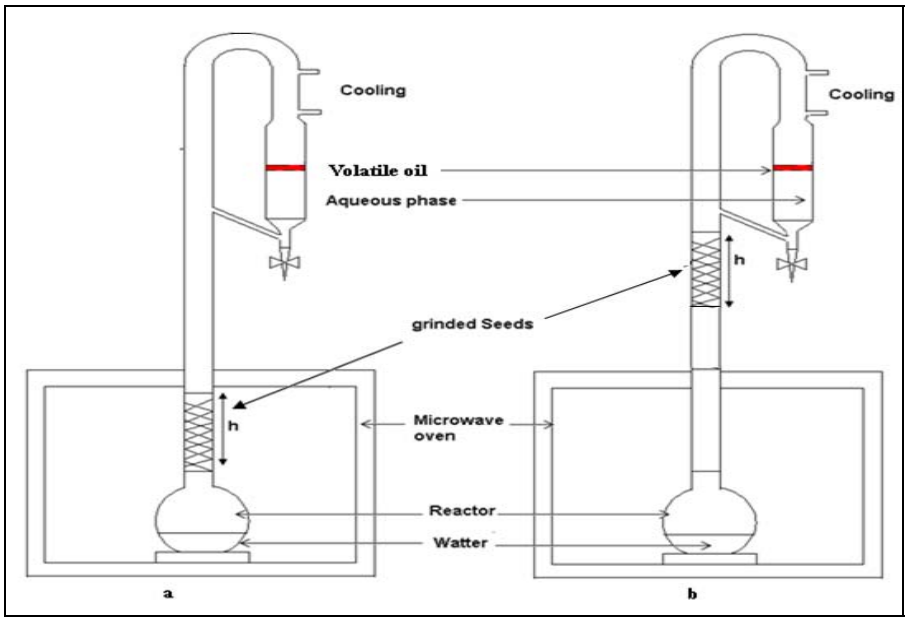

Fig 1: a-Microwave steam distillation MSD1, seeds inside of oven apparatus, b- Microwave steam distillation MSD2, seeds outside of oven apparatus

2.3 GC and GC-MS Analysis

GC analysis was performed on a HP 6890 standard model using the following conditions: fused-silica-capillary column with a non polar stationary phase HP5-MS (60 m, 0.25 mmi.d, 0.25 µm film, 5% biphenyl, 95% dimethylpolysiloxane), detector used FID, carrier gas Helium (0.03 MPa, flow rate 0.5 ml min^{-1}), injector and detector temperature are respectively regulated at 280 and 300 °C. The splitless injection mode was used; injection volume for all

samples 0.2 µl ; the oven temperature was programmed at 60 °C for 10 min, then progressed from 60 to 250 °C at 5 °C min^{-1} and was held at 250 °C for 10 min. Percentages of the constituents were calculated by electronic integration of FID peak areas.

The essential oil samples were injected in a HP 6890 chromatograph connected to a Hewlett-Packard 5973 mass-selective detector.

Oven temperature progression, column operating conditions, injection mode, carrier gas conditions and injector temperature were similar to GC ones. The volume injection for all samples was 1 µl (1% in hexane).

The temperature interface of the mass spectrometer was fixed to 280 °C; the solvent delay time was 4 min. The source temperature was 230 °C. The instrument was operated in electron-impact (EI) with at 70 eV, and scanned in the 30-550 m/z range.

The homologous *n*-alkanes series (relative to C7– C28 on the HP5 column) injected in GC and GC-MS in the same conditions as the essential oils were used to calculate the retention indices. Peak area percentages were calculated by using the normalization method. The component identification used the comparison of the mass spectral fragmentation patterns with those stored in the database (Nist 2002, Wiley 7) and with the previously published spectra. The comparison of the linear retention indices (L.R.I) of the essential oil constituents compared with those of the published index data [20, 17] confirmed the identification. Analyses were performed at least three times, and the mean values were reported.

2.4 Antioxidant activity

1, 1-Diphenyl-2-picrylhydrazyl (DPPH) and superoxide anion radical-scavenging activity

The effect of the tested essential oils on DPPH degradation was estimated according to the method described by [21, 22] with small modifications. Each essential oil was diluted in pure methanol at different concentrations, and then 2 ml were added to 0.5 ml of a 0.2 mmol/l DPPH methanolic solution. The mixture was shaken vigorously and left standing at room temperature for 30 min. The absorbance of the resulting solution was then measured at 517 nm measured after 30 min. The antiradical activity (three replicates) was expressed as IC_{50} (µg/ml), the antiradical dose required to cause a 50% inhibition. A lower IC_{50} value corresponds to a higher antioxidant activity of essential oil [23]. The ability to scavenge the DPPH radical was calculated using the following equation: DPPH scavenging effect (%) = $((A_0-A_1) \times 100)/A_0$ (eq.1)

Where A_0 is the absorbance of the control at 30 min, and A_1 is the absorbance of the sample at 30 min.

