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Effect of Black Cumin (*Nigella sativa L.L.*L.) Seeds on Some Fluconazole-Resistant and Biofilm-Forming Genes on Strains of *Candida albicans strains (C1, C2, CAF2 and CAI4)*

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Abstract: The rise in drug resistance has become a serious health issue globally; as such the world health organization has since 1981 encouraged nations on the search for phytomedicines. *Nigella sativa* L. extracts have been widely studied and proved effective in the treatment of both metabolic and infectious diseases. This study was carried out to determine the effect of black cumin seeds (*Nigella sativa* L.) at molecular level (fluconazole-resistant genes in *Candida albicans*' strains). Aqueous and metabolic extracts of the *N sativa* were prepared, while the oil extract was obtained from herbal shop and their phytochemical contents were determined. *Candida albicans* strains *C1* (wild type), *C2* a randomly selected clinical isolate and *CAI4* and *CAF2* (homogenous and heterogenous *URA* gene deficient respectively) strains were used for this study. 28 out of 50 (56%) *Candida albicans* isolated from HVS of suspected PID patients and 3 laboratory strains were resistant to fluconazole. Fluconazole resistance pattern and mode of action of each extract on the different strains were studied. Genes implicated in antifungal resistance and biofilm formation in *C. albicans* (*EGR11, MDS3* and *MDR1)* were amplified (PCR), and the effects of the three extracts of *N. sativa* on the genes were studied. The aqueous extract had the highest concentrations of the phytochemicals followed by the methanolic extract and then the oil. The aqueous and methanolic extracts were found to have fungicidal effect at 100mg/ml and 250mg/ml respectively and oil at 100% only. While the oil and some concentration of the methanolic extracts have fungistatic effects. *CAI4* and *CAF2*strains showed resistance to all the concentrations of the aqueous and methanolic extracts. The PCR result showed variation genes implicated in stress and pathogenicity, they were modulated on exposure to the plant extracts. It is recommended that the aqueous and oil of *N. sativa* be used in the treatment of multidrug resistant *Candida* infections alongside conventional antimicrobials. More extensive study should be carried out on the effect of the seed extracts of *N. sativa* on *CAF2* strains *CAI4* strains to study then role of *URA3* gene on antifungal resistanceand mutagenesis in *C. albicans*.

Keywords: Drug, Resistant, Fluconazone, Treatment, Infectious, Disease, Black Cumin, Candida Albicans

1. Introduction

There has been a high increase in the prevalence of fungal infections in the past few years, which has increased the number of vulnerable patients, especially those with impaired immunity [8]. More so, lots of antifungal drugs used for the prevention and treatment of these diseases cause an increase in drug-resistant species, hence encouraging researchers to search for botanical drugs [8]. Candidiasis, a popular fungal infection in humans, is mostly caused by *C. albicans*, *C. tropicalis*, *C. parapsilosis* and *C. glabrata* with *C. albicans* being responsible for 40% of its mortality rate [1, 3]. Although the global prevalence of candidiasis is not clear, it has been reported that this infection becomes about twice more prevalent due to changes in human demography and behaviour coupled with drug resistance and causing over 10

million people to visit physicians annually [3].

There are a number of classes of compounds that comprise the arsenal used to treat *Candida* infections including; polyenes, azoles, echinocandins, nucleoside analogues, and allylamines, with varying degrees of effectiveness depending on the type and location of infection and how sensitive the *Candida spp* happens to be [17, 18]. The most often administered antifungal drug used for most *C. albicans* infections is fluconazole, which is a member of the azole antifungal class [17]. Azoles inhibit 14-α-sterol demethylase, encoded by the *EGR11* gene, which is an enzyme involved in the biosynthesis of the fungal-specific membrane, sterol ergosterol. As some Non-*Albicans Candida* (NAC) species exhibit intrinsic resistance to azoles, the use of the drug is likely a contributing factor to the more frequent incidence of infections caused by these NAC species [1, 8]. However, there are many studies that have documented the ability of *Candida* to develop high-level resistance to azole antifungals [16, 19] Perlin, 2015; [13, 1] Apparently, apart from *C. albicans* infections, other species that do not respond to treatment with common drugs such as clotrimazole and fluconazole are on the increase [16, 13]. However, the use of antifungal agents are raising concerns about their potential in choosing and spreading resistant fungal strains or species. Studies have documented an increased incidence of infections caused by yeasts that either have acquired resistance or are intrinsically resistant to the drug in use [7, 1, 11].

