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Full Length Research Paper

Chemical composition, antibacterial and antifungal properties of Tunisian *Nigella sativa* fixed oil

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We investigated the chemical composition (by gas chromatography) and anti-microbial activity of the Tunisian *Nigella sativa* fixed oil against different standard Gram-negative and Gram-positive strains and Candida isolates by disc diffusion method, and determined the minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC) and minimal fungicidal concentration (MFC) values. Twenty three fatty acids were identified in *N. sativa* fixed oil. Palmitic acid was the major saturated fatty acids (SFA) accounted for 12.2% of the total fatty acid (TFA). For monounsaturated fatty acids (MUFA), oleic acid was the major one with 21.67% of TFA. Linoleic acid has the most important percentage (58.73% of TFA) and was the major polyunsaturated fatty acids (PUFA). For the antibacterial activity, the best inhibition was seen against *Staphylococcus aureus* ATCC 25923 (16.66 mm) and *Salmonella typhimurium* ATCC 14028 (15.33 mm). The best antifugal activity was found against *Candida parapsilosis* ATCC 22019 (13.33 mm) and *Candida glabata* ATCC 90030 (12 mm). This fixed oil can be used as an antibacterial and antifungal agent each time the MIC values have a good effect on the antibacterial and antifungal activities of the Tunisian *N. sativa* fixed oil. Our results demonstrate important antibacterial and antifungal activities of the Tunisian *N. sativa* fixed oil; these effects further validate the traditional use of *N. sativa* in the folk medicine against bacterial and fungal infection.

Key words: *Nigella sativa*, fixed oil, gas chromatography (GC), antibacterial activity and antifungal activity.

INTRODUCTION

A variety of plants and plant extracts were used throughout human history for medicinal purposes (Ali and Blunden, 2003). Of these *Nigella sativa*, an annual herbaceous plant belonging to the *Ranunculaceae* family that produces seeds commonly known as "black seed" was used for more than 2000 years in the Middle East and Indian subcontinent for treating a variety of diseases (Aljabre et al., 2005). It was shown that *Nigella* extracts and oils mediate diverse biological activities, including anti-tumor, anti-inflammatory, anti-oxidant, anti-diabetic,and antibiotic activities (Burtis and Bucar, 2000). Accordingly, Nigella were recommended as supplement in the treatment of diverse ailments, ranging from asthma, chronic headache (including migraine), chest congestion, dysmenorrhoea, obesity, paralysis, hemiplegia, back pain, rheumatism, hypertension and gastrointestinal problems. Furthermore, it was proposed to be used as a stimulant, diuretic, emmenagogue, lactagogue, anthelmintic and carminative agent (Cowan, 1999).

Black seeds contain 36 to 38% fixed oil by weight; the remainder consisting of proteins, alkaloids, saponins, and essential oils (Denning et al., 1997). Within fixed oils,

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Nickavara et al. (2003) reported that palmitic acid was the major saturated fatty acids (SFA), accounting for 12.5% of total fatty acids (TFA). On the other hand, oleic acid was the major mono-unsaturated fatty acid (MUFA), accounting for 21.67% of TFA, while linoleic acid was the most important polyunsaturated fatty acid, with an estimated 55.6% of TFA. Despite their potential role as antimicrobial agents, limited interest in the use of plants or plant extracts was seen in the last century (Gali-Muhtasib et al., 2000), which has changed in recent years, and ethnobotanical research field has expanded (Ghedira, 2006).

Nigella crude extracts were reported to have a promising effect on multi-drug resistant organisms, including *Staphylococcus aureus*, *Shigella, Vibrio cholera* and *Candida albicans* (Salman et al., 2008). The antibacterial effect of *N*. sativa was further tested on Gram-positive and Gram-negative bacteria. While black seed extract and oil were shown to possess antibacterial activity, Gram-positive bacteria were more susceptible to the black seeds oil, whereas Gram-negative organisms were more sensitive to the black seeds extracts (Gulluce et al., 2003).

In the present study, we investigate the chemical composition, along with the anti-bacterial and the anti-fungal activities of Tunisian *N. sativa* fixed oil.

MATERIALS AND METHODS

Plant material

Seeds of *N. sativa* used in the present work were harvested in 2009 from the agricultural province of El-Gobba, located in the region of Menzel-Temime (northeast Tunisia). The latitude of the region is 36.74°, the altitude is 45 m, the annual precipitation average is 500 mm/year, and the monthly temperature average is 19.9°. Following harvest, seeds were stored at 4° pending extraction.

