Antimicrobial Activity by a Unique Composition of Cold Pressed Nigella Sativa Seed (Black Cumin) Oil

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Received: 15 October 2021; Accepted: 19 November 2021


ABSTRACT

The oil produced from Nigella sativa seeds (black cumin) is a known folklore remedy for treatment of various health conditions, including the treatment of microbial infections. The microbes Malassezia furfur, Candida albicans and Staphylococcus aureus are commonly found in healthy skin and gut microbiota, but can also be infection-inducing microorganisms upon overgrowth, capable of disrupting the homeostasis of the microbiome. Treatment with antibiotics has led some of these microbes to evolve antibiotic-resistant strains. Several Nigella sativa oils produced by cold pressing of agricultural crops to contain high or low amounts of the active molecule, thymoquinone as well as high or low amounts of free fatty acids were tested for their antifungal and antibacterial properties. The growth of Malassezia furfur and an antibiotic-resistant strain of Candida albicans were most inhibited by a combination of high amounts of Thymoquinone (3%) and low amounts of free fatty acids (2%). While the growth of Staphylococcus aureus is strongly inhibited by Nigella sativa oil, it seems that another component, yet to be identified, is responsible for the antibacterial effect as all oil compositions tested presented similar and strong inhibition of the bacterial growth. According to the results, Nigella sativa oil may be used as an alternative safe antimicrobial agent, and perhaps even as a preventative care for maintenance of microbiome balance and diversity.

Keywords
Nigella sativa seed oil, Thymoquinone, Antimicrobial, Candida albicans, Malassezia furfur.

Introduction

The use of medicinal herbs for the treatment of various medical conditions has been common since ancient times. The plant Nigella sativa (N. sativa, family Ranunculaceae) is an annual flowering plant native to south-eastern Europe, western Asia, including the Middle East and northern Africa. It is cultivated in different parts of the world for nutritional purposes, traditional medicine as well as for a source of its unique constituents [1,2]. For the past few decades, N. sativa has been the focus of researchers due to its qualities that are perceived as beneficial for improved nutrition and as a natural remedy. Deciphering of the major chemical components of N. sativa [3], particularly its seed (also termed black cumin seed) composition [2,4-6], has resulted in a growing interest in N. sativa. More recently, there has been a revival of interest in the use of N. sativa seeds and of various oils, including preparations derived therefrom, for the treatment of multiple conditions and as a supplement for use in the maintenance of good health.

Black cumin seeds are rich with oils, including fatty acids and several essential oils [7]. Many, but not all, of the agents that are present in the oil contained within the Nigella sativa seed are pharmacologically active. In fact, the seeds of this plant contain two distinguishable oil fractions: fixed oil and essential oil, the latter containing a mixture of highly active volatile agents. Among these volatiles, one of the most active component is the monoterpene thymoquinine (TQ), (Figure 1) and many of the pharmacological activities of Nigella sativa oil-derived compositions are attributable to this agent, including antioxidant and anti-inflammatory qualities [8,9].
In Maulidiani et al. [10], a study defining and differentiating *Nigella sativa* seeds from four different origins resulted in distinct composition, biological molecules and biological activity. For example, the Ethiopian sample exhibited high DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging and nitric oxide (NO) inhibition activities, theorized to be related to the presence of higher levels of TQ and thymol. Alternatively, the Qasemi and Syrian samples exhibited high α-glucosidase inhibitory activity, which was correlated to the high fatty acid contents. The composition and ratio between active molecules in the *N. sativa* seed oil appears to play a major role on the bioactivity of these oils.