It has so far turned out that the effort to understand the resistant mechanisms and develop different antifungal drugs, which are expensive and time-consuming, has apparently become needless, as these pathogens quickly develop resistance to the drugs in recent times. This has led to an increase in searching for more efficient substitutes with a different mode of action on the pathogens. Hence, medicinal plants happened to be the best optional source for new antimicrobial drugs [22].

*Nigella sativa L.L.*Linn (NS) (shown in Figure 1) is a herbal plant belonging to the family Ranunculaceae, commonly known and found in different parts of the world and its seeds are usually used as condiments [27]. In South Asia, it is known as '*kalonji*', "Black Cumin" in the west while its Arabic name is '*Habatul Sauda*'. There is a popular saying by Arabian herbal medical practitioners that "blac*k seed is a treatment of every disease but death"*. With reference to this saying, Asian herbalists largely use black seeds for different varieties of ailments either synergistically or singly with other herbs or antibiotics [23]. This herb possesses several active compounds that might contribute to their promising effects on various kinds of ailments. Among the important active compounds are thymoquinone (30-48%), thymohydroquinone, dithymoquinone, p-cymene (7-15%), carvacrol (6-12%), 4-terpineol (2-7%) and 1-4% t-anethol [27]. The exhibiting potentials of this plant are what drive the present study with the aim of evaluating its antifungal properties against clinical strains of *C. albicans* and its mutants (*CF1*, *CF2*, *CAF2* and *CAI4*) with inference to their genetic makeup [9].

2. Materials and Methods

2.1. Study Area

The study area was Kaduna State which is located in the North-west geopolitical zone of Nigeria which is between longitude 6^{o} and 9^{o}E and latitude 9^{o} and $11\text{-}30^{o}$ N. It has distinctive wet and dry seasons which start and ends in April to October and November to March in that order. The State shares boundaries with Zamfara and Katsina States to the north; Kano State, to the North West; Niger State, to the West; Federal Capital Territory, to the south; Plateau and Bauchi States to the East. Kaduna State has a land area of about 48473.25sq Km with a human population of over 6.06 million according to the census figure of 2006 (NPC, 2006). It comprises of 23 Local Government Areas of which Kaduna North Local Government Area where the Nigerian Defence Academy is situated, is selected as the focal point of this research.

The study was conducted in the Department of Biological Sciences, Nigerian Defence Academy, Kaduna, while the molecular analysis was carried out in DNA Laboratories in Kaduna.

2.2. Isolation and Identification of C. albicans

Selective isolation technique described by Jasim *et al*. (2016) which was adopted to isolate *C. albicans* from the HVS samples of some women. The swab sample was streaked on Sabouraud Dextrose Agar and incubated at 24°C for 48hrs. Cultural feature such as distinctive yeast smell and creamy pasty colony was used to presumptively identify isolates as *Candida spp*. [20].

2.3. Germ Tube Test

Candida spp were subjected to germ tube test to distinguish *albicans* from non-*albicans Candida* following the method of 19 Jasim *et al*. (2016) with slight modification. All isolates were inoculated with 2ml fresh plasma and incubated at 37°C for 2 hours. Loop full of the solution was aseptically placed on a clean grease-free glass slide and viewed microscopically using oil immersion for wet mount and the presence of germ tubes.

2.4. Herbal Material (Black Cumin)

2.4.1. Source of Herb

The seeds and oil of Black Cumin (*Nigella sativa*) Linn was purchased from Islamic Herbal Medical Center at Tudun-Wada, in Kaduna State, Nigeria.

2.4.2. Preparation of Seeds of Black Cumin

The seeds of Black cumin (*Nigella sativa*) were sanitized and washed in a 6% sodium hypochlorite solution (50 ppm), (Reckitt Benckiser, Nig. Ltd) as described by Ritenour *et al*., (2011). 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 5minutes.

2.4.3. Preparation of Seed Extract of Black Cumin

One hundred grammes (100g) of dried ground seeds of *Nigella sativa* (weighed by Fisher Scientific scale) was weighed in 3000ml conical flasks, and 1500ml of each solvent (distilled water or methanol) was added to each flask making a ratio of 1 to 15 of the seeds and in procedure solvents; described by Parish and Davidson [14]. Each mixture was placed on an orbital shaker (Stuart Orbital incubator, S1500) and left to extract for 2nmhours at a speed of about 100rpm at room temperature (25°C). Extract was then filtered using a conical flask with side arm, a filter funnel (size 2), and a 90mm diameter filter paper (Whatman No. 1) as described [4]. Filtered extract was then poured into a weighed 500mL round bottom flask.