Plant extracts

Extraction and analysis of fixed oil

Extraction of crushed seeds (250 g) was made in petroleum ether for 4 h in a Soxhlet apparatus as described by Nickivara el al. (2003). After concentration under reduced pressure, 1 ml was dissolved in 20 ml petroleum ether, and 2 ml of methalonic KOH (2M) was added. The mixture was shaken for 2 min, and was allowed to stand for 10 min. The fatty acid methyl esters-rich upper layer was removed, washed with water, and analyzed by GC using Hewlett-Packard (HP) 5890 chromatograph, a split/splitless injector, and a flame-ionization detector (FID) linked to an HP Chemstation integrator. A DB23 fused silica capillary column (60 m length × 0.32 mm i.d × 0.25 µm film thickness; HP-Agilent Technologies, Wilmington, DE) was used with nitrogen as the carrier gas, the flow rate of which being set at 0.44 mL/min. The temperature of the flame was maintained at 280° for the ionization detector and at 270° for the injector as per: 6.5°/min: from 130 to 170° at 2.8°/min: from 170 to 215°, 12 min isotherm, 230 at 40°/min and 20 min isotherm. The standard fatty acids methyl esters were run under the same conditions (Freese et al., 1973).

Assessment of antibacterial activity.

Bacterial strains

The antimicrobial activity of *N. sativa* fixed oil was investigated using the following reference strains: Gram-positive cocci: *Staphylococcus epidermidis* CIP106510, *S. auras* ATCC 25923, *S. aureus* ATCC 29213, *Micrococcus luteus* NCIMB 8166, *Enterococcus feacalis* ATCC 29212 and the Gram-negative bacteria: *Escherichia coli* ATCC 35218, *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella thyphymirium* ATCC 14028, *S. typhimurium* ATCC 1408, and *Listeria monocytogenes* ATCC 19115.

The antibacterial activity was assessed using the agar-disk diffusion assay (Hannan et al., 2008). Briefly, bacterial cultures were grown overnight (18 to 24 h) on Mueller-Hinton agar (MH) plates at 37°. Isolated bacterial colonies were then transferred into API suspension medium (Biomérieux, France), and adjusted to the 0.5 McFarland turbidity standard with a Densimat (Biomérieux, France). Bacteria inoculate was plated on MH agar plates, and dried for 5 mn at room temperature. A sterile 6 mm Whatman No. 3 filter paper was placed on the MH agar seeded with bacteria, and *N. sativa* fixed oil (10 µl) was dropped onto each paper disk, followed by incubation at 37° for 1 h, and a further overnight (18 to 24 h) at 37°. The antibacterial activity was evaluated by measuring the zone of growth inhibition around the disks. Standard disks of Gentamycin (10 µg) served as positive anti-bacterial controls (CASFM 2005 guidelines).

Assessment of antifungal activity

Four reference Candida species were used: *C. albicans* ATCC 90028, *Candida Krusei* ATCC 6258, *Candida parapsilosis* ATCC 22019, and *Candida glabrata* ATCC 90030. The anti-*Candida* spp. activity was achieved by the agar-well diffusion method. All *Candida* reference strains were inoculated onto Sabouraud dextrose agar and incubated for 18 h at 37°. The yeast cultures were harvested, suspended in sterile saline, and the cell density adjusted to 10^7 cells/ml (OD_{540} = 0.5). For the antifungal activity of essential oil investigated, three sterile 6 mm Whatman paper (N°3) discs were impregnated with 10 µl of *N. sativa* fixed oil, placed on the inoculated surface, and incubated at 37° for 18 to 24 h. The diameter of the zones of inhibition around each disc were examined after 24 h, and recorded as the mean diameter (mm) of complete growth-inhibition. Amphotericin B (Fungizone, BioBasic INC; 10 µg) was used as positive control. Testing was done in triplicates; results being expressed as the mean of three determinations (Table 2).

Minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC), and minimal fungicidal concentration (MFC) assays

Minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC), and minimal fungicidal concentration (MFC) values were determined for bacterial and fungal strains used, as described (Khan et al., 2003). The microbial strains inocula were prepared from 12 h broth cultures and suspensions, and were adjusted to 0.5 McFarland standard turbidity. The 96-well plates were prepared by dispensing into each well 95 µl of nutrient broth, and 5 µl of the inoculum. Fixed oil (100 µl) concentrations were added into respective wells, followed by transfer of 100 µl from the serial dilutions were into eleven consecutive wells. The last well containing 195 µl of nutrient broth without the fixed oil and 5 µl of the inoculum on each strip was used as negative control. The plates were incubated at 37° for 18 to 24 h. The MIC was defined as the lowest concentration of the compounds able to inhibit the

Table 1. Fatty acid compositions of Tunisian *N. sativa* fixed oil.

