Antifungal effects of *Nigella sativa L.* (Black cumin) seed extracts and seed oil on selected *Candida albicans* strains

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Abstract
The seed extracts of *Nigella sativa* (black cumin*)* have been used in different civilizations around the world for centuries to treat various animal and human ailments. *C. albicans* is a member of the normal human microbiome, but under certain circumstances it can cause infections that range from superficial infections of the skin to life-threatening systemic infections. Resistance to antifungal drugs in *C. albicans* has been on the increase in recent years. Several factors such as biofilm formation and overexpression of genes for efflux drug pump have been identified to contribute to the pathogenic potential of this fungus. This research examined antifungal effects of black cumin seed extracts and seed oil on *C albicans* wild-type (C1 and C2) and URA3 delete (CAF2 and CAI4) strains. Effects of the extracts on the key genes (MDS3 and MDR1) involved in drug resistance in *C. albicans* were also investigated. Aqueous and methanol extracts of the plant seeds were prepared using standard procedure. Aqueous and methanol extracts of *N. sativa* seeds, as well as the seed oil, were used for antimicrobial screening and for the analysis of effects on drug resistance genes using agar well diffusion technique, while fluconazole was used as a standard drug. The seed oil, as well as the aqueous and methanolic seed extracts of *N. sativa* greatly inhibited the wild-type clinical isolates C1 and C2. Interestingly, the URA3-delete strains were only inhibited by the oil. The amplification of the target genes in the various strains presented some intriguing results, with the gene(s) detected only prior to exposure or after exposure to the agent or at both instances. *N. sativa,* particularly the seed oil, has proven to have antifungal effects on fluconazole-resistant *C. albicans* with varying effects on the resistant genes.

Keywords: *Nigella sativa,* seed extract, seed oil, *Candida albicans*, antifungal, phytochemical

Introduction
Candida albicans is a normal commensal colonizing several body locations like the skin, genital tract, and gastro-intestinal tract in humans (Calderone, 2002). However when the immune system is altered, *C. albicans* can cause extensive mucosal colonization and systemic disease. In recent years, the prevalence of fungal infections has significantly increased, increasing the number of susceptible individuals, especially those with weakened immune system (Marquez and Quave, 2020).

Given the similarity of drug targets in fungi with that of human eukaryotic cells, the number of clinical antifungal agents available is limited, in contrast to antibacterial drugs. Coupled with the increased cases of antifungal resistance including resistance to azoles, which are common medications used to treat infections caused by *Candida* species. These drugs have a high likelihood of relapse and serious side effects. According to Pfaller *et al.* (2007), the upregulation of the expression of *EGR11* gene caused azole resistance in *C. albicans*. Similar research was conducted for *CaMDR1* and over-expression of this transporter led to an increase in resistance, more so to fluconazole than to other azoles (Pfaller *et al*., 2007; Khandelwal *et al*., 2019). Genome-wide profile study of match isolate pairs of fluconazole-susceptible and fluconazole-resistant counterparts that overexpressed multidrug efflux transporter (*MDR1)* was used to identify the mechanism of *MDR1* over-expression in azole resistance (Kalkandelen *et al*., 2015). These drug efflux pumps contribute to azole resistance in *C. albicans* by efflux of azole drugs from the cells. In fluconazole-susceptible isolates of *C. albicans*, efflux pumps are usually expressed in a low or non-detectable level but expression is stimulated in response to azoles and other chemicals (Khandelwal et al., 2019). Overexpression of *MDS3*, which is involved in *C albicans* hyphal growth under alkaline pH, has been discovered in pathogenicity and virulence (Adib-Hajbaghery and Rafiee, 2018).