The solvent was evaporated with a rotary evaporator (SearchTech Instruments, RE52-1), in a water bath at 40°C, for 5-10minutes. 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 using volumetric flasks, after which they were sterilized by filtration using 0.45µm aqua membrane nylon filter disk (Becton, Dickinson Company).

Reconstituted and filtered extracts of the seeds, and the oil of *Nigella sativa* which served as stock solutions were stored in the freezer of a regular refrigerator (Haier Thermocool, Deluxe series HRF-350N) set at 4^{o}C as described by Adeniran and Sonibare (2013) in the Laboratory Refrigerator.

2.5. Concentration of Antimicrobial Agent

Highest concentration of working solutions for each of the test antimicrobial agents; fluconazole (40mg/ml), aqueous extract (400mg/ml) and methanolic extract (500mg/ml) of seeds of Black cumin was made from the stock. Undiluted oil of Black cumin served as the highest (100%) concentration. Each concentrate was further diluted to 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.

2.5.1. Antimicrobial Screening

The potency of the seeds and oil of *Nigella sativa* as inhibitory agent to microbial growth was determined using modified method of agar well diffusion described by James and Mary (2009). Tube dilution method used by De-Paiva *et al*. (2003) was adopted to determine the minimum inhibitory concentration and minimum bactericidal concentration of the extracts against test organisms.

2.5.2. Test Fungal Isolates

A total of twelve (12) clinically significant strains of *C. albicans* were subjected to susceptibility test in the course of this research. Three (*CAF2, C1* and *CAI4*) of the isolates were type strains gotten from the Department of Biological Sciences at Nigerian Defence Academy, Kaduna and the other 9 strains were isolated in Laboratory of the Microbiology, Barau Dikko Teaching Hospital, Kaduna, from high vaginal swab (HVS) sample that was collected from females that have attained reproductive age.

2.5.3. Agar Diffusion Method of Antifungal Assay

Twenty milliliters (20ml) of sterile Mueller H Agar (MHA) were poured and let to set before 100 µl of microbial suspension (at 0.5 McFarland turbidity standards) for each *C. albicans* strain was spread on to the surface, using sterile spreader as described [10]. The plates were incubated at 37°C for 30 minutes 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 filled with 0.2ml including conventional antifungal drugs (fluconazole 200mg), at the same concentrations (%). Plates were kept on working bench at room temperature for 1 hour (to allow extract diffusion) before incubation at 25°C for 24 hours [28]. Inhibition zones were measured to determine the effectiveness of the extract against each organism and results were expressed in millimeters [12]. Diameters of zones of inhibition (mm) were compared with Interpretation standard for the activity of antifungal agent (Table 1).

2.6. Minimum Inhibitory Concentration (MIC) Determination

The minimum inhibitory concentration (MIC) of the seed and oil of *Nigella sativa L.L.* was determined against *Candida* strains that were susceptible to antifungal effect of the extracts and oil. The MIC was determined by tube dilution method described by Moghim *et al*. (2015), with slight modification. To each of the ten concentrations (%) of the seed extract and oil of the herb, 0.1ml each of test strains was respectively introduced. Solution in each tube was thoroughly mixed and incubated for 18-24 hours at 24°C. All tubes were examined macroscopically for turbidity changes indicating growth of the strains. Concentration of extract/oil of *Nigella sativa* in the tube that precedes the one without sign of growth was taken as the MI*C.* Solutions (in tubes) with no sign of growth were selected for the determination of minimum fungicidal concentration (MFC).

2.7. Minimum Fungicidal Concentration (MBC) Determination

The minimum concentration of the seed extracts and oil of *Nigella sativa L.L.* needed to kill 99.9% of the initial Inocula of *C. albicans* was determined by selecting the concentrations with inhibitory activity MIC test (i.e., those that did not have any turbidity on the MIC determination) [14]. A loopfull from mixture of selected tubes was inoculated onto molten sabouraud dextrose agar (SDA) plates divided into quadrants. Plates were incubated at 25°C for 24 hours, and then *Candida* growth was evaluated. Last quadrant (with the lowest concentration of plant extract) that

showed no growth was taken as the MBC.

Table 1. *Interpretation standard for activity of antifungal agent.*

Susceptible	Intermediate	Resistance
≥ 19	13-18	≤ 12

Source: CLSI (2012)

2.7.1. Phytochemical Screening

The presence and/or absence of alkaloids, saponins, tannins, flavonoids and other phytochemicals in the seed extract and oil of *Nigella sativa* were evaluated using standard techniques as described by Patil and Paikrao (2012) [15].