*N. sativa* oil was also found to possess antimicrobial properties [11,12]. Bacterial or fungal skin infections and systemic infections are a common health burden that can impair the quality of life for many individuals, and in some cases can develop into life-threatening conditions. For example, even though it is a natural part of a healthy skin microbiome, upon overgrowth *Malassezia* yeasts are associated with several cutaneous pathologies including tinea versicolor, atopic dermatitis and seborrheic dermatitis [13]. Moreover, *Malassezia* yeasts may also cause systemic infections that can lead to severe illnesses when penetrating the bloodstream, potentially being more threatening than originally presumed by health care professionals [14]. *Candida albicans* (*C. albicans*) is a fungus found in the gut, skin and epithelial tissues that causes frequent infections, referred to as cutaneous candidiasis when present as a skin infection and as candidiasis when present as a systemic infection. *C. albicans* is also present in mucosal tissues and can cause oral infections [15]. The available, conventional anti-fungal therapies primarily include three classes of agents: azoles, polyenes and echinocandins. Azoles and polyenes are frequently used to treat *Malassezia* and *Candida* species, however, their frequent use contribute to the development of resistant strains [16]. *Staphylococcus aureus* (*S. aureus*) is a Gram-positive bacterium and the most common pathogen involved in skin infections such as impetigo, cellulitis and infected wounds. Due to its high prevalence, especially in health care institutions, a number of antibiotic-resistant strains of *S. aureus* have evolved, including methicillin-resistant *S. aureus* (MRSA), a dangerous strain that is resistant to most antibiotic treatments [17]. The development of antibiotic-resistant strains and resulting prevalence of infections emphasizes the need to find alternative or additional anti-microbial therapies. Furthermore, maintaining homeostasis of the skin microbiome may be perturbed upon impaired regulation of key organisms growth [18-20]. Black cumin oil may have the potential to both inhibit overgrowth and support maintenance of a healthy skin and gut microbiome. To date, several works have demonstrated antimicrobial activities by black cumin oil [21]. This current research aims to demonstrate the importance of standardization of cold pressed black seed oil to achieving this biological effect, primarily through standardization of the level and the ratio of the active components within the oil, namely the TQ and free fatty acids (FFA) levels. Specifically, a unique *N. sativa* oil’s TQ and FFA composition has shown to have a significant role in the efficacy of the oil’s antifungal (*M. furfur* and *C. albicans*) and anti-bacterial (*S. aureus*) activities, including against antibiotic-resistant strains.

**Results and Discussion**

*N. sativa* seeds oil composition of tested crops

The thymoquinone content in *N. sativa* seeds generally occurs at concentrations of 0.3% to 1.0% (w/w), with differences observed between seeds obtained from different countries and regions. The cold-pressed oil obtained from *N. sativa* seeds predictably contains on average TQ levels generally in the range of 0.5% to 1.5% (w/w).

Various procedures have been developed for obtaining TQ-rich fractions from *N. sativa* seeds. However, these procedures are often complex, lengthy, and expensive, being selective for enriching the TQ level at the expense of losing other important oil components. In many cases the procedures employ the use of solvent extraction or CO$_2$ extraction steps which can result in selective extraction, concerns related to the use of chemicals or exorbitantly expensive extraction costs [10,22,23].

The manufacturing process used for the preparation of the oils in the current study is a cold press process, which is an environmentally friendly, physical process that doesn’t require the use of organic solvents or supercritical CO$_2$. A cold pressed oil maintains the inherent composition of the oil as well as the purity, allowing consistent delivery of a full-spectrum oil with optimal strength and potency. The oil content in the *Nigella sativa* seeds is around 30% as well as the yield, with 1000 kg seeds resulting in about 300 kg oil.

The oils used in the current study are sourced from *N. sativa* crops that have been bred over generations to produce a higher concentration of TQ, the key active molecule in the black seed oil. These levels of TQ are higher than is generally found in oil from average *Nigella sativa* seeds [10,23-27], and allows for standardization of the oils with TQ concentrations up to 3%. Table 1 summarizes the composition of oils used in this study. For simplicity, the values of FFA in oils are rounded to 2% or 10% for low or high content, respectively, and amounts of TQ are rounded to 3% or 0.5% for high or low content, respectively.
Table 1: Composition of *N. sativa* oils evaluated in this study. FFA amounts are expressed as Oleic acid equivalent.