Fatty acids	Retention time	Percentage
C14:0	5.069	0.307417
C14:1	5.782	0.036341
C16:0	6.845	12.206697
C16:1 w7	7.167	0.558833
C18:0	9.468	2.460320
C18:1 w9	9.869	21.671780
C18:1 w7	10.288	1.278507
C18:2 w6	10.758	58.731533
C18:3 w3	11.779	0.591862
C20:0	12.712	0.008544
C20:1 w9	13.012	0.448121
C20:2 w6	13.481	0.640796
C20:3 w6	14.101	0.078654
C20:4 w6	15.145	0.025675
C20:5 w3 (EPA)	16.177	0.002180
C22:0	17.995	0.052505
C22:1	20.493	0.006047
C22:4 w6	21.350	0.001687
C22:5 w3	22.220	0.109123
C24:0	23.991	0.003782
C22:6 w3 (DHA)	25.527	0.007143
C24:1	26.769	0.252455

growth of the microorganisms. The MBC and MFC values were interpreted as the highest sample dilution (lowest concentration), which showed clear fluid with no development of turbidity and without visible growth (Table 3).

RESULTS AND DISCUSSION

Twenty three fatty acids were identified in the Tunisian *N. sativa* fixed oil, which represented about 99.93 % of the total fatty acid (Table 1). The extract was consisted of six saturated fatty acids (SFA) (15.51%), seven monounsaturated fatty acids (MUFA) (24.24%) and nine polyunsaturated fatty acids (PUFA) (60.18 %). Palmitic acid was the major SFA (12.2% of TFA), which was in accord with the findings of Nickavara et al. (2003). In contrast, the oleic acid content among TFA fraction (21.67%) was lower than to that reported by Nickavara et al. (2003) (23.5% of TFA), while linoleic acid was detected at higher concentrations (58.73% of TFA) than (55.6%) reported by Nickavara et al. (2003).

An alarming increase in antibiotic-resistant bacterial strains necessitates that search for more effective antibiotics, which depends on localization of bioactive photochemical. Insofar as plants were long used for preventing, or even curing infectious conditions, several plant products were documented to inhibit growth of pathogenic bacteria (Knapp and Melly, 1986). Of these, black seed extract was extensively studied for its antimicrobial activity against a wide range of bacterial, fungal and parasitic organisms (Manoj and Pradeep, 2005). Here we investigate Tunisian *N. sativa* fixed oil anti-bacterial and anti-*Candida* activities, which were reported as inhibition zones, along with "*in vitro*" activities (MIC, MBC and MFC).

N. sativa fixed oil displayed significant antimicrobial activity, but with varied efficiency dependeing on the strains tested, highlighted by range in the zones of inhibition (IZ) from 7 to 16.66 mm (bacteria), and 8 to 13.33 mm (*Candida*), of which *S. aureus* ATCC 25923 (IZ = 16.66 mm) and *S. typhimurium* ATCC 14028 (IZ = 15.33 mm) showing the best inhibition. Weak anti-bacterial activity was seen for *S. epidermidis* CIP 106510 (IZ = 6 mm) and *E. coli* ATCC 25922 (IZ = 7 mm). This was reminiscent of the finding of Manoj and Pradeep, (2005) in which the inhibitory effect of black seed oil on *L. monocytogenes* was reportedly more than twice that of gentamicin.

Long-chain unsaturated fatty acids were previously shown to possess anti-bacterial activity, and their inclusion as food additives reportedly inhibited the outgrowth of microorganisms (McCutcheon et al., 1995).

For example, linoleic acid and oleic acid were the anti-bacterial components contained in herbs (McGaw et al., 2002). In addition, long-chain unsaturated fatty acids are bactericidal to pathogenic microorganisms, including

Table 2. Antibacterial and antifungal activity of Tunisian *N. sativa* fixed oil.

Strains	IZD*	Gentamycin (10 µg/disk)	Amphotericin B (10 µg)
L. monocytogenes ATCC 19115	14.66 ± 0.577	6 ± 0	-
E. feacalis ATCC 29212	9.33 ± 0.577	6 ± 0	-
S. epidermidis CIP 106510	6 ± 0	8 ± 0	-
S. aureus ATCC 25923	16.66 ± 0.577	22 ± 0	-
S. aureus ATCC 29213	13.66 ± 0.577	22 ± 0	-
M. luteus NCIB 8166	11.33 ± 0.577	24 ± 0	-
E. coli ATCC 35218	13.66 ± 0.577	18 ± 0	-
E. coli ATCC 25922	7 ± 0	20 ± 0	-
S. typhimurium ATCC 1408	11.66 ± 0.577	24 ± 0	-
S. typhimurium ATCC 14028	15.33 ± 0.577	14 ± 0	-
P. aeruginosa ATCC 27853	12.33 ± 0.577	13 ± 0	-
C. albicans ATCC 90028	10.33 ± 0.577	-	11 ± 0
C. glabata ATCC 90030	12 ± 0	-	14.33 ± 0.577
C. Krusei ATCC 6258	8 ± 0	-	10.33 ± 0.577
C. parapsilosis ATCC 22019	13.33 ± 0.577	-	10.33 ± 0.577

*: Inhibition zone in Diameter (mm ± SD) around the discs Impregnated with 10 µl (10 mg/ disc) of fixed oil.