Superoxide anion scavenging activity was assessed using the method described by [24]. The reaction mixture contained 0.2 ml of essential oil has different concentration, 0.2 ml of 60 mM PMS stock solution, 0.2 ml of 677 mM NADH, and 0.2 ml of 144 mM NBT, all in phosphate buffer (0.1 mol/l, pH 7.4). After incubation at ambient temperature for 5 min, the absorbance was read at 560 nm against a blank. The value of the antioxidant activity was based on IC_{50}. The IC_{50} index value was defined as the amount of antioxidant necessary to reduce the generation of superoxide radical anions by 50%. The IC_{50} values (three replicates per treatment) were expressed as µg/ml. As for DPPH, a lower IC_{50} value corresponds to a higher antioxidant activity of plant extract. The inhibition percentage of superoxide anion generation was calculated using the following formula: Superoxide (%) = $((A_0-A_1) \times 100)/A_0$) where A_0 and A_1 having the same meaning as in Equation (eq.1).

2.5 Reducing power

The ability of the extracts to reduce Fe^{3+} was assayed by the method of Oyaizu [25], 1 ml of *Nigella sativa* essential oil were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% $K_3Fe(CN)_6$. After incubation during 50 °C for 25 min, 2.5 ml of 10% trichloroacetic acid was added and the mixture was centrifuged at 650 g for 10 min. Then, 2.5 ml of the upper layer was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% aqueous $FeCl_3$. The absorbance was measured at 700 nm. The average absorbance values were plotted against concentration and a linear regression analysis was carried out. Increased absorbance of the reaction mixture indicated increased reducing power. EC_{50} value (mg/ml) is the effective concentration at which the absorbance was 0.5 for reducing power. Ascorbic acid was used as positive control.

2.6 Antimicrobial activity

2.6.1 Microorganisms

The test microorganisms included the following Gram-positive bacteria: *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* CIP 106510, *Micrococcus luteus* NCIMB 8166, *Bacillus cereus* ATCC 11778 (a), *B. cereus* ATCC 14579 (b) and Gram-negative bacteria: *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* LT2 DT104, *Listeria monocytogenes* ATCC 19115, *Enterococcus faecalis* ATCC 29212. The antibacterial effect was also tested against four *Candida* species (Strains) as: *Candida albicans* ATCC 90028, *Candida parapsilosis* ATCC 22019, *Candida glabrata* ATCC 90030 and *Candida krusei* ATCC 6258. These micro-organisms were provided from the Department of microbiology (Pasteur Institute of Algiers).

2.6.2 Disc-diffusion assay

Antimicrobial activity test was done according to the protocol described by Vuddhakul *et al*. [26] with slight modification. For the experiments, a loopful of the microorganisms working stocks were enriched on a tube containing 9 ml of Mueller-Hinton broth (for bacteria) and Sabouraud Chloramphenicol broth (for *Candida*), then incubated at 37 °C for 18-24 h. The overnight cultures were used for the antimicrobial activity of the essential oil used in this study and the optical density was adjusted at 0.5 McFarland turbidity standards with a DENSIMAT (Biomérieux). The inocula of the respective bacteria and fungus were streaked onto MH or SB agar plates using a sterile swab.

Sterile filter discs (diameter 6 mm, Whatman Paper No. 3) were impregnated with 10 µl of essential oil placed on the appropriate agar mediums (SB, MH and MH + 1% NaCl). Gentamycin (10 µg/disc) and amphotericin B (20 µg/disc) were used as positive reference standards to determine the sensitivity of one strain/isolate in each microbial species tested.

The antibiotic susceptibility was determined by using the Kirby–Bauer method and Mueller–Hinton agar plates supplemented with 1% NaCl as described by [27]. After incubation at 37 °C for 18-24 h, the diameter of the inhibition zone was measured with 1 mm flat rule and the diameters were interpreted according to the CSFA (Comité de la Société Française de l'Antibiogramme [28].

The dishes were incubated at 37 °C for 18-24 h for microbial

strains. The diameter of the zones of inhibition around each of the discs was taken as measure of the antimicrobial activity. Each experiment was carried out in triplicate and the mean diameter of the inhibition zone was recorded.

2.6.3 Micro-well determination of MIC and MBC

The minimal inhibition concentration (MIC) and the minimal bactericidal concentration (MBC) values were determined for all bacterial strains used in this study as described by Gulluce *et al*. [29]. The inocula of the bacterial strains were prepared from 12 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. The essential oils of *Nigella sativa* dissolved in 10% dimethylsulfoxide (DMSO) + 90% water, were first diluted to the concentration (20 mg/ml) to be tested, and then serial two-fold dilutions were made in a concentration range from 0.05 to 50 mg/ml in 5 ml sterile test tubes containing nutrient broth. The well plates were prepared by dispersing into each well 95 µl of nutrient broth and 5 µl of the inocula. A 100 µl aliquot from the stock solutions of each essential oil was added into the first wells. Then, 100 µl from the serial dilutions were transferred into 11 consecutive wells. The last well containing 195 µl of nutrient broth without essential oil and 5 µl of the inocula on each strip was used as the negative control. The final volume in each well was 200 µl. The plates were incubated at 37 °C for 18-24 h. The essential oils tested in this study were screened two times against each organism. The MIC was defined as the lowest concentration of the compounds to inhibit the growth of the microorganisms. The BMC values were interpreted as the highest dilution (lowest concentration) of the sample, which showed clear fluid with no development of turbidity and without visible growth. The tests were performed in triplicate.