<table>
<thead>
<tr>
<th>Black seed oil formulation 1.8% TQ low FFA</th>
<th>Thymoquinone (TQ) %</th>
<th>P-cymene %</th>
<th>Free Fatty Acids (FFA) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black seed oil 3% TQ low FFA</td>
<td>3.21</td>
<td>1.33</td>
<td>1.80</td>
</tr>
<tr>
<td>Black seed oil 3% TQ high FFA</td>
<td>3.08</td>
<td>1.21</td>
<td>10.30</td>
</tr>
<tr>
<td>Black seed oil 0.5% TQ low FFA</td>
<td>0.53</td>
<td>0.21</td>
<td>2</td>
</tr>
<tr>
<td>Black seed oil 0.5% TQ high FFA</td>
<td>0.42</td>
<td>0.23</td>
<td>9.80</td>
</tr>
</tbody>
</table>

Figure 2: *N. Sativa* oils containing 3% TQ inhibit *M. furfur* growth. Antifungal activity of four oils diluted or not in Jojoba oil after 48 hr culture. Zinc pyrithion (ZPT) was used as a positive control. (A-D) Representative Pictures of the microbial plates. (E-H) The zone of inhibition was measured using ImageJ software. Results are presented as diameter means ± SEM, n=3, */**/**p<0.05/0.01 for significant differences from control group (empty microbial disc), n/s statistically non-significant.
Antifungal and antibacterial properties

Four oils with either high or low content of TQ (estimated 3% or 0.5%, respectively), and low or high content of FFA (2% or 10%, respectively), were tested for their antifungal qualities. The oils were tested at four concentrations: undiluted (100% oil) or diluted with jojoba oil to 10%, 5%, and 1% (v/v). A disc diffusion assay was used to assess the ability of N. Sativa oil to inhibit M. furfur growth. Three of the undiluted oils (100%) inhibited the growth of M. furfur in a TQ and FFA dose dependent manner, except the oil composed of 0.5% TQ and 10% FFA which did not inhibit growth (Figure 2). Interestingly, the oils diluted in jojoba oil did not present antifungal properties and presented similar results as the negative control (empty disc). This may imply that jojoba oil, which has a similar chemical structure to sebum, is promoting the growth of M. furfur, having a cancelling effect on the growth inhibition effects of TQ. M. furfur is known to be nourished by oily skin fractions like sebum. Further study is needed to confirm this explanation. The oil containing 0.5% TQ and 2% FFA moderately inhibited fungal growth in a similar manner as zinc pyrithione (ZPT, 0.01%), a known antifungal agent [28], which was used as a positive control. However, the oils containing 3% TQ showed the most potent inhibition of M. furfur growth. This shows that as a single factor, 3% TQ was the most significant factor to affect the inhibition of M. furfur growth. The comparison of efficacies presented by four undiluted oils is shown in Figure 3 which illustrates that the presence of 3% TQ in the oil allows for strong antifungal activity. Interestingly, among the two oils containing 3% TQ, the one containing 2% FFA had enhanced potency compared to the one containing 10% FFA. This observation was strengthened by similar results while tracking inhibition of Fluconazole-resistant C. albicans growth (Figure 4). As expected, Fluconazole did not inhibit the growth of Fluconazole-resistant C. albicans. In the C. albicans series ZPT was used as the positive antifungal control. As before, undiluted oils containing 3% TQ presented the strongest antifungal activity, with the oil possessing a combination of 3% TQ and low 2% FFA exhibiting the strongest inhibition effect. The antifungal effect of oils containing 0.5% TQ was mild. Interestingly, the oil containing 1.8% TQ and 2% FFA showed a moderate inhibition effect, with a higher potency than the oil with 0.5% TQ concentration and a similar potency to the oil with 3% TQ and 10% FFA composition.

Collectively, these findings are confirmation that the antifungal activity of black seed oil is dependent on the concentration of TQ and that a low level of FFA is correlated with maximum efficacy. The higher levels of FFA may contribute to the degradation of TQ in the black seed oil, possibly through the pro-oxidant activity of FFAs. This conclusion is further emphasized in the statistical comparison of tested oils (Figure 5) that demonstrates that TQ is the most active single component factor and that TQ activity is maximized at the lowest FFA levels. Though FFAs are known for their antibacterial and antifungal activities [29,30] it seems that at higher concentrations FFAs impair oil quality which directly impacts the ability of TQ to inhibit fungal growth.