Nevertheless, pursuit for new cell targets, has increased exponentially and natural products from plants hold great promise in the development of potent antifungal drugs. Medicinal plants have been noted as one of the best alternative sources for developing novel antibacterial medications (Adeosun *et al*., 2016). When used at the proper quantities, herbal remedies are generally thought to be safer and have fewer adverse effects than contemporary drugs (Vermani and Garg, 2002). Herbal medicines are more easily tolerated by patients, have less side effects, and there is less drug resistance.

Nigella sativa L. (Ranunculaceae), among other therapeutic plants, has long been revered as one of the world's most nutrient-rich herbs. Several scientific investigations have been conducted to support the alleged traditional medicinal applications of this species' seed (Assi *et al*., 2016; Adib-Hajbaghery and Rafiee, 2018). In Indonesia, *N. sativa* is often referred to as black cumin (Vermani and Garg, 2002).

Pharmacological studies have demonstrated that *N. sativa* seed extracts have antibacterial activity (Hosseinzadeh *et al*., 2007), anti-dermatophyte (Aljabre *et al*., 2005), antiviral activity against cytomegalovirus (Salem *et al*., 2000), effective against nasal abscesses, orchitis, eczema (Abd El-Hack *et al*., 2016; Nodoushan *et al*., 2009; Bukhari, 2018).

Phytochemical tests of *N. sativa* revealed the presence of numerous phytoconstituents, primarily alkaloids, saponins, sterols, and essential oils (0.4-0.45%), which includes trans-anethole, p-cymene limonene, carvone, -thujene, thymoquinone (TQ), thymohydroquinone (THQ) (Ainane *et al*., 2014). Therefore, the documented therapeutic potentials of seed extracts of *N. sativa* necessitated the investigation of their effects against *C. albicans* wild-type and URA3-delete (reduced for virulence) strains, as well as some genes implicated in drug resistance.

Materials and Methods

Collection of Seeds and Seed Oil of *N. sativa* (Black Cumin)

The seeds and seed oil of *N. sativa* (black cumin) was purchased from Islamic Herbal Medical Centre, Tudun-Wada, Kaduna. The

samples were wrapped in clean foil paper and transferred to the Department of Biological Sciences, Nigerian Defence Academy, Kaduna, for analysis.

Preparation of *N. sativa* (Black cumin) Seeds

The seeds of *N. sativa* were washed and sanitized in a 6% sodium hypochlorite solution (50 ppm, Reckitt Benckiser, Nig. Ltd). After washing, the materials were left in the biological hood (LABGARD Laminar Flow Biological Safety Cabinet) to get rid of excess water. Seeds were then crushed and grounded using 12-speed blender (Excella) for 5 min. One hundred grams (100 g) of dried ground seeds was weighed into 3000 mL conical flasks, and 1500 mL of each solvent (distilled water or methanol) was added to each flask in a ratio of 1 to 15 of the seeds and solvents as described by Parish and Davidson (1993). Each mixture was placed on an orbital shaker (Stuart Orbital incubator, S1500) and left to extract for 24 h at a speed of about 100 rpm at room temperature (25^{o}C). Extract was then filtered using a conical flask with side arm, a filter funnel (size 2), and a Whatman filter paper (No. 1) (Eleazu *et al*., 2013). Filtered extract was then poured into a weighed 500 mL round bottom flask. The solvent was evaporated with a rotary evaporator (SearchTech Instruments, RE52-1), with a water bath working at 40^{o}C, for 5-10 min. After evaporation of solvent, the weight of extract was obtained by subtracting the weight of flask from the weight of the flask and sample. Dimethyl sulfoxide (DMSO) was used to reconstitute the extracts in order to make a stock solution, after which the reconstituted extracts were sterilized by filtration using 0.45 µm aqua membrane nylon filter disk (Becton, Dickinson Company). Reconstituted and filtered extracts of the seeds, and the seed oil of *N. sativa* which served as stock solutions were stored in the laboratory Refrigerator (Haier Thermocool, Deluxe series HRF-350N) at -20^{o}C (Adeniran and Sonibare, 2013).