3. Results

3.1 Extraction

Table 1 lists the yields, extraction times and energy consumption of the MSD1, MSD1-CG, MSD2, and MSD2-CG essential oils. The high values were attributed to the seeds from MSD1-CG (1.06%) and MSD2-CG (0.9%), followed by MSD1 (0.73%) and MSD2 (0.72%).

These percentages are higher compared to HD and HD-MO essential oils (respectively isolated by conventional hydrodistillation and hydrodistillation assisted by microwave previously studied [30].

Table 1: Yields, extraction time, energy consumption and environmental impactof *N. sativa* seed essential oils of MSD1, MSD1-CG, MSD2, and MSD2-CG compared to those obtained in literature

	MSD1	MSD1-CG	MSD2	MSD2-CG
Time extraction (min)	5	5	5	5
Yields (%)	0.73	1.06	0.72	0.90
Energy consumption/g of essential oils (KWh)	0.054	0.038	0.055	0.050
CO_2 released g CO_2/g of essential oil	43.2	30.4	44	40

In addition, these yields are widely higher or comparable than all those indicated by some authors (0.2 to 1.3%) [31-33]. Extracted essential oils were of a dark yellow colour. All oils had a characteristic odor, especially those isolated by the MSD1-CG and MSD2-CG techniques which were relatively fresher. They tend to crystallize partially at a lower temperature (- 8 °C) after 72 to 96 hours of freezing, and acquire a lightly greenish and black-colored crystalline structure probably due to the thymoquinone. Thus, the cryogrinding process allows extracting more amount of essential oil compared to the classical grinding. In addition the major quantity of oil was obtained during the two first minutes.

As shown in table 1, an extraction time of 5 min (MSD) provides high yields compared to those obtained by means of HD (2.5 h) or HD-MO (10 min) [30]. As noted previously, the reduced cost of extraction is clearly advantageous for the MSD method, particularly with cryogrinding in terms of time, energy and speed to reach the extraction temperature.

Regarding environmental impact, the calculated quantity of carbon dioxide released in the atmosphere is higher in the case of conventional [30] (5185 g CO_2/g of essential oil) than for MSD1, MSD2, MSD1-CG, MSD2 and MSD2-CG (43.2, 30.4, 44 and 40 g CO_2/g of essential oil respectively). Thus, the cryogenic grinding permitted to reduce lightly (MSD1-CG and MSD2-CGthe CO_2 released in the atmosphere compared to the classical grinding.

These calculations have been made according to literature [34]. To obtain 1 kWh from coal or fuel, 800 g of CO_2 will be released into the atmosphere during combustion of fossil fuel. From where SDAM techniques allowed to reduce significantly environmental burden (less CO_2 released into the atmosphere).

3.2 Chemical composition

Analysis of the oils by GC and GC/MS revealed 46 compounds (All identified) in the N. sativa seed oils samples (Table 2).

Substantially higher amounts of oxygenated compounds and lower amounts of monoterpene hydrocarbons are present in the essential oils of the seeds extracted by MSD1 and MSD2 in comparison with MSD1-CG and MSD2-CG (Figure 2). Monoterpene hydrocarbons are less valuable than oxygenated compounds in terms of their contribution to the fragrance of the essential oil.

Conversely, the oxygenated compounds are highly odoriferous and, hence, the most valuable. The greater proportion of oxygenated compounds in the MSD and MSD-CG essential oils is probably due to the diminution of thermal and hydrolytic effects, compared with HD-MO and particularly with HD [22] which uses a large quantity of water and is time and energy consuming.

The unidentified peak reported by many authors (m/z (% rel.int.) 125 (100), 153 (88), 85 (85), 72 (82), 93 (80), 121 (42), 81 (74), 136 (37), 168 (2)) [17] was tentitatively elucidated as the dehydrosabinaketone present in all samples studied.