2.7.2. Test for Alkaloids

Extract (0.5g) was diluted to 10 ml with acid alcohol, boiled and filtered. 2mL of diluted ammonia was added to 5 ml of the filtrate, followed by the addition of 5 ml of chloroform. The mixture was shaken in a gentle manner to extract the alkaloidal base, and the chloroform layer was extracted with 10 ml of acetic acid. The chloroform layer was divided into two portions. Mayer's reagent was added to one portion and Draggendorff's reagent to the other. The formation of a cream (with Mayer's reagent) or reddish-brown precipitate (with Draggendorff's reagent) was regarded as positive for the presence of alkaloids.

2.7.3. Test for Saponins

The ability of Saponin to produce frothing in aqueous solution was used as screening test for Saponin. 5 ml of distilled water was added to 0.5 g of extract in a test tube. The solution was shaken vigorously and observed for a stable persistent froth. The froth was mixed with three drops of olive oil and shaken vigorously, after which it was observed for the formation of an emulsion indicating the presence of saponins.

2.7.4. Quantitative Determination of Saponin

The spectrophotometric method of Brunner (1984) was used for Saponin determination. A total of2g of the finely grinded sample was weighed into a 250 ml beaker and 100 ml of Isobutyl alcohol or (But-2-ol) was added. Shaker was used to shake the mixture for 5 hours to ensure uniform mixing. The mixture was then filtered with No 1 Whatman filter paper into 100 ml beaker that contains 20 ml of 40% saturated solution of magnesium carbonate ($MgCO_3$). The mixture obtain again was filtered though No 1 Whatman filter paper to obtain a clean colourless solution. 1ml of the colourless solution was taken into 50 ml volumetric flask using pipette, 2 ml of 5% iron (iii) chloride ($FeCl_3$) solution was also added and made up so as to the mark with distilled water. It was then be allowed standing for 30 min for the colour to develop. The absorbance was read against the blank at 380 nm.

2.7.5. Test for Flavonoids

Three methods were used to test for flavonoids.

Exactly 5 ml of diluted ammonia was added to a portion of an aqueous filtrate of the extract and 1mL of concentrated sulphuric acid was then added. A yellow colouration that disappeared on standing indicated the presence of flavonoids. Some few drops of 1% alluminium solution was put into a portion of the filtrate. A yellow colouration indicated the presence of flavonoids. A portion of the extract was heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered, and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. A yellow colouration indicated the presence of flavonoids [25].

2.7.6. Quantitative Determination of Flavonoid

The total flavonoid content of the extract was determined using a colorimeters assay developed by Bao (2005). 0.2 ml of the extract was added to 0.3 ml of 5% $NaNO_3$ at zero time. After 5min, 0.6 ml of 10% $AlCl_3$ was added and after 6 min, 2 ml of 1M NaOH was added to the mixture followed by the addition of 2.1ml of distilled water. Absorbance was read at 510 nm against the reagent blank and flavonoid content was expressed as mg rutin equivalent.

2.7.7. Test for Terpenoids

Crude extract was dissolved in 2ml of chloroform and evaporated to dryness. 2mL of concentrated H_2SO_4 was added to the suspension and heated for about 2 minutes. A grayish colour indicated the presence of terpenoids.

2.7.8. Quantitative Determination of Terpenoids

The procedure described by Sofowora (1993) was used. A portion of 0.5 g of finely grounded sample was weighed into a 50 ml conical flask 20 ml of chloroform: methanol 2:1 was added and the mixture was shaken thoroughly and allowed to stand for 15min at room temperature. The suspension was centrifuged at 3000 rpm the supernatant was discarded and the precipitate was re-washed with 20 ml chloroform: methanol 2:1 and then re-centrifuged again. The precipitate was dissolved in 40 ml of 10 % SDS solution and 1ml of 0.01M ferric chloride was added and allowed to stand for 30min before taking the absorbance at 510nm.

2.7.9. Test for Tannin

Exactly 0.5 g of the extract was stirred with 100ml of distilled water, filtered and ferric chloride reagent was added to the filtrate a blue-black green or blue green precipitate was taken as evidence for presence of tannin.