Isolation and Identification of *C. albicans*

C. albicans strains were isolated from high vaginal swab (HVS) samples by streaking the swabbed samples on Sabouraud Dextrose Agar (SDA) and incubation at ambient temperature for 48 h. Cultural features such as distinctive yeast smell and creamy pasty colony were used to presumptively identify isolates as *Candida* spp. (Raju and Rajappa, 2011). The presumptive isolates were subjected to germ tube test to distinguish *albicans* from non-*albicans Candida* following the method of Jasim *et al*. (2016) with slight modification. All isolates were inoculated into tubes containing 2 mL fresh plasma and incubated at 37^{o}C for 2 h. Loopful of the solution was aseptically placed on a clean grease-free glass slide and viewed under the microscope at x10 and X40 magnification for the presence of germ tubes.

Preparation of stock and working solutions of *N. sativa* extracts and fluconazole

Highest concentration of working solutions for each of the test antimicrobial agents; fluconazole (40 mg/mL), aqueous extract (400 mg/mL) and methanol extract (500 mg/mL) of seeds of black cumin was made from the stock. The undiluted seed oil of *N. sativa* served as the highest (100%) concentration. Each concentrate was further diluted in percentages (following the order of 100%, 75%, 50%, 25%, 12.5%, 6.25% and 3.125%) to make the working concentrations. The respective diluent (distilled water or methanol) was used as negative control.

Test Fungal Isolates

Two (2) wild-type clinical *C. albicans* strains (C1 and C2) and two mutant *C. albicans* strains [single URA3-delete (CAF2) and double URA3-delete (CAI4)] were subjected

to susceptibility test in the course of this research.

Antimicrobial Screening

The potency of *N. sativa*'s aqueous and methanolic seed extracts, and seed oil as inhibitory agent to *C. albicans* was determined using modified method of agar well diffusion described by James and Mary (2009).

Agar diffusion method of antifungal assay

Twenty milliliters (20 mL) of sterile Mueller Hinton agar (MHA) was poured into petri dishes and allowed to set before 100 µL of microbial suspension (at 0.5 McFarland turbidity standard) for each *C. albicans* strain was spread on the surface, using sterile spreader as described by Moghim *et al*. (2015). The plates were incubated at 37^{o}C for 30 min to allow excess fluid be absorbed and agar dried. With the aid of a 6 mm diameter cork-borer, five (5) cups were bored separately at equidistance on the agar. The borer was sterilized before and after every use. Each cup was separately filled with 0.2 mL of *N. sativa* oil, aqueous extract, methanolic extract and the conventional antifungal drug (fluconazole 200 mg), at corresponding concentrations (%). Plates were kept on the work bench at room temperature for 1 h (to allow extract diffusion) before incubation at 25^{o}C for 24 h (James and Mary, 2009). Inhibition zones were measured to determine the effectiveness of the extract against each organism and results were expressed in millimeters (Moses *et al*., 2013). Diameters of zones of inhibition (mm) were compared with control/standard for the activity of antifungal agent and data obtained was interpreted as susceptible ≥ 19, intermediate 13-18 and resistance ≤ 12 according to CSLI (2012).

Analysis of Effects of *N. sativa* Seed Oil, and Aqueous and Methanolic Seed Extracts on Fluconazole-Resistant Genes

Genomic DNA extraction

Genomic DNA of both fluconazole-resistant *C. albicans* strains, as well as strains susceptible to the effect of *N. sativa* were extracted using the phenol/chloroform method described by Sambrook *et al*. (1989). Extracted DNA was quantified by using spectrophotometer (DU-640, Beckman, Germany).