The MSD1 oils could be distinguished from the MSD2 oils (Figure 2) by their richness in ketones (475.31 mg MSD1 vs. 360.8 mg MSD2) and alcohols (33.33 mg MSD1 vs 22.7 mg MSD2). Additionally, MSD2 oils could be also differentiated from the MSD1 oils by their greater richness in monoterpene hydrocarbons (262.65 mg MSD1 vs. 374.1 mg MSD2). The sesquiterpene hydrocarbon percentages are similar (23.01 mg MSD1 vs. 20.35 mg MSD2). The same observations were noted when we used the

cryogenic grinding with high percentage in ketones (470.11 mg MSD1-CG vs 319.9 mg MSD2-CG) and of alcohols (55.31 mg MSD1-CG vs 20.54 mg MSD2-CG) by MSD1-CG compared to MSD2-CG and high amount of monoterpene hydrocarbons (589.94 mg MSD1-CG vs 588.94 mg MSD2-CG) in MSD2-CG compared to MSD1-CG.Consequently, the best and the most natural oils as MSD1-CG and MSD2-CG are richer in monoterpene hydrocarbons than other oils MSD1-CG and MSD2-CG.

The MSD1-CG oils could be also differentiated from the MSD2-CG oils by their greater richness in sesquiterpene hydrocarbons (31.11 mg vs18.59 mg respectively).

In the same way, notable amounts of sesquiterpene hydrocarbons were identified in the oils essentially in MSD1 (23.01 mg) and MSD1-CG (31.11 mg) oils dominated mainly by longifolene, α-longipinene and Z-γ-bisabolene. Conversely, only small amounts of aldehydes were isolated, in many oils not exceeding 1.15 mg/100 g of seeds. On the other hand, no trace of diterpene hydrocarbons was detected in our oils compared to the conventional hydrodistillation HD and HD-MO [35] where a moderate amount of the diterpene hydrocarbons (Pimaradiene) was identified.

Globally, the cryogenic grinding accelerates the extraction phenomena, enhances the global essential oil yields and considerably reduces thermal reactions as the transformation of thymoquinone (TQ) to thymohydroquinone (THQ) as reported in other works [35] or polymerization of thymohydroquinone to dithymoquinone (DTQ) (Figure 3). Indeed, the longer extraction techniques as HD [56] or solvent extraction procedure followed by steam distillation (SESD) showed a high amount of thymohydroquinone varying from 5.43 to 24 mg/100g of seeds [6] with HD and from 50 to 250 mg/100 g of seeds with SESD [35]. Conversely, with the MSD and especiallyMSD2-CG, only small amounts of THQ were detected, not exceeding 6.35 mg.

The main components in MSD1, MSD1-CG, MSD2 and MSD2-CG oils were thymoquinone

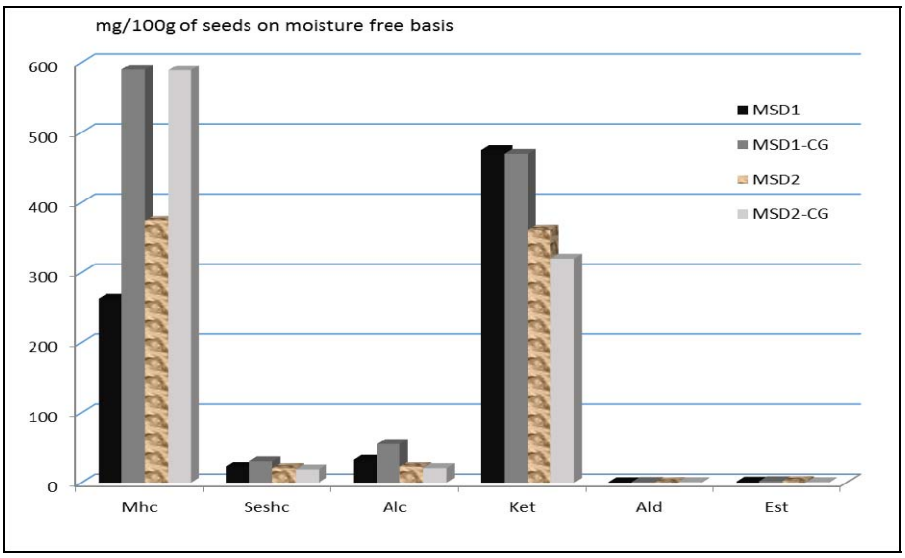

Fig 2: Percentages of different chemical families according to the technique used
Mhc: monoterpene hydrocarbons, Seshc: sesquiterpenes hydrocarbons, Alc: alcohols, Ket: ketones, Ald: aldehydes, Est: esters

(443.55, 413.57, 331.82 and 272.95 mg respectively), p-cymene (181.71, 374.40, 244.17 and 272.95 mg respectively), dehydro-sabina ketone (24.60, 50.69, 25.83 and 44.02 mg respectively) and carvacrol (10.32, 12.67, 10.96 and 3.91 mg respectively) followed by longifolene (11.90, 19.58, 16.43 and 12.72 respectively). Nevertheless, Nickavar *et al*. [36] reported that trans-anethole (38.3%), p-cymene (14.8%), and limonene (4.3%) were recognized in N. sativa as the major compounds of the volatile oil.