2.7.10. Quantitative Determination of Tannin

A total About 0.2g of finely ground sample was weighed into a 50 ml sample bottle. 10 ml of 70 % aqueous acetone was added and properly covered. The bottles were put inside an ice bath shaker and shaken for 2 hours at 30°C. Each solution was then centrifuged and the supernatant stored in ice. 0.2 ml of each of the solutions was pipetted into the test tube and 0.8 ml of distilled water was then added. Standard tannin acid solutions were prepared from a 0.5 mg/ml of the stock and the solution made up to 1ml with distilled water. 0.5 ml of Folin ciocateau reagent was added to both sample and standard and followed by 2.5 ml of 20 % Na_2CO_3. The solutions were then vortexed and allowed to incubate for

40minutes at room temperature; its absorbance was read at 725 nm against a reagent blank concentration of the same solution from a standard tannic acid curve prepared (Makkar and Goodchild, 1996).

2.7.11. Test for Cardiac Glycosides

The followings were carried out to test for cardiac glycosides:

Legal's test- Exactly …. Of the extracts were dissolved in ….. pyridine and ……. drops of 2% sodium nitroprusside with ……. drops of 20% NaOH were added. A deep red colouration which faded to a brownish yellow indicates the presence of cardenolides.

2.7.12. Keller- killiani's Test

A total of 0.5g of the extract was dissolved in 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was then under layered with 1ml of conc. H_2SO_4, a brown colour obtained at the Interface indicate the presence of a deoxy sugar, characteristic of cardenolides.

Salkowski's test- A total of 0.5g of the extract was mixed with 20 ml of chloroform and filtered, 3 ml of conc. H_2SO_4 were added to the filtrate to form a layer. Reddish brown colour at the Interface was observed which indicates the presence of steroidal ring.

2.7.13. Quantitative Determination of Cardiac Glycosides

The procedure described by sofowora (1993) A total of was used 10 ml of the extract was pipetted into a 250 ml conical flask. 50 ml chloroform was added and shaken on a vortex mixer for 1hour. The mixture was filtered into 100 ml conical flask. 10 ml of pyridine and 2 ml of 29% of sodium nitroprusside were added and shaken thoroughly for 10 min. Then 3 ml of 20 % NaOH was added to develop a brownish yellow colour, Glycoside's standard (Digitoxin) was prepared using concentrations which ranged from 0 – 50 mg/ml from stock solution, the abs was read at 510 nm.

2.8. Molecular Analysis of Fluconazole Resistant Forming Genes

2.8.1. Genomic DNA Extraction

Genomic DNA of both fluconazole resistant *C. albicans* strains and strains susceptible to the effect of *Nigella sativa* were extracted using the phenol/chloroform method described by Sambrook [21]. Extracted DNA was quantified by using spectrophotometer (DU-640, Beckman, Germany) on the basis of optical density 260:280 ratio.

2.8.2. Amplification of EGR11, MDR1 and Mds3 Genes in C. albicans

Using the extracted genomic DNA as template, *Mds3, EGR11*, and *MDR1*genes were amplified by Polymerase Chain Reactions (PCR) techniques using the primer described in Table 2. The PCR assay was done in 30 µl volumes containing 6µL 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°C till terminated. The amplicons were shipped to Macrogen InC., South-Korea, for purification and sequencing.

2.8.3. Agarose Gel Electrophoresis

After the PCR amplification, 5 µl of the PCR reaction was analyzed in 1% agarose gels containing 1µg/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). 100 bp ladder was used as molecular weight marker and loading dye (0.25% xylene cyalon, 0.25% bromophenol blue, 30% glycerol and 1mM EDTA) were employed.

3. Statistical Analysis

All data acquired from this research were 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 2. *PCR primers used for amplification of MDS3, MDR1 and EGR11 genes in C. albicans.*

Gene	Forward primer and sequence	Tm (°C)	Reverse primer and sequencesszx	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
EGR11	5-CACGGGGTTGCCAATGTTATG-3	51	5-GAATTGGCTTTAGCAGCAGCA-3	55	~761

4. Results

4.1. Phytochemical Composition of Seed Extract and Oil of Nigella Sativa

The phytochemical screening result for the presence of bioactive components in the oil and seed extract of *Nigella sativa* are presented in Table 3. The result indicated the presence of cardiac glycoside, glycoside, steroids, tannins, alkaloids and flavonoids. Saponins was absent in the oil of *Nigella sativa* while anthraquinones were absent in both oil and seed of the herb.