Assessment of the antibacterial properties of N. sativa oils painted a different picture. N. sativa oils strongly inhibited the growth of Methicillin-resistant S. aureus (MRSA), however, no preference was observed for different levels of TQ or FFAs and no significant differences were observed between the inhibition abilities of the different oils (Figure 6). The fact that all N. sativa oils inhibited the growth of S. aureus support the anti-bacterial benefits of the oils, but there is a need to further investigate which part or parts of the oil is functional in order to optimize and calibrate this benefit.

Figure 3: Enhanced antifungal activity by a unique composition of 3% TQ and 2% FFA. Comparison of antifungal activities by undiluted oils. The main ability to inhibit fungal growth is attributed to higher TQ content. The combination of 3% TQ and 2% FFA is much more potent than the combination of 3% TQ and 10% FFA. Results are presented as diameter means ± SEM, n=3.
Figure 4: *N. Sativa* oils containing 3% TQ inhibit *C. albicans* growth. The antifungal activity of five oils following 48 hr of culture. ZPT (0.5%) was used as a positive control. (A-B) Representative pictures of the microbial plates. (C) The zones of inhibition are presented in the graph as diameter means ± SEM; n=3.

Figure 5: Enhanced inhibition of *C. albicans* growth composition of 3% TQ and 2% FFA. Comparison of antifungal activities according to oil compositions. (A) The importance of TQ in the ability to inhibit fungal growth. (B) The amounts of FFAs affect the ability of TQ to inhibit *C. albicans*. n/s statistically non-significant.
Figure 6: *N. Sativa* oils inhibit *S. aureus* growth. The ability of five *N. sativa* oils to inhibit *S. aureus* growth was examined in Disc diffusion assay. The zones of inhibition are presented in the graphs as diameter means ± SEM; n=3.

Figure 7: *N. sativa* oil present potent antimicrobial properties. The ability of low amounts of *N. sativa* oil containing 3% TQ and 2% FFAs to inhibit M. furfur (A-B) growth or *S. aureus* (C-D) was tested using MIC assay. Results are presented as percent of control by means ± SEM, n=3, */**p<0.05/0.01 for significant differences from control group.
In view of the strong observed antimicrobial activity of oils rich with TQ and with the aim to assess if it is possible to reduce the oil concentration while maintaining the antimicrobial activity of N. sativa oils, the minimum inhibitory concentration (MIC) assay, a more sensitive antimicrobial method, was performed. The ability of black seed oil with 3% TQ and 2% FFA diluted in Dimethyl sulfoxide (DMSO, v/v) to inhibit M. furfur and S. aureus growth, separately, was examined. The black seed oil with 3% TQ and 2% FFA was diluted to 2% oil in DMSO, v/v, followed by serial dilution in microbial growth medium. A final concentration of 0.002% formulation inhibited the growth of M. furfur by more than 30%, and the growth of S. aureus by almost 20% (Figure 7). The amount of TQ in the diluted formulation is as low as 0.00006% TQ, demonstrating a very powerful antimicrobial ability for TQ. It can be inferred that the qualities of the oil (or at least its antifungal properties) are enhanced by its unique composition and low amounts of FFAs, enabling significant TQ activity at very low concentrations in the essential oil.

Regulating the growth of S. aureus, C. albicans and M. furfur, all dominant commensals, may play an important role in maintaining homeostasis of the skin microbiome. C. albicans also plays an important role in the gut microbiome [31]. The three organisms are directly or indirectly linked with numerous pathological conditions. For example, S. aureus outgrowth is reverse-correlated with reduced diversity in the skin microbiome and the severity of relapses in atopic dermatitis patients [32,33]. S. aureus is the main organism to generate bacterial biofilm, together with Pseudomonas aeruginosa in chronic wounds. Slow healing or chronic wounds is a condition prone to occur with systemic risk factors, such as diabetes mellitus, cancer and malnutrition [34]. This is supported by the fact that the gut microbiome is an important regulator of health which can also affect skin integrity as well as the skin microbiome [35]. Finally, the correlation of M. furfur to seborrheic dermatitis is already known with its correlation with severity of psoriasis relapses being researched yet still inconclusive [36].