As reported in the literature, the valuable constituents that have beneficial chemical effects were principally thymoquinone [16, 11], p-cymene [37], carvacrol [38], and 4-terpineol [31] (Table 3). They represent a global amount of 639.55 mg of ground seed in MSD1, 809.86 mg in MSD1-CG, 590.86 mg in MSD2 and 651.55 mg in MSD2-CG. From these results, it is obvious that N. sativa seed essential oil is highly bioactive oil and its consumption could be beneficial to human health especially the MSD1-CG and MSD2-CG essential oils.

Furthermore, it is interesting to note that the previously characterized dimer dithymoquinone (or nigellone) [39] was not detected in all oils seen by the authors, as confirmed by Burits and Bucar [31].

The two methods of steam distillation assisted by microwave allowed to isolate a great amount of ketones and monoterpene hydrocarbons compared to the hydrodistillation (HD) and hydrodistillation assisted by microwaves (HD-MO) techniques previously studied [35, 17]. However the alcohols, esters and acids percentages are more important in HD and HD-MO [29]. In our oils, no trace of acids was identified as indicated in Table 2.

According to the data values (Table 2), it must be pointed out that the cryogrinding effect on the composition is very important. The chemical composition of oils is closely dependent to the physical structure (dimension of particles) of the grinded seeds and the current thermal reactions during the classical grinding. Thus, the high amount of oxygenated compounds in the MSD oils doesn't necessarily mean they are more natural and identical to the essential oil contained in in-vivo plants than MSD-CG oils.

There are slightly fewer compounds present in the chromatograms of essential oils extracted by MSD and MSD-CG compared to those obtained by HD and HD-MO previously studied [35, 17]. The loss of some compounds in MSD and MSD-CG compared with HD and HD-MO techniques [35, 17]. is probably not that these compounds are not extracted but rather that the reduction in

extraction time and the amount of water in the MSD and MSD-CG methods reduces the degradation of compounds by hydrolysis, trans-esterification or oxidation, and hence there are fewer degradation products noted in the analysis. Besides it is reported that the super critical CO_2 extraction of N. sativa oil at different experimental conditions of pressure, temperature and time revealed that the yield, antioxidant capacity and thymoquinone quantity could vary with the changes in the operating conditions which show that they had significantly influenced the extract yield and composition [40].

Table 2: Chemical composition of Algerian (Sahara) *N saliva* seeds essential oils extracted by different techniques

N°	Compounds	RI ref	RI	mg/100 g of seeds on moisture free basis			
				MSD1	MSD1-CG	MSD2	MSD 2-CG
1	α-Thujene	930	932	39.67	109.44	57.91	73.37
2	α-Pinene	939	937	10.32	27.65	14.87	75.33
3	α-Fenchene	952	951	tr	tr	0.78	0.98
4	Camphene	954	955	tr	tr	tr	0.98
5	Sabinene	975	977	5.55	3.46	7.83	24.46
6	β-Pinene	979	980	12.70	43.78	23.48	21.52
7	β-Myrcene	990	991	tr	tr	tr	0.98
8	α- Phelandrene	1002	1004	tr	tr	0.78	tr
9	α-Terpinene	1017	1018	tr	1.15	1.57	tr
10	p-Cymene	1024	1026	181.71	374.40	244.17	369.80
11	1,8-Cineole	1031	1033	3.17	3.46	3.13	2.93
12	Limonene	1029	1034	8.73	13.82	14.09	9.78
13	Benzene acetaldehyde	1042	1044	tr	tr	tr	tr
14	<(E)-β-> Ocimene	1050	1051	tr	tr	tr	19.57
15	γ-Terpinene	1059	1058	1.59	12.67	8.61	12.72
16	+<o>Tolualdehyde ti	1062	1063	tr	tr	tr	tr
17	cis-Sabinene hydrate	1070	1071	tr	tr	tr	tr
18	Terpinolene	1088	1090	tr	tr	0.78	tr
19	p-Cymenene	1089	1092	-	tr	1.57	-
20	trans-Sabinene hydrate	1098	1097	3.97	9.22	-	-
21	2-Isopropyl -5-mthyl-(2-Z)-	1114	1116	-	1.15	0.78	0.98
22	Dehydro-sabina ketone ti	1120	1122	24.60	50.69	25.83	44.02
23	β-Fenchol	1121	1124	0.79	1.15	0.78	tr
24	Mentha-2.8-diene-1-ol <-cis-	1137	1138	0.79	1.15	-	-
25	β-Pinene oxide	1159	1160	1.59	4.61	1.57	2.93
26	4-Terpineol	1177	1178	3.97	9.22	3.91	4.89
27	p-Cymen-9-ol	1205	1206	6.35	12.67	tr	7.83
28	Thymol methyl ether ti	1235	1237	-	tr	-	-
29	Thymoquinone	1252	1260	443.55	413.57	331.82	272.95
30	Trans-sabinene hydrate	1253	1265	0.79	1.15	0.78	tr
31	Linalool acetate	1257	1268	tr	tr	0.78	tr
32	Catechol<3-methyl>	1258	1270	tr	tr	1.57	tr
33	(E)-Anethole	1284	1286	tr	1.15	0.78	0.98
34	Bornyl acetate	1288	1290	0.79	1.15	0.78	0.98
35	Carvacrol	1299	1301	10.32	12.67	10.96	3.91
36	α-Longipinene	1352	1354	6.35	4.61	1.57	2.93
37	Eugenol	1359	1363	2.38	4.61	3.91	2.93
38	Iso-longifolene	1390	1392	tr	tr	tr	tr
39	β-Longipinene	1400	1402	tr	tr	tr	tr
40	Longifolene	1407	1409	11.90	19.58	16.43	12.72

41	(Z)-Caryophyllene	1404	1409	tr	tr	tr	tr
42	(E)-Caryophyllene	1419	1421	2.38	4.61	0.78	0.98
43	ar-Curcumene	1480	1482	tr	tr	0.78	tr
44	Z-γ-Bisabolene	1515	1516	2.38	2.30	0.78	1.96
45	6-Methyl-α-(E)-ionone	1521	1522	0.79	tr	0.78	tr
46	Thymohydroquinone	1555	1557	6.35	5.76	2.35	0.98

RI ^{ref}: Retention indices of reference [20]

RI: calculated retention indices in this work

tr = trace, less than 0.5 mg/100 g of seeds on moisture free basis,

ti: Tentitatively identified,

(-): not detected,

MSD1: microwave steam distillation technique 1 with classical grinding of *N. sativa* seeds

MSD1-CG: microwave steam distillation technique 1 with cryogrinding of *N. sativa* seeds

MSD2: microwave steam distillation technique 2 with classical grinding of *N. sativa* seeds

MSD2-CG: microwave steam distillation technique 2 with cryogrinding of *N. sativa* seeds

Table 3: Antioxidant activity of essential oils extract from Algerian variety of N. sativa seeds: scavenging activity (expressed as IC_{50} values: µg/ ml), DPPH, superoxide radicals and β-carotene bleaching test Reducing power was expressed as EC_{50} values (µg/ml)

	MSD1	MSD1-CG	MSD2	MSD2-CG	BHT
DPPH IC_{50} (µg ml^{-1})	203	250	220	275	12.1
O_2^- IC_{50} (µg ml^{-1})	9	11	10	14	1.7
PR EC_{50}(µg ml^{-1})	11	13	12	18	81
β-Carotenes IC_{50} (µg) ml^{-1})	21	24	22	27	73

3.3. Antioxidant activities

DPPH is a free radical that accepts an electron or hydrogen radical to become a stable molecule. DPPH radical scavenging is one of the important methods to evaluate antioxidant activity of essential oils and phenolic extracts. Table 3 illustrates scavenging of the DPPH radical by Nigella sativa essential oils according the techniques used. The scavenging effect of essential oils and standard (BHT) on the DPPH radical expressed as IC50 values was 203, 250, 220 and 270 µg/ml for MSD1, MSD1-CG, MSD2 and DME2-CG respectively, and 12.1 µg/ml for BHT. In fact, Burits and Bucar [37] reported Nigella sativa essential oils notably reduced the concentration of DPPH free radical, with an efficacy lower than that of reference compound, carvacrol with an IC50 of 28.8 mg/ml. The performance of Eugenia caryophyllata essential oil was better than that of Nigella sativa [41].

Otherwise, the antioxidant activity of CLG (Classical grinding) essential oils is more important compared to those of the CG essentials. This difference is probably due to the abundance of oxygenated compounds in CLG oils as thymoquinone and carvacrol. Whereas, oils with higher monoterpenic abundance were reported to be ineffective. These results are in agreement with the poor performance given by the oils with similar patterns and by single monoterpenic hydrocarbons [42].

The superoxide anion is the most common free radical generated *in vivo*. Under oxidative stress, the concentration of this species can increase dramatically in all cells, inducing several pathophysiological processes, due to its transformation into more reactive species [43].

The decrease in absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture. Table 3 showed that the results of the inhibiting capacities of superoxide were very interesting for the Nigella sativa essential oils (MSD1: 9, MSD1-CG: 11, MSD2: 10 and DME2-CG: 14 µg/mL), but are inferior as compared to BHT (1.7 µg/mL).

Looking at the effects of the essential oils and their percentage of the major and antioxidants compounds as thymoquinone, carvacrol and 4-terpineol [31], and comparing the results, it seems that the three compounds are not the only radical scavenging compounds in the oils. At least in the peroxidation test the essential oils possessed a strong antioxidative effect that cannot just be achieved by summarizing the activities of the three compounds. It is possible that there are synergistic effects, as they were demonstrated to exist in drugs used for the cure of gastric diseases and plants with radical scavenging properties [44], but there might also be another active principle not yet identified in the essential oils.

Another reaction pathway in electron donation is the reduction of an oxidized antioxidant molecule to regeneratethe ‘‘active” reduced antioxidant. As showed in Table 3, the reducing power of N. sativa essential oils, expressed as CE50, was clearly more important (MSD1: 11, MSD1-CG: 13, MSD2: 12 and MSD2-CG: 18 µg mL-1) than that of positive control BHT (81 µg mL-1).

Peroxide value of oil is a valuable measure of oil quality.In our case, β-carotene undergoes rapid discoloration in the absence of an antioxidant. The presence of an antioxidant such as a phenolic can hinder the extent of β-carotene destruction by ‘‘neutralizing’’ the linoleate free radical and any other free radicals formed within the

system [45]. Table 3 summarizes the inhibition of β-carotene bleaching by the Nigella sativa essential oils and by BHT. The values of IC50 are 21, 24, 22 and 27µg/mL for MSD1, MSD1-CG, MSD2 and DME2-CG respectively, and 73 µg/mL for BHT. Finally, the best results were globally attributed specially for the essential oils treated by classical grinding containing high amount of oxygenated compounds.

Practically, no previous works were found to compare our results. But many authors have investigated the peroxide value of Nigella sativa fixed oils as Cheikh-Rouhou *et al*. [46] and Atta *et al*. [47] who reported interesting values of N. sativa oils from different origins.

These antioxidants properties (DPPH radical-scavenging activity, superoxide anion radical-scavenging activity, reducing power, β-carotene linoleate system) of essential oils are important for food industry in order to find possible alternatives to synthetic preservatives as BHT and BHA. In fact, N. sativa essential oils provide interesting results in terms of ability to neutralize free radicals and prevent unsaturated fatty acid oxidation.

3.4. Antimicrobial activity

The antimicrobial activities of *Nigella sativa L*. essential oils against microorganisms examined in this work and its potency were qualitatively and quantitatively assessed by the presence or the absence of inhibition zone diameter (MIC and MBC values). The results given in Table 4 had substantial antimicrobial activity against 10 bacteria and five yeasts species tested. In fact, the data obtained of zones of growth inhibition (mm) scored in Mueller–Hinton agar demonstrated that Gram-positive bacteria exhibited the highest diameters of growth inhibition (between 9.5 and 35 mm). In the whole, *Nigella sativa* essential oils were particularly effective against *M. luteus* with a diameter of inhibition varying from 30 to 35 mm. In addition, Gram-negative bacteria were less sensitive to *Nigella sativa* essential oils with a diameter of growth inhibition ranging from 8.6 (*Enterococcus faecalis*) to 14 mm (*E. coli*).

MIC and MBC values indicate that the essential oils of *N. sativa* were efficient against all tested bacteria with MIC about 0.046 to 0.1mg/ml for Gram-positive bacteria, from 0.07 to 0.17 mg/ml for Gram-negative bacteria. The MBC values were also important and low concentration of *Nigel sativa* essential oils were sufficient to eliminate the growth of *M. luteus* (MBC: 0.17 to 0.25 mg/ml) and E. faecalis and E. coli (MBC: 0.65 to 0.7 mg/ml).

As showed in Table 4, the high antimicrobial activity of MSD-CG essential oils compared to the MSD essential oils is probably duo to their high content in monoterpene hydrocarbons, the *p*-cymene having a substantial antimicrobial effect [37]. To our knowledge, this work represents the first attempt to study the chemical composition and the biological activities of Algerian variety of *N. sativa* growing in arid zone (Sahara-Adrar), and especially to study his antibacterial effect against several pathogen microorganisms. In this context, *N. sativa* essential oils gave interesting results in terms of both antimicrobial activity and ability to neutralize free radicals and prevent unsaturated fatty acid oxidation. Globally, these findings may confirm the interesting potential of this spice as a valuable source of natural bioactive molecules and valorise its important role to prevent against various contaminations after consumption of some food products.

Nevertheless, essential oils must be used carefully combined with antibiotics since their combination might also imply antagonistic effects. Thus, some examples as *Menthapiperita* L. (Lamiaceae), *M. Elaleuca alternifolia* Cheel (Myrtaceae), *Thymus vulgaris* L. (Lamiaceae) and *Rosmarinus officinalis* L. (Lamiaceae) essential oils showed antagonistic effects in combination with amphotericin B against *C. albicans*. The additive, synergistic and antagonistic effect was often linked to the percentage in which the essential oil and the antibiotic were applied [48].

The extract of black seed as well as its oil have been reported to possess antibacterial activity; however Gram-positive bacteria are more susceptible to the action of the oil, whereas Gram-negative organisms are more sensitive to the extracts of black seeds [20].

Characterization of black seed oil composition by gas chromatography-mass spectrometry analysis [30] has revealed the presence of a variety of compounds possessing antimicrobial properties, including carvacrol [49, 50], thymol [51], thymohydroquinone [52], thymoquinone [30], limonene, carvone [53], *p*-cymene and γ-terpinene [54]. In addition, previous results indicate that black seed oil possesses significant antimicrobial activity against *Listeria* [55].

4. Conclusion

The proposed extraction methods MSD and MSD-CG are an original combination of microwave heating and steam distillation. They provide more valuable essential oils and allow substantial saving of energy. Additionally, these methods offer important advantages over traditional alternatives, namely: shorter extraction times (5 min for MSD method against 10 min and 150 min for HD-MO and HD respectively [30], substantial savings of energy, and a reduced environmental burden (less CO_2 released into the atmosphere). All these advantages make MSD a good alternative for the extraction of essential oils from aromatic plants.

A diverse range of yield and chemical composition has been demonstrated by the kind of essential oils isolated according the techniques and the nature of grinding used and from *Nigella sativa* species.

We also noted that the antibacterial, antifungal and antioxidant properties were closely related to the technique extraction and the kind of the grinding used.

On the whole, the use of microwaves as energy source and the steam distillation allows to isolate essential oils preserving all of the nutritional components.

In perspective, we are studying techniques permitting to extract essential oils less than one minute in order to eliminate completely all chemical alterations, testing at the same time the antioxidant and biological activities.

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Table 4: Antibacterial and antifungal activity of essential oil of Algerian N. sativa essential oils against human pathogenic bacteria and yeast strains using agar disc diffusion method and determination of MIC (mg/ml) and MBC (mg/ml) values.

	MSD1 (mm ± SD)			**MSD1-CG (mm ± SD)**			**MSD2 (mm ± SD)**			**MSD2-CG (mm ± SD)**			**Ant**
Microorganisms	IZ	MIC	MBC	IZ	MIC	MBC	IZ	MIC	MBC	IZ	MIC	MBC	
Bacterial strains													
Gram-positive bacteria													
Staphylococcus epidermidis	10.8± 0.5	0.09	0.41	12.67± 0.50	0.082	0.33	9.67± 0.58	0.09	0.45	11.45± 0.55	0.08	0.36	22.67± 0.7
Staphylococcus aureus	9.5± 0.8	0.092	0.75	12± 0.22	0.085	0.67	10± 0 .5	0.10	0.69	11± 0.3	0.09	0.63	34.54± 0.51
Micrococcus luteus	30± 0.6	0.05	0.23	34± 0.3	0.042	0.17	32± 1	0.053	0.25	35± 1.0	0.046	0.20	26.67± 1.42
Bacillus cereus (a)	20.67± 0.55	0.09	0.71	24.62± 0.5	0.08	0.64	19.2± 0.44	0.09	0.75	22.62± 0.65	0.08	0.69	28± 1.3
Bacillus cereus (b)	21.64± 0.55	0.095	0.69	24.67± 0.61	0.09	0.65	20.67± 0.48	0.099	0.74	22.62± 0.45	0.085	0.72	27± 1.5
Gram-negative bacteria													
Escherichia coli	11± 1.0	0.07	0.73	14± 2	0.08	0.66	10± 1	0.08	0.75	12± 0.8	0.075	0.66	29.21± 0.5
Listeria monocytogenes	12± 0.55	nd	nd	11± 0	nd	nd	10± 0.65	ND	ND	10± 0	ND	ND	39.60± 0.5
Enterococcus feacalis	9± 0.5	0.075	0.68	9± 0.35	0.08	0.65	9± 0.33	0.08	0.73	8.6± 0	0.070	0.70	28± 1.1
Pseudomonas aeruginosa	11.65± 0.56	nd	nd	13.7± 0.43	nd	0.51	10.55± 0.56	nd	nd	11.79± 0.54	nd	nd	32.1± 0.50
Salmonella typhimirium	10.5± 0.5	0.15	1.41	12± 0.1	0.17	1.25	10± 0.1	0.17	1.45	11±0.5	0.15	1.35	23± 1.2
Yeast strains													
Candida albicans	17± 1	0.043	0.42	15± 1	0.042	0.33	16± 0.9	0.05	0.49	17± 1	0.039	0.38	12± 0
Candida glabrata	21.61± 0.50	0.015	0.025	24.35± 0.45	0.01	0.023	20.67± 0.56	0.02	0.03	22.65± 0.37	0.01	0.028	13.67± 0.36
Candida parapsilosis	14± 0.2	0.08	0.42	15± 0.3	0.08	0.34	13± 0.2	0.088	0.48	14± 0	0.082	0.42	11.1± 0.5
Candida krusei	21± 1	0.006	0.052	22± 0.4	0.005	0.041	20± 0.9	0.008	0.058	21.5± 1	0.005	0.053	13± 0.5
Saccharomyces cerevisae	16.42± 0.55	0.01	0.021	18.1± 0.45	0.01	0.015	16.33± 0.45	0.01	0.027	17.66± 0.57	0.01	0.020	18± 1

Ant: Antibiotics, MIC: minimum inhibitory concentration, MBC:, minimum bactericidal concentration, IZ: Inhibition zone in diameter (mm ± SD) around the discs impregnated with 10 µl of essential oil SD: standard deviation, Gen: gentamycin (10 µg/disc), AB: amphotericin B (10 µg/ml), nd: not determined

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