4.2. Phytochemical Concentration of Seed Extract and Oil of Nigella Sativa

The mean of total bioactive compound concentrations

(ranging from 2.34mg/g to 3.77mg/g) recorded for the seed extracts of *Nigella sativa* were notably higher than the concentration (1.36mg/ml) recorded for the seed oil in Table 4 (P<0.05). Aqueous extract of the seed of Nigelia Sativa was recorded to contain highest amount of these bioactive compounds in the following order; phenol with average of 5.20mg/g, followed by tannin with 4.10mg/g, flavonoid with 4.04mg/g, alkaloids with 3.03mg/g and saponin with 2.47mg/g which was the least among the bioactive compounds detected in the aqueous extract of the seed. The average concentrations of the active compounds in the methanolic extract of seed of Nigelia Sativa were as follow; phenol 3.40mg/g, flavonoid 2.90mg/g, alkaloid 2.30mg/g, saponin 1.58mg/g and tannin 1.50mg/g.

Table 3. *Phytochemical content of oil and seed extract of Nigella sativa.*

SN	Constituent	Oil extract	Methanolic extract	Aqueous extract
1	Quinones	+	+	+
2	Glycosides	+	+	+
3	Cardiac Glycosides	+	+	+
4	Saponins	-	+	+
5	Tannins	+	+	+
6	Steroids	+	+	+
7	Interference	+	+	+
8	Flavonoids	+	+	+
9	Alkaloids	+	+	+

Keys: Present (+)
Absent (-)

4.3. Antifungal Assay

4.3.1. Antifungal Effect of Fluconazole

The results obtained from the evaluation of antifungal activity of conventional drug, fluconazole (200mg) against twelve (12) clinical strains of *C. albicans* are shown in Table 5. Although, all the strains of *C. albicans* in this study showed certain degree of sensitivity to the effect of fluconazole (depending on the concentration), the zone of inhibitions still fell within resistance value. The zone of inhibition recorded for fluconazole at highest concentration (100%) against *C. albicans* strains ranged between 2 mm and 12 mm; and 0mm and 12 mm at the least concentration (25%).

4.3.2. Antifungal Activity of Seed Extracts of Nigella Sativa L.L. Against Selected Strains of Candida Albicans

The results obtained from the evaluation of antibacterial activities of both seed extract and oil of *Nigella sativa L.L.* against four (4) fluconazole resistant strains of *C. albicans*, are presented in Tables 4 and 5 respectively. Different concentrations of the components of Nigelia Sativa exhibited varying antifungal actions in a dose dependent manner against strains of *C. albicans* tested in this study.

From the result shown in Table 6, the oil of *Nigella sativa* exhibited better inhibitory effect against most of the test pathogens with least inhibition zone (13.33±2.08mm) above the stipulated standard (≤ 12mm 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 oil of *Nigella sativa* against *C1* and *CAI4* strains and also between *C2* and *CAF2* strains of *C. albicans*. Highest values ranging from 20.33±7.51mm to 32.67±7.09mm of inhibition zones for the oil were recorded against the four test strains of *C. albicans* at 100% concentration in Table 6.

The antifungal activity of the methanolic and aqueous extracts of the seed of *Nigella sativa* was not profound in this present study compared to that of the oil. The seed extracts of the herb exhibited no inhibitory activity against *CAF2* and *CAI4* strains of *C. albicans* at every concentration in Table 7. Except against *C. albicans C1* at 25% concentration, 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). Inhibition zones of 10.67mm and 16mm were recorded for the methanolic extract of *Nigella sativa* seed against *C2* and *C1* strains respectively. However, at 50% concentration, there were no significant differences (at p-0.05) between the inhibitory effect of both aqueous and methanolic extract of *Nigella sativa* seed against *C1* and *C2* strains in Table 7.

Table 4. *Antifungal sensitivity of C. albicans against fluconazole.*

S/N	Test *Candida* strain	% Zone of Inhibition (mm) by Concentrations (**40mg/ml)				
		0 (DW)	25	50	75	100
1	*C. albicans C1*	0	9	11	11	12
2	*C. albicans CAI4*	0	7	8	8	8
3	*C. albicans CAF2*	0	10	12	11	10
4	*C. albicans C2*	0	7	8	10	12
5	*C. albicans* C3	0	8	11	9	10
6	*C. albicans* C4	0	4	6	7	7
7	*C. albicans* C5	0	10	10	11	11
8	*C. albicans* C6	0	0	2	2	2
9	*C. albicans* C7	0	6	8	10	11
10	*C. albicans* C8	0	10	10	10	12
11	*C. albicans* C9	0	7	9	10	10
12	*C. albicans C1*0	0	8	7	7	6

Keys: Millimeter (mm)
Containing only the diluent [Sterile distilled water] (DW)
Initial concentration (**)