Efforts to find alternatives to antibiotic for the treatment of varied pathological conditions are constantly ongoing. The development of antibiotic resistant microbial strains makes the efforts even more challenging. The ability to strongly inhibit the growth of key microorganisms responsible for the most common skin infections suggests an important role for cold-pressed N. sativa oil as an alternative and safe antimicrobial agent that is also able to assist in balancing skin and gut microbiome for health and wellness support.

Material and Methods

Plant material origin and processing

*Nigella Sativa* seeds originate from crops that went through a hybridization and selection process aiming at increasing levels of TQ in the plant seeds. These proprietary plants were grown in an agricultural farm in the western Negev, Israel and the harvested seeds underwent cold press process yielding approximately 30% oil content (1000 kg seeds yield 300 kg oil)

**Determination of FFA levels**

FFA levels were determined using the AOAC Official Method 940.28 (1999) Fatty Acids (Free) in Crude and Refined Oils Titration Method [37]. The values are expressed as Oleic acid.

**Measurement of TQ and p-cymene levels by GC-FID**

The sample of oil was dissolved in acetone and injected into a Gas Chromatograph (GC) system equipped with FID detector, autosampler and an integrating program (Thermo Trace 1310). Column: GC Capillary Column Rxi 5ms: 30m*0.25 mm*.25 µm. Detector: FID, Temperature 2700C. Injector: SSL, 2500C. Hydrogen: 35 ml/min. Air: 350 ml/min. Carrier Gas: Helium. Flow rate: 2 ml/min. Split: 1:10. Oven: 1050C; Hold 5 min; 250C/ min to 2600C; Hold 3.8 min at 2600C. Time run: 15 minutes. Quantitation was calculated against the quantified reference standard materials: p-Cymene (Acros, Code 111765500) and TQ (Aldrich. Cat. No. 274666, Purity 99%). **Typical Retention times:** p-Cymene 2.8 min; TQ 6.6 min.

**Disc diffusion assay**

Starter culture of *C. albicans* or *M. Furfur* were grown at 32°C for 24 hr. in YM Medium or modified Dixon (mDixon) medium, respectively. Then, cultures were split and grown to mid log phase (0.35, 600nm). 150 µl of the culture was spread evenly throughout YM agar or mDixon agar plates. Starter culture of MRSA was grown at 37°C for 24 hr. in LB medium. Then, culture was split (OD=0.1, 600nm). 150 µl of the culture was spread evenly throughout LB agar plates. Paper disks saturate with oil or antibiotics were carefully placed on top of the agar. Plates were incubated for 48 hr until fungi have grown out completely. The area of inhibited fungal growth (zone of inhibition) was measured using the ImageJ software.

**MIC assay**

A starter culture of *S. aureus* was grown in LB broth at 37°C for 24 hours. Then, the culture was diluted to mid-log phase (0.05; 600 nm). The bacteria were incubated with black seed oil at a final volume of 200 µl in U-shape 96-well plates for 5 hr and absorbance of culture was documented (600nm). Ampicillin was used as a positive anti-bacterial control (Sigma aldrich, 10 µg/ml). A starter culture of *M. Furfur* was grown in mDixon broth at 32°C for 96 hr. Then, the culture was diluted and grown for 3 hr to mid-log phase (0.35; 600 nm). The fungus was incubated with black seed oil at a final volume of 200 µl in U-shape 96-well plates at 32°C under constant shaking (50 RPM) for 48 hr. Zinc pyrithione (Sigma aldrich, 0.01%) was used as a positive control. Following incubation, 40 µl of AlamarBlue (final concentration 0.004%) were added to all wells at the end of incubation. Following 20 min, fluorescence was read (Excitation 545; Emission 590).

**Statistical analyses**

Significance analyses were calculated by student's t-test in Microsoft Excel software.
Acknowledgments
We thank Patricia O’Connell for her valuable contribution reviewing the manuscript.
Navit Ogen-Shtern is partially funded by the Israeli Ministry of Science and Technology. This research was partially funded by TriNutra Ltd..

References