Amplification of *MDR1* and *MDS3* Genes in *C. albicans*

Using the extracted genomic DNA as template, *MDS3* and *MDR1*genes were amplified by Polymerase Chain Reactions (PCR) techniques using the primers described in Table 1. The PCR assay was done in 30 µL volumes containing 6 µL of RedLoad (Jena Bioscience Jena, Germany) PCR mix, 0.3 µL of each primer, 5 µL of DNA extract and 18.4 µL of PCR grade water. Thermal cycling was done using the GeneAmp 9700 (Applied Biosystems, USA) thermal cycler. Cycling conditions were 94°C for 3min; followed by 30 cycles of 94°C for 30 seconds, 54°C for 30 seconds and 60°C 90 seconds. After that, a final extension of 72°C for 7 min was done and the products were held at 4^{o}C till terminated.

Agarose gel electrophoresis

A volume of 5 µL of the PCR reaction was mixed with 3 µL loading dye (0.25% xylene cyalon, 0.25% bromophenol blue, 30% glycerol and 1 mM EDTA) and analyzed in 1% agarose gels containing 1 µg/mL of ethidium bromide. The electrophoresis was done at 100 V for 35 min, observed under UV light on a trans-illuminator and photographed with the gel electrophoresis image system (Gel-Doc). A 100bp ladder was used as molecular weight marker.

Statistical analysis

Data acquired from this research was subjected to one-way analysis of variance (ANOVA) with 95% confidence level and 5% significance level; and difference between means were determined by Duncan's new multiple range test.

Table 1: PCR primers used for amplification of *MDS3* and *MDR1* genes in *C. albicans*

Gene	Forward primer sequence	Tm (°C)	Reverse primer sequence	Tm (°C)	Amplicon Size (bp)
MDS3	5-CCAACTCCGAATCCAGCTCA-3	53	5-GCTGCAATCAGACCCTTCCT-3	50	~209
MDR1	5-CCATCACCGGTAACGACAGA-3	55	5-GCACCAAACAATGGACCCAC-3	50	~460

Results

Antifungal effect of fluconazole

The results obtained from the evaluation of antifungal activity of fluconazole (200 mg) against *C. albicans* strains used in this study are presented in Table 2. The zone of inhibitions obtained shows that the strains are resistant to fluconazole even at the highest concentration. The zone of inhibition recorded for fluconazole at highest concentration (100%) against *C. albicans* strains ranged between 8 mm and 12 mm; and 7 mm and 10 mm at the least concentration (25%).

Table 2: Antifungal sensitivity of fluconazole against *C. albicans*

Test *Candida* strains	Zone of Inhibition (mm) by Concentrations				
	0% (SDW)	**25%**	**50%**	**75%**	**100%**
C. albicans C1	0	9	11	11	12
C. albicans C2	0	7	8	10	12
C. albicans CAI4	0	7	8	8	8
C. albicans CAF2	0	10	12	11	10

Key: Sterile distilled water (SDW)

Antifungal activity of seed extracts and seed oil of *N. sativa* against strains of *C. albicans*

The results obtained from the evaluation of antibacterial activities of aqueous and methanolic seed extracts, and seed oil of *N. sativa* against the fluconazole-resistant strains of *C. albicans* show that different concentrations of the components of *N. sativa* exhibited varying antifungal

actions in a dose-dependent manner as shown in Table 3. From the result, the seed oil of *N. sativa* exhibited better inhibitory effect against most of the test pathogens with least inhibition zone (13.33±2.08 mm) above the stipulated standard (≤ 12 mm for resistance) for the conventional drug (fluconazole). At 25% concentration, there were no significant differences (at p-0.05) between the inhibitory effect of the seed oil of *N. sativa* against *C1* and *CAI4* strains, and also between *C2* and *CAF2* strains of *C. albicans*. Highest values ranging from 20.33±7.51 mm to 32.67±7.09 mm of inhibition zones for the seed oil were recorded against the four test strains of *C. albicans* at 100% concentration in Table 3.

The antifungal activity of the methanolic and aqueous extracts of the of *N. sativa* seeds was not profound compared to that of the seed oil. The aqueous and methanolic extracts of the herb exhibited no inhibitory activity against *CAF2* and *CAI4* strains of *C. albicans* at every concentration as shown in Table 3. Inhibition zones recorded against *C1* and *C2* strains at different concentrations of the aqueous extract of the seed were however above the standard for the conventional drug (fluconazole). At 25% concentration, inhibition zones of 12.00 mm and 14.00 mm were recorded for the methanolic extract of *N. sativa* seeds against *C2* and *C1* strains respectively.

Table 3: Antifungal activity of seed oil and seed extracts of *N. sativa* against fluconazole-resistant *C. albicans*

***C albicans* strain**	**Seed Oil**			**Methanol Extract**			**Aqueous Extract**		
	Zone of Inhibition (mm) ±SD by Concentration (%)								
	25	**50**	**100**	**25**	**50**	**100**	**25**	**50**	**100**
C1	13.33±2.08^{a}	17.33±1.53^{a}	20.33±7.51^{a}	12.00±2.00^{b}	14.33±1.15^{b}	19.33±1.53^{b}	20.00±1.00^{b}	17.00±2.65^{b}	13.67±1.53^{b}
C2	20.67±1.53^{b}	25.00±3.61^{b}	28.33±3.79^{ab}	14.33±0.58^{c}	15.67±1.53^{b}	24.67±1.15^{c}	23.00±2.65^{c}	16.00±4.58^{b}	17.00±1.00^{c}
CAF2	21.67±2.08^{b}	18.67±3.06^{a}	32.67±1.15^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
CAI4	15.00±1.00^{a}	17.33±2.52^{a}	23.67±7.09^{ab}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}

Key: Millimeter (mm); Standard Deviation (SD)
Note: Values with the same superscript in the same column are not significantly different from each other (p>0.05)

Effect of *N. sativa* seed oil and methanol and aqueous seed extracts on fluconazole-resistant *C. albicans* strains

The results of the amplification of *MDS3* and *MDR1* genes from the fluconazole-resistant *C. albicans* strains before and after exposure to seed extracts and seed oil of the plant are presented

in Figures 1 and 2. Interestingly, *MDS3* was detected in strains *CAF2* (209 bp) before the treatment but was not detected after exposure to the seed oil of the plant. Also, the gene was absent in strains C1, C2 and *CAI4* before the treatment but were detected after the treatment with the extracts for strains C1 and C2 and seed oil for strain *CAI4*. Furthermore, *MDR1* gene of about 460 bp amplicon size was also detected in strains *C2*, *CAF2* and *CAI4* before exposure to the seed extracts and seed oil of *N. sativa*, however, strains *CAI4* and *C2* retained the gene even after exposure to the seed oil and methanolic extract of *N. sativa* seeds respectively, while the gene was not detected in strain C2 after treatment with the seed oil only as shown in Figure 2.

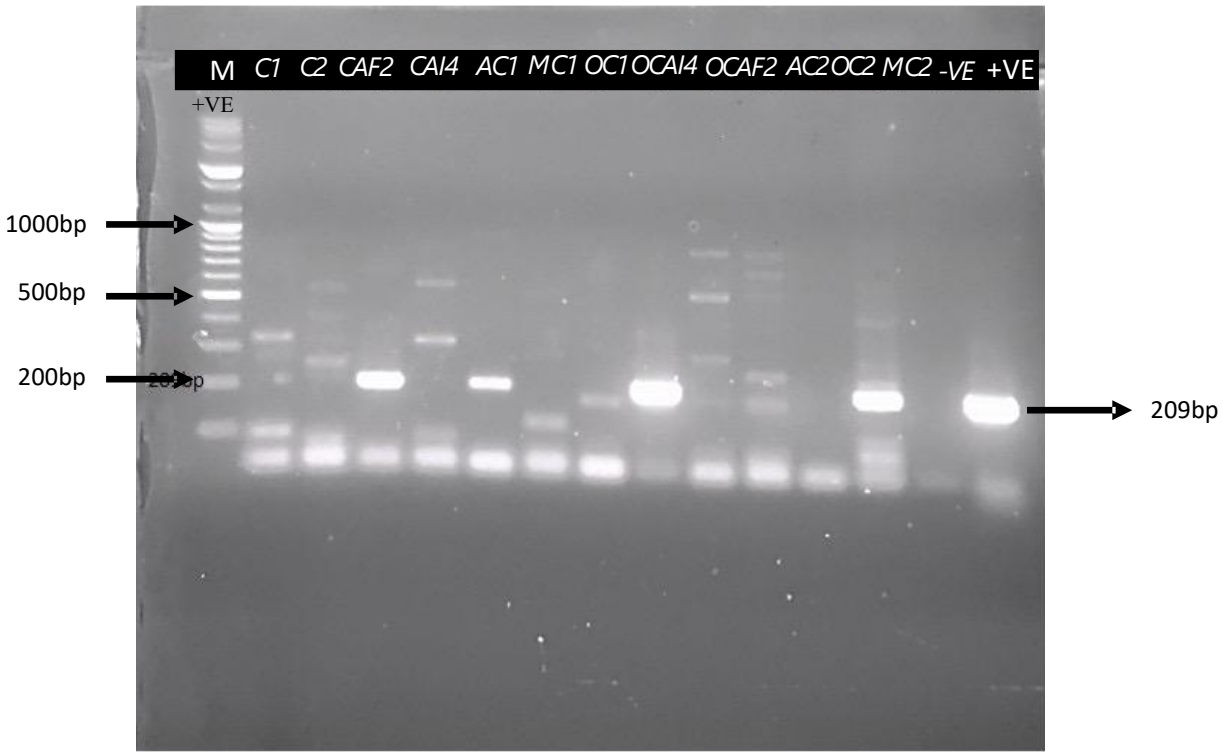


Figure 1: Gel electrophoresis result showing the ~209bp amplicon encompassing the complete *MDS3* gene in fluconazole-resistant strains of *C. albicans* before and after subjected to antifungal effect of *N. sativa*

Keys: Positive control (+ve): Wild-type strain treated with 200 mg fluconazole; Negative control (-ve): Sample without DNA*; AC1-C1:* after treatment with aqueous extract of *N. sativa; MC1-C1:* after treatment with methanolic extract of *N. sativa; OC1-C1:* after treatment with *N. sativa* Oil*; OCAI4-CAI4:* after treatment with *N. sativa* Oil*; OCAF2-CAF2:* after treatment with *N. sativa* Oil*; AC2-C2:* after treatment with aqueous extract of *N. sativa; OC2-C2:* after treatment with *N. sativa* Oil*; MC2-C:2* after treatment with methanolic extract of *N. sativa*