Table 3. MIC and MBC values of Tunisian *N. sativa* fixed oil.

Microorganisms tested	*N. sativa* fixed oil (mg/ml)	
	MIC	MBC
L. monocytogenes ATCC 19115	3.125	6.25
E. feacalis ATCC 29212	3.125	6.25
S. epidermidis CIP 106510	3.125	12.5
S. aureus ATCC 25923	1.562	25
S. aureus ATCC 29213	1.562	6.25
M. luteus NCIB 8166	6.25	12.5
E. coli ATCC 35218	6.25	25
E. coli ATCC 25922	3.125	6.25
*S. typhimuriu*m ATCC 1408	6.25	25
S. typhimurium ATCC 14028	3.125	6.25
P. aeruginosa ATCC 27853	6.25	50

MIC: Minimal inhibitory concentration; MBC: Minimal bactericidal concentration.

Methicillin-resistant *S. aureus* (Nadkarni, 1976), H. pylori (Nickavara et al., 2003), and *Mycobacteria* (Nilsson, 1978). In our hands, linoleic acid and oleic acid were the major constituents of *N. sativa* fixed oil. However, the possibility that other compounds in the oil that may act in an additive or synergistic fashion in mediating anti-bacterial properties of *N. sativa* fixed oil cannot be excluded at present.

The increased predisposition to fungal infections precipitated by several pathological conditions necessitated the development of effective and safe anti-fungal drugs (Phillips, 1992). Anti-fungal activity tests demonstrated that *N. sativa* fixed oil showed varied growth inhibition of *Candida* strains, evidenced by effective inhibition of *C. parapsilosis* ATCC 22019 (IZ = 13.33 mm) and *C. glabata* ATCC 90030 (IZ = 12 mm), and marginal inhibitory activity against *C. Krusei* ATCC 6258 (IZ = 8 mm). Our findings were in agreement with those of Aljabre et al. (2005) which recommended the use of *N. sativa* as a natural antifungal drugs in the treatment of dermatophytes skin infections, and with the findings of Khan et al. (2003) in which the aqueous extract of *N. sativa* seeds possessed anti-candidal activity in a murine model with no visible adverse effects on the tissue architecture, indicating a strong antifungal activity.

Table 4. MIC and MFC values of Tunisian *N. sativa* fixed oil.

Microorganisms tested	*N. sativa* fixed oil (mg/ml)		Amphotericin B (mg/ml)	
	MIC	MFC	MIC	MFC
C. albicans ATCC 90028	0.781	3.125	0.097	0.781
C. glabrata ATCC 90030	0.781	6.25	0.195	1.562
C. Krusei ATCC 6258	1.562	3.125	-	-
C. parapsilosis ATCC 22019	0.195	6.25	0.195	0.39

MIC: Minimal inhibitory concentration; MFC: Minimal fungicidal concentration.

The MIC, MBC and MFC values of the *N. sativa* fixed oil against different strains are summarized in Tables 3 and 4. The strongest anti-bacterial activity was seen against *L. monocytogenes* ATCC 19115, *E. feacalis* ATCC 29212, *E. coli* ATCC 25922, and *S. typhimurium* ATCC 14028 (MIC: 3.125 mg/ml; MBC: 6.25 mg/ml). The strongest anti-candidal activity was seen against *C. albicans* ATCC 90028; MIC: 0.781 mg/ml; MFC: 3.125 mg/ml). The anti-fungal amphotericin B was also active against all *Candida* reference strains (zone of inhibition range: 10.33 to 14.33 mm; MIC range: 0.097 to 0.195 mg/ml).

N. sativa fixed oil possesses appreciable antimicrobial activity against most of the bacteria and fungi used here, evidenced by the MIC MBC and MFC values. This is likely to be attributed to the presence of high concentrations of Linoleic (58.73% of TFA) and oleic acids (21.67% of TFA), as was also demonstrated elsewhere (Cutcheon et al., 1995). The development of resistance to current antibacterial continues to be a serious difficulty in the treatment of infectious diseases, and thus development of newer antibiotics has become a high priority in biomedical research (Sun et al., 2003). On the other hand, the frequency of invasive fungal infection has risen substantially with the increasing numbers of immunocompromised patient, such as those infected with HIV, those receiving cancer chemotherapy, immunosuppressive therapy, or broad-spectrum treatment (Walsh et al., 2004).

Conclusions

Our results showed that *N. sativa* fixed oil has an important antimicrobial activity. It remains a good natural source for the production of new antimicrobial drugs, thereby supporting the broader use of *N. sativa* as natural medicine for microbial infections.

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