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Chemical Composition and Antifungal Activity of *Nigella Sativa L.* Oil Seed Cultivated In Morocco

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ABSTRACT: *Nigella sativa L. commonly called black seed belongs to the family Ranunculaceae is known for its indigenous medicinal uses since immemorial time. The present study was carried out in order to characterize the Nigella seed oil cultivated in Morocco for its fatty acid and sterol composition using gas chromatography. The results indicated the presence of linoleic acid (58.5%) and oleic acid (23.7%) as unsaturated fatty acids and palmitic acid (13.1%) as saturated fatty acid while β-sitosterol and stigmasterol were among the major components, together constituting about 69 % of total sterols. Nigella seed oil has a strong antifungal activity compared to the conventional fungicides. Our findings demonstrate that Nigella seed oil has an antifungal activity that might be a natural potential source of antifungal used in food, in cosmetics and in pharmaceuticals products.*

KEY WORDS*: Nigella Sativa L., Fatty acids, sterol, Antifungal activity, Candida species*

I. INTRODUCTION

Nigella sativa L. (NS) commonly called as black seed belongs to the family *Ranunculaceae* is cultivated and distributed in various parts of the world especially in Asian, Middle Eastern, Far Eastern and East Mediterranean countries. The seeds of NS are known for its carminative, stimulatory, protective, curative and diuretic activities and are generally employed in various recipes as spice and condiment due to its aroma particularly in Middle East, Asia, Italy, Germany and France [1].Extensive studies in literature have revealed that fixed oil of NS seeds is rich in linoleic, oleic and palmitic acids [2 - 3] while several authors have reported the essential oil isolated and identified active constituents in NS seeds with beneficial clinical applications [4 - 5].The seeds of NS have been subjected to a range of pharmacological investigations in recent years. Those studies have shown a wide spectrum of activities such as antibacterial [6 - 7], antitumor [8], anti-inflammatory [9 - 10], exhibited central nervous system (CNS) depressant and analgesic [11], hypoglycemic [12-13], smooth muscles relaxant [14 - 15] cytotoxic and immunostimulant [16]. *Candida albicans* is the most frequently isolated yeast pathogen, but *C. glabrata* is rapidly emerging as a common cause of nosocomial yeast infection. Other pathogenic yeast species isolated from clinical samples included *C. tropicalis*, *C. parapsilosis*, *C. kefyr*, *C. famata*, *C. lusitaniae*, *C. guilliermondii*, *C. norvegensis*, etc.However, these are isolated less frequently in comparison to *C. albicans* and *C. glabrata* [17 -20]. A new species of Candida with similar phenotypic characteristics to those of *C. albicans* was identified in 1995 and was named *C. dubliniensis* that was less sensitive than *Candida albicans* to azole antifungals especially fluconazole [20- 22].In recent years, some researchers have focused on the use of components made from plant's extracts that exhibit *in vitro* and *in vivo* biological activity, thereby, justifying based research on traditional medicine for their activity characterization

looking for molecules that are effectively antifungal, with fewer side effects, very tolerable by the human body and costly less [23 - 24].
Literature data on chemical composition of NS seeds oil (cold press) are very limited [25]. Although, no biological evaluation or virtual screening have been done in Morocco. The aim of this work was to characterize the NS seed oil for its fatty acid and sterol composition and to study the oil antifungal activity compared with conventional fungicides effect.

II. MATERIALS AND METHODS

Plant material : Fully ripened seeds used in this work were harvested in the year 2012 from the agricultural province of Had-Kort located at the region of Gharb-Chrarda-Bnihssan region in the west of Morocco. After harvest, the seeds were stored at 4 °C until oil extraction.Oil extraction was carried out using cold-presses (IBG Monforts Oekotec GmbH, Mönchengladbach, Germany) (26-27).

Determination of oil composition

Fatty acid composition : Fatty acids (FAs) were converted to fatty acid methyl esters (FAMEs) before analysis by shaking a solution of 60 mg oil and 3 mL of hexane with 0.3 mL of 2 N methanolic potassium hydroxide [28]. They were analysed by gas chromatograph (Varian CP-3800, Varian Inc.) equipped with a FID. The column used was a CP- Wax 52CB column (30 m×0.25 mm i.d.; Varian Inc., Middelburg, The Netherlands).The carrier gas was helium, and the total gas flow rate was 1 mL/min. The initial column temperature was 170 °C, the final temperature 230 °C, and the temperature was increased by steps of 4 °C/min. The injector and detector temperature was 230 °C. Data were processed using Varian Star Workstation v 6.30 (Varian Inc., Walnut Creek, CA, USA). The results were expressed as the relative percentage of each individual fatty acid (FA) presents in the sample.

Sterols composition : Sterol composition was determined after trimethylsilylation of the crude sterol fraction using a Varian 3800 instrument equipped with a VF-1 ms column (30 m 9 0.25 mm i.d.) and helium (flow rate 1.6 mL/mn) as carrier gas, column temperature was isothermal at 270 °C, injector and detector temperature was 300 °C. Injected quantity was 1 µL for each analysis [29]. Data were processed using Varian Star Workstation v 6.30 (Varian Inc., Walnut Creek, CA, USA).

Oxidative Stability of Seed Oils : The oxidative stability of each sample was determined as the induction period (IP, h) recorded by a 743 Rancimat (Metrohm, Switzerland) apparatus using 3 g of oil sample [30]. Samples placed into Rancimat standard tubes were subjected to the normal operation of the test by heating at 110 °C with an air flow of 20 L/h. The polyphenol content was determined using the Folin–Ciocalteu spectrophotometrically according to the Singleton method [31] using caffeic acid as standard. In an alkaline medium, polyphenols reduce phosphomolybdic acid of Folin–Ciocalteu reagent [32]; this reduction is reflected by the appearance of a dark blue color. The color produced, (whose maximum absorption is between 725 and 765 nm) is proportional to the amount of polyphenols present in plant extracts [33].

Isolation and identification of microorganisms : The 6 isolates of *Candida* studied in this work include *Candida albicans* (n = 12), *Candida dubliniensis* (n = 2), *Candida glabrata* (n = 7) and *Candida krusei* (n = 5). All *Candida* species were clinically isolated from infected patients in the laboratory of parasitology-mycology and bacteriology Avicenna military hospital at Marrakech in Morocco. On Sabouraud chloramphenicol agar plates and identified by the germ tube test and an API 20 C AUX (Bio-merieux, marcy-l'etoile, France) according to the Manufacturer's recommendations and chromogenic medium CandiSelect 4 (Bio-RAD, Marnes-la-Coquette, France). For this experiment, all isolated strains were tested and yeast cells were harvested from Sabouraud dextrose agar, supplemented with chloramphenicol, and then counted and adjusted density in serum glucose sterile 5% of 2.16 x 10^5 Cells/mL to 5.22 x 10^5 Cells/mL with counting with haemocytometer for each repetition.

Drugs used as controls : The used drugs usually eradicate fungal infections such as those belonging to the family of imidazole agents like Fluconazole and those belonging to the family of polyenes, agents such as Amphotericin B. These antifungal agents are dissolved in 2 mL dimethyl sulfoxide (DMSO) 10% to give the following stock solutions: Fluconazole 75 mg/mL and Amphotericine B 33 mg/mL.

Preparation of fungal suspension : A fresh overnight culture, in log phase, of the tested yeasts was used to prepare the cell suspension by inoculating 5 mL of serum glucose 5% broth with an appropriate yeast strain and incubating for 24 hour at 37°C to ensure that yeast cells were actively dividing, then adjusted between: 2.16 x 10^5 Cells/mL to 5.22 x 10^5 Cells/mL for fungal strains with counting with haemocytometer for each repetition.

Antifungal activity

Determination of the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

The broth macro dilution method was used to determine the minimum inhibitory concentration (MIC) according to NCCLS M38P [34] for yeasts. The fungal suspension was then used to inoculate the tubes in the test group. The test groups were prepared with 1 mL of medium containing 10 µL of DMSO 10%, the final concentration never exceeded 2%, and 200 µL of each strain of yeast suspension previously adjusted. 10 µl of *Nigella sativa L.* seeds oil (700 mg / mL), 10 µl of fluconazole (75 mg / mL) and 10µl of amphotericin B (33 mg / mL) were respectively added to tubes containing culture medium in the test groups, then adding a sufficient amount of glucose 5% to a final volume of 1 mL and serial dilutions and concentration gradient is established as follows: *Nigella sativa L.* seeds oil of 7 mg / mL to 0.875 mg / mL, Fluconazole 0.75 mg / mL to 0.09375 mg / mL and Amphotericine B of 0.33 mg / mL to 0.04125 mg /mL . The test tubes were incubated at 37 ° C on a shaker ELISA plates for 48 hours, and the MICs were determined. The MIC was defined as the lowest concentration of the oil at which the microorganism did not demonstrate visible growth. The growth of micro-organisms is evaluated by counting them with haemocytometer for each repetition. To determine the minimum fungicidal concentration (MFC), aliquots (20 µl) of broth were taken from each negative after reading MIC tube, and cultivated in the Sabouraud agar plates (SDA) then incubated at 37 °C for 48 h. The MFC was defined as the lowest concentration of the essential oil at which the incubated microorganisms were completely killed. Each test was performed in triplicate. Fluconazole and amphotericin B were used as positive controls antifungal.

For each strain tested, the growth conditions and the sterility of the medium were checked in two control tubes. The safety of DMSO was also checked at the highest tested concentration. All experiments were performed in triplicate.

III. RESULTS AND DISCUSSION

Chemical Composition of Nigella sativa L. Seeds

Fatty acid composition : Data regarding fatty acid composition of NS oil is presented in Table 1. which shows that linoleic, oleic and linolenic acids account for more than 80% of the total fatty acids. They represent the main unsaturated fatty acids. The ratio of linoleic acid to oleic acid was more than 2:1. This value agreed with those reported in case of soybean oil (C18:2 = 52%, C18:1 = 25%) and in corn oil (C18:2 = 59%, C18:1 = 27%) [34]. The ratio of saturated to unsaturated fatty acids (S/U%) was 49%. These ratios were higher than those reported by Ramadan and Mösel [35] for black cumin seed oil (26%). Atta [36] showed that the oils of black cumin varieties contained oleic and linoleic acids at relatively high levels (19–20 and 48–49, respectively) but these are lower than those corresponding to the Moroccan variety (24 and 59, respectively) (Table I).

Table I. Fatty acid composition of the Moroccan Nigella sativa seeds oil.

Fatty Acid (%)	% of total F.A	Fatty Acid (%)	% of total F.A
Myristic Acid (C14:0)	1 ± 0.1	Linolenic Acid (C18:3)	0.4 ± 0.1
Palmitic Acid (C16:0)	13.1 ± 0.1	Arachidic Acid (C20:0)	0.5 ± 0.1
Palmitolic Acid(C16:1)	0.2 ± 0.1	Gadoleic Acid (C20:1)	ND
Stearic Acid (C18:0)	2.3 ± 0.1	Total saturated F.A (TSFA)	16.8 ± 0.5
Oleic Acid (C18:1)	23.8 ± 0.1	Total unsaturated F.A (TUFA)	82.9± 0.5
Linoleic Acid (C18:2)	58.5 ± 0.1	……	…….

Values are given as means of three replicates ± SD.

In this study, saturated fatty acids accounted for 17 % of total fatty acids. Among them, the main saturated normal chain fatty acids were palmitic, stearic, myristic and arachidic. In this study the Behenic and the Eicosenoic acids were not observed while they were reported in the Tunisian, Iranien and pakistanian seed oil. The source of variability may be genetic (plant cultivar, variety grown), seed quality (maturity, harvesting-caused damage and handling/storage conditions), oil processing variables, or accuracy of detection, lipid extraction method and quantitative techniques [35].

Sterol Composition : We reported total Sterols of NS seed oil 2915 mg/kg of dry matter weight (Table II). sterols were composed of cholesterol representing a minority with 0.88% of Total Sterol (TS), campesterol with 13.1 %, Δ5-avenasterol forming 12.4%, Δ7-avenasterol 2.1%, β-sitosterol and stigmasterol as major sterols representing 49.41 and 17.8%, respectively. As for cholesterol, it has been also observed by Atta [36] in black cumin seeds from Egypt where it was represented 7.2% of (TS) while this component was not detected by Ramadan and Mörsel [35] in NS seeds from Turkey. However, these authors reported the presence of lanosterol,

representing 3.4% of (TS). On the other hand, it is known that the ratio of β-sitosterol/campesterol could be used as an index to identify the oil purity and authenticity. In our findings, the β-sitosterol/campesterol ratio is of 3.7, which is lower than the value (5.8) reported by Nergiz and Otles [37] and Harmouni-Sellami [38] in black cumin seeds from Turkey and Tunisia respectively.

Table II. Sterol composition of the Moroccan Nigella seeds oil.

Sterols	% of total sterols	Sterols	% of total sterols
Cholesterol	0.88 ± 0.1	Beta Sitosterol	49.4± 0.1
Brassicasterol	Nd	Delta 5 avenasterol	12.4± 0.1
Campesterol	13.1± 0.1	Delta 7 stigmasterol	0.64± 0.1
Stigmasterol	17.8± 0.1	Delta 7 avenasterol	2.11± 0.1

Oxidative stability

The oxidative stability is an important parameter for evaluate the oil and fats quality; it gives a good estimation of their susceptibility to oxidation.

Table III. Rancimat (induction time) and Polyphenol content of the Moroccan NS seeds oil.

Induction time (h)	13±3
Polyphenols (as mg gallic acid/kg of oil)	320±6

Induction time evaluated by the Rancimat accelerated method [39], the results of the Rancimat test was about 13 hours (Table III). This value was higher than that found for sunflower (8.5 h) and rapeseed oils (5h) [40]. But the stability of NS seeds oil is more sensitive to oxidation than argan (32 h) and olive oils (26 h) [41]. A high direct correlation was observed between total phenol content and oxidative stability by Rancimat [42; 43].

The NS oil had high phenolic compounds content 320 mg/kg (table III), which mainly determine a greater resistance to auto-oxidation [44, 45]. The amount of phenols in crude seeds oils is an important factor when evaluating the oil quality, because these compounds have been correlated with sensorial quality, the oil shelf life, and particularly its resistance to oxidation [46].

Antifungal activity

Determination of minimum inhibitory and fungicidal concentration : The results presented in the table 4 show that the Nigella seed's oil has a strong antifungal activity compared with the conventional fungicide. The MIC_{50}, MIC_{90} and the Minimal Fungicidal Concentration (MFC) were respectively 2.453; 4.916 and 6.360 (mg/mL) against *Candida albicans, 2.955; 5.183 and 6.360 (mg/mL) against Candida dubliniensis,3.630; 5.992 and 6.360 against Candida glabrata, and 2.724; 4.939 and 6.360 against Candida krusei*, all strains are isolated from nosocomial infections in hospitals (Table 4). We note a very important antifungal activity especially against strains *Candida albicans* species, this activity extends even to species resistant to conventional antifungal used as control. The antifungal activity of Nigella seed's oil may be attributed to the presence of β-sitosterol and oleic acid as the main components in the oil composition of *Nigella sativa L.*, several studies have shown that long chain fatty acid has a fungistatic effect against a few strains of *Candida* [47, 48]. Also another study showed that different components such as β-sitosterol and stigmasterol has antifungal activity against pathogenic fungi to humans, *Aspergillus flavus, A. Niger, Geotrichum candidum*, *Candida tropicalis* and *C. albicans* [49]. A fraction composed of other β-sitosterol, isolated from the hexane extract of *Lantana hispida* showed significant antimycobacterial even among strains of *Mycobacterium tuberculosis* multidrug-resistant activity [50] and as antibacterial [51]. Also, previous studies showed that β-sitosterol have antifungal activity, and other oils rich in β-sitosterol as oil of *vitex agnus castus L.*, who showed very strong antifungal activity against Candida species [52]; in agreement with the current results [53]. Although the high content of polyphenols of Nigella seeds oil, may be responsible for its significant antioxidant activity and responsible for its effectiveness on the inhibition of growth of certain bacteria and yeasts including *Candida albicans* [54]. In another study conducted by Sitheeque et al, it was shown that all the polyphenols of black thea showed anti-Candida activity against all tested Candida species as C. *albicans* and C. *glabrata* that was found to be the most sensitive species followed by C. *parapsilosis*, C. *albicans*, C. *krusei* and C. *tropicalis* [55]. Also, Herrera et al. [56] concluded that all propolis extracts rich in polyphenols in varying concentrations evaluated are able to inhibit the growth of *Candida* species, all presenting significant differences in the concentration of polyphenols present and the corresponding antifungal activity.

Table IV. Minimum Inhibitory concentrations (MIC50 and MIC_{90} [mg/mL]) and Minimum Fungicidal Concentration MFC ([mg/mL]) of *Negilla sativa L.* seeds oil against *Candida* strains.

MIC and MFC (mg/mL) at 48 h					
Species (Number of isolates)	Nigelle oil & Antifungal agent	Range	MIC_{50}	MIC_{90}	MFC
Candida albicans (n=12)	Nigelle Oil	6.36-0.39	2.453	4.916	6.36
	FLC	0.75-0.012	0.012	0.112	0.112
	AMB	0.33-0.005	0.117	0.238	0.238
Candida dubliniensis (n=2)	Nigelle Oil	6.36-0.39	2.955	5.083	6.36
	FLC	0.75-0.012	0.39	0.711	0.75
	AMB	0.33-0.005	0.005	0.022	0.022
Candida glabrata (n=7)	Nigelle Oil	6.36-0.39	3.63	5.992	6.36
	FLC	0.75-0.012	0.431	>0.75	>0.75
	AMB	0.33-0.005	0.171	0.295	0.295
Candida krusei (n=5)	Nigelle Oil	6.36-0.39	2.724	4.939	6.36
	FLC	0.75-0.012	0.478	>0.75	>0.75
	AMB	0.33-0.005	0.175	0.296	0.296

Fluconazole (FLC); Amphotericine B (AMB); *Nigella sativa L.* Seeds oil (Nigelle oil).

IV. CONCLUSION

The antifungal activity of the *Nigella sativa L.* seed's oil has been demonstrated even in *Candida* species responsible for nosocomial infections resistant to certain antifungal synthesis , which suggests its use in the future as adjuvant to conventional antifungal therapy. However, this oil should be tested to evaluate its effectiveness in different environments and cropping systems and also assess its toxicity and safety in clinical and pharmacological experiments. This study demonstrates the composition and the antifungal activity of the Nigella seeds oil which could be a new source of edible oil and consolidate the idea of using Nigella sativa L. oil in food, in cosmetics and in pharmaceutical products.

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