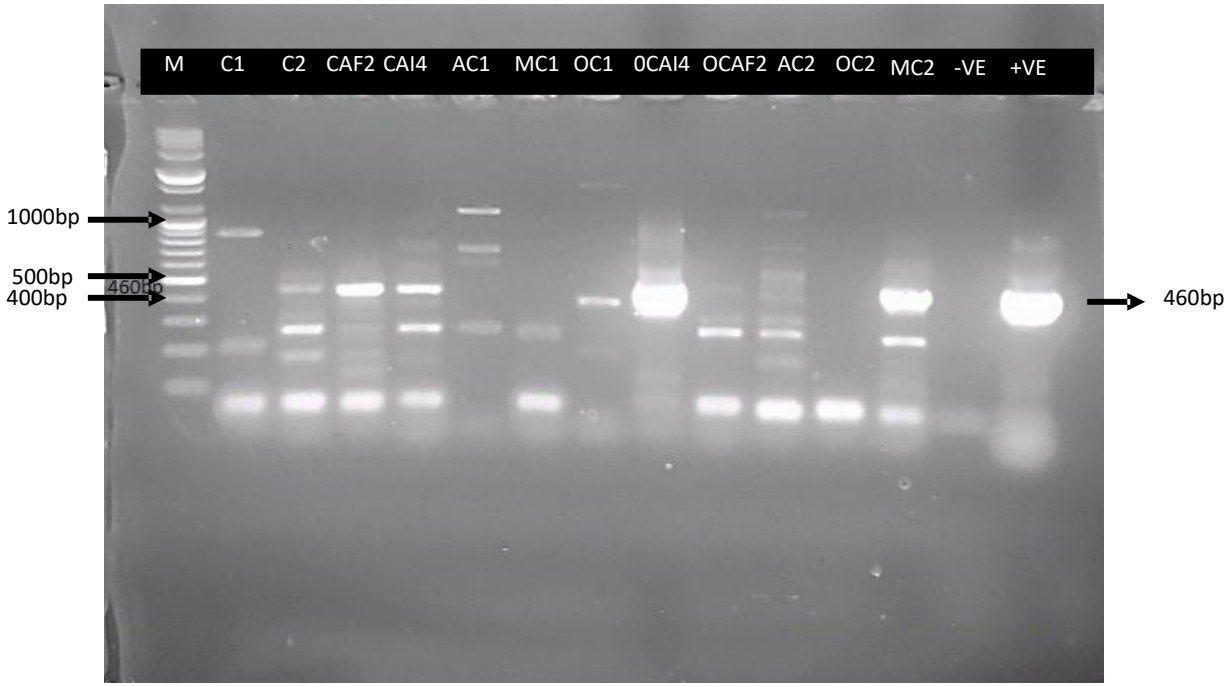


Figure 2: Gel electrophoresis result showing the ~460bp amplicon encompassing the complete *MDR1* gene in fluconazole-resistant strains of *C. albicans* before and after subjected to antifungal effect of *N. sativa*

Keys: Positive control (+ve): Wild-type strain treated with 200 mg fluconazole; Negative control (-ve): Sample without DNA*; AC1-C1*: after treatment with aqueous extract of *N. sativa; MC1-C1* after treatment with methanolic extract of *N. sativa; OC1-C1* after treatment with *N. sativa* Oil*; OCAI4-CAI4* after treatment with *N. sativa* Oil*; OCAF2-CAF2* after treatment with *N. sativa* Oil*; AC2-C2* after treatment with aqueous extract of *N. sativa; OC2-C2* after treatment with *N. sativa* Oil*; MC2-C2* after treatment with methanolic extract of *N. sativa*

Discussion

C. albicans is a highly adaptable microorganism, being able to develop resistance following prolonged exposure to antifungals. Several studies have documented the resistance of *C. albicans* to fluconazole and other antifungal drugs. This could be due to various mechanisms including but not limited to biofilm formation that decreases antifungal drug accessibility, increased expression of multidrug efflux pumps, selection of spontaneous target mutations, target expression deregulation, and alteration in ergosterol biosynthesis pathway related to important genes such as, *MDS3, MDR1* and *EGR11* (Pfaller *et al*., 2012; Whaley *et al*., 2017; Benedetti *et al*., 2019).

The results of this study revealed that the *C. albicans* strains *C1*, *CAF2* and *CAI4* were tolerant to the highest concentration of fluconazole used in this study (40 mg/mL). This observation supports the findings from previous published work (Pfaller *et al*., 2010; Pfaller *et al*., 2012; Fothergill *et al*., 2014;

Whaley *et al*., 2017;) which reported that infections caused by *C. albicans* and other non albicans Candida species are associated with varying levels of fluconazole resistance. While all the strains showed sensitivity to the oil extract of *N. sativa*, the CAF2 and CAI4 strains of *C. albicans* exhibited total resistance to the effect of the methanolic and aqueous extracts of the plant.

CAF2 and CAI4 are *URA3* single and double delete mutants of the hospital strain of *C. albicans*. *URA3* encodes orotidine 5-phosphate decarboxylase, an enzyme involved in de novo synthesis of pyrimidine ribonucleotides. It has been shown that strains lacking *URA3* are reduced for virulence (Kirsch and Whitney 1991), but intriguingly these URA3-delete strains are resistant to the *N. sativa* methanol and aqueous extracts which were found effective against the wild-type clinical isolates, C1 and C2, meaning that *URA3* plays a role in the antifungal response of *C. albicans* to *N. sativa* extracts.

The study on the effect of the oil and seed extracts of *N. sativa* on the antifungal resistant genes of *C. albicans* strains holds promise in the elucidation of mechanism of action of *N. sativa* against *C. albicans.* The knowledge of the antifungal resistance mechanisms can allow the design of alternative therapeutical options in order to modulate or revert the resistance. (Costa-de-Oliveira and Acácio, 2020).

The results of this study show that the *MDR1* gene was amplified in *C2, CAF2* and *CAI4* prior to treatment with *N. sativa* oil and extracts. However, the intensity of the band was more pronounced in *CAI4* and C2 after exposure to the plant extract and also detected post-exposure of C1 strain. This is consistent with the findings of Morschhauser (2002) who found that in many fluconazole-resistant *C. albicans*, the reduced drug accumulation was due to high expression of the *MDR1* gene. The amplifications of *MDR1* in these three strains are also consistent with the findings of Morschhauser (2002) who observed that *MDR1* is over-expressed in fluconazole-resistant *C. albican*s.

The *MDS3* gene, known for its role in pH-regulated morphogenesis and biofilm formation, was amplified in *CAF2* before treatment with the plant extract and in C1, CAI4 and C2 after treatment with the plant extract. The formation of filaments and biofilms has been reported to confer resistance to antifungal drugs in *C. albicans*. Biofilms are much more resistant to antimicrobial agents and host immune factors compared to dispersed yeast cells (Fanning and Mitchel, 2012). The factors responsible for increased resistance include the complex architecture of biofilms which serves as a shield to the cells in a biofilm, and metabolic plasticity (Fanning and Mitchel, 2012).

The likely explanation for the lack of detection of *MDS3* gene in the other strains before exposure to the oil and extracts of the plant, but detection after treatment, could be that the extract induced transient duplication, resulting in increased copy numbers of the genes. This observation is supported by the work of Bhattacharya *et al*. (2019) in which the qPCR analysis of genomic DNA from cells of *C. auris* showing resistance to fluconazole indicated that transient gene duplication of *CDR1* and *ERG11* caused the observed age-dependent enhanced fluconazole tolerance in *C. auris* strains. Differences observed in the genetic composition of strains of *C. albicans* exposed to oil and seed extracts of *N. sativa* compared with the same strains prior to exposure could be the case of gene duplication or mutation

reversal in response to the effect of the plant extracts.

Conclusion

The *C. albicans* strains used in this study exhibited varying degrees of resistance to fluconazole but showed good susceptibility to the oil of *N. sativa* seeds. While the two wild-type clinical strains were sensitive to the oil, methanolic and aqueous extracts of *N. sativa*, the URA3-delete strains showed sensitivity only to the oil, suggesting that *URA3* may play a key role in the sensitivity of *C. albicans* to *N. sativa* extracts. It will be interesting to elucidate the role of *URA3* in the response of *C. albicans* to *N. sativa* extracts and perhaps other antifungal preparations.

The *MDR1* and *MDS3* genes were amplified in all the isolates before or after exposure to the plant extracts or both. Overall, the study showed that *N. sativa* extracts have inhibitory effects on the wild-type clinical isolates of fluconazole-resistance *C. albicans.*

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Conflict of Interest: The authors declare no conflict of interest.

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