Table 5. *Antifungal activities of Oil of Nigella sativa L.L. against fluconazole resistant C. albicans.*

Test *Candida* strain	Zone of Inhibition (mm) ±SD by Concentration (%)				
	0	25	50	75	100
C. albicans C1	0.00±0.00	13.33±2.08^{a}	17.33±1.53^{a}	20.00±5.29^{a}	20.33±7.51^{a}
C. albicans C2	0.00±0.00	20.67±1.53^{b}	25.00±3.61^{b}	24.67±1.15^{ab}	28.33±3.79^{ab}
C. albicans CAF2	0.00±0.00	21.67±2.08^{b}	18.67±3.06^{a}	29.67±1.53^{b}	32.67±1.15^{b}
C. albicans CAI4	0.00±0.00	15.00±1.00^{a}	17.33±2.52^{a}	22.00±2.65^{a}	23.67±7.09^{ab}

Key: Millimeter (mm)
Standard Deviation (SD)
Containing only the diluent [Dimethyl-Sulfoxide] (*)
Values in the same column with same superscripts do not vary significantly from each other, (P > 0.05) according to DMRT

Table 6. *Antifungal activity of seed extract of Nigella sativa L.L. against fluconazole resistant C. albicans.*

Test *Candida* strain	Zone of Inhibition (mm) ±SD by Concentration (%)									
	Aqueous extract (***400mg/ml)					Methanolic extract (***500mg/ml)				
	0	25	50	75	100	0	25	50	75	100
C. albicans C1	0.00±0.00	12.00±2.00^{b}	14.33±1.15^{b}	15.67±1.15^{c}	19.33±1.53^{b}	16.00±2.00^{c}	20.00±1.00^{b}	17.00±2.65^{b}	17.67±2.08^{c}	13.67±1.53^{b}
C. albicans C2	0.00±0.00	14.33±0.58^{c}	15.67±1.53^{b}	18.00±2.00^{b}	24.67±1.15^{c}	10.67±1.53^{b}	23.00±2.65^{c}	16.00±4.58^{b}	12.33±3.21^{b}	17.00±1.00^{c}
C. albicans CAF2	0.00±0.00	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}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
C. albicans CAI4	0.00±0.00	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}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}

Key: Millimeter (mm)
Standard Deviation (SD)
Containing only Initial concentration (***)
Values in the same column with same superscripts do not vary significantly from each other, (P > 0.58) according to DMRT

4.4. Minimum Inhibitory Concentrations (MIC) of Seed Extract and Oil of Nigella Sativa

The minimum concentration of both seed extract and oil of *Nigella sativa* that is required to inhibit the growth of selected strains of *C. albicans* were evaluated and the results are presented in Table 7. The MIC of the aqueous and methanolic extracts of the seed were not evaluated against *CAF2* and *CAI4* strains as they did not show positive results in the antifungal assay. The oil of *Nigella sativa* exhibited very good inhibitory property against *C1* and *CAF2* strains of *C. albicans* with a very low MIC of 6.25% in each strain.

4.5. Minimum Fungicidal Concentration (MFC) of Seed Extract and Oil of Nigella Sativa

The result as summarized in Table 8 showed that 25% of 400mg/ml is the minimum concentration of aqueous extract of *Nigella sativa* seed required to totally eradicate the growth of both *C. albicans C1* and *C2*. However, 50% of the concentration (500mg/ml) of methanolic extract is recorded as MFC against *C. albicans C1* while 100% concentration of the oil of *Nigella sativa* was recorded as MFC against *C. albicans C2*, showing no fungicidal property against other test strains of *C. albicans*.

4.6. Molecular Assay

Fluconazole resistant genes (*MDR1 and EGR11*) and biofilm forming gene (*Mds3*) amplified from the DNA of the four selected fluconazole resistant strains of *C. albicans* before and after sensitivity test against the seed extract and oil of *Nigella sativa L.L.* were electrophoresed and the results are presented in Figures 1 through 3.

4.7. Amplification of Mds3 Gene in C. albicans

The result of amplification of *Mds3* gene with about 209bp amplicon size in the fluconazole resistant strains of *C. albicans* is shown in Figure 3. According to the gel, strain *CAF2* shows the presence of *Mds3* gene (~209bp) in well 3 (before the sensitivity test) which becomes absent after being subjected to the antifungal effect of the oil of *Nigella sativa* (well 9). The *Mds3* gene was not detected in well 1, 2 and 3 of strain *C1*, *C2* and *CAI4* respectively (before sensitivity test), but was however detected in each strain after subjected to sensitivity test against seed extract (for strain *C1* and *C2*) and oil (for strain *CAI4*) of *Nigella sativa*. The positive control, which is a reference strain (in well +ve) showed the presence of *Mds3* gene while the negative control (well –ve) showed the absence of the gene.

4.8. Amplification of EGR11 and MDR1 Genes in C. albicans

Amplification of *MDR1* gene of about 460bp amplicon size in the fluconazole resistant strains of *C. albicans* in Figure 4 shows that strains *C2*, *CAF2* and *CAI4* (in well 2, 3 and 4 respectively) possess the *MDR1* before exposure to the seed extract and oil of *Nigella sativa*, however, strains *CAI4* and *C2* retained the gene with more Intensity after exposure to the oil and methanolic extract of seed of *Nigella sativa L.L.* respectively. *MDR1*gene which was previously not detected in *C. albicans C1* strain before exposure of the strain to the oil of N *sativa* was detected after exposure to the drug.

EGR11 gene which is responsible for fluconazole resistance in fungi were only amplified in *C. albicans* strain *C1* and *CAI4* (well 1 and 4 respectively) before exposure to

seed extract and oil of *Nigella sativa*. Exposure of the *CI* strain to the oil and aqueous extract of *N sativa* resulted in increased intensity of the *ERGII* bands as seen on agarose gel. (wells 7 and 5 in Plate 3).

Table 7. *Minimum Inhibitory Concentration (MIC) of seed extract and oil of Nigella sativa L. against fluconazole resistant C. albicans.*

Test *Candida* strain	Minimum inhibitory concentration (%)		
	Seed extract		Oil
	Aqueous (**400mg/ml)	Methanolic (**500mg/ml)	
C. albicans C1	12.5	6.25	6.25
C. albicans C2	6.5	25	25
C. albicans CAF2	NE	NE	6.25
C. albicans CAI4	NE	NE	12.5

Key: Initial concentration (**);
Not evaluated (NE)

Table 8. *Minimum Fungicidal Concentration (MFC) of seed extract and oil of Nigella sativa L.L. against fluconazole resistant C. albicans.*

	Minimum Fungicidal concentration (%)		
	Seed extract		Oil
	Aqueous (**400mg/ml)	Methanolic (**500mg/ml)	
C. albicans C1	25	50	NFE
C. albicans C2	25	NFE	100
C. albicans CAF2	NFE	NFE	NFE
C. albicans CAI4	NFE	NFE	NFE

Key: Initial concentration (**)
Not evaluated (NE)
No Fungicidal effect (NFE)

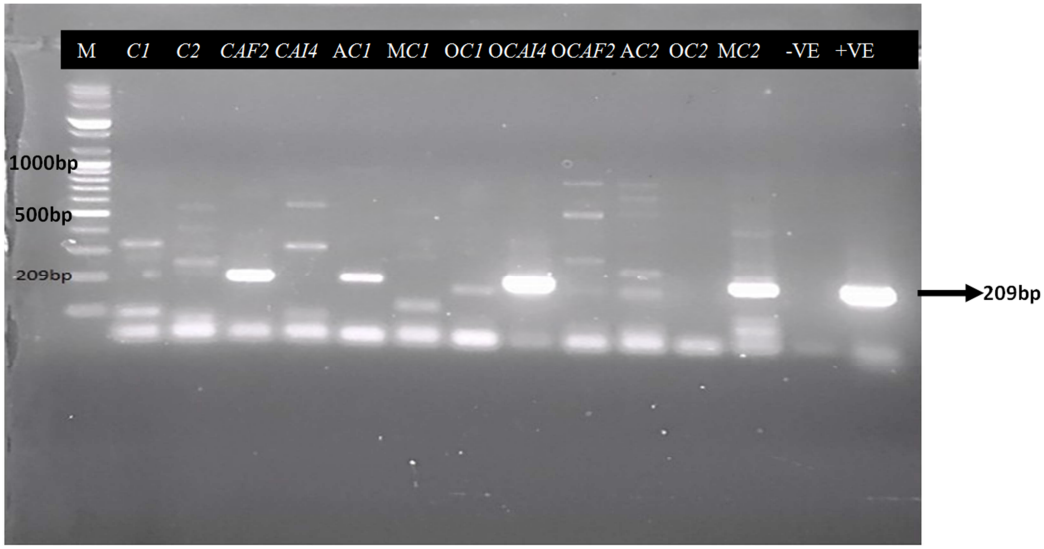

Figure 1. *Gel electrophoresis result showing the ~209bp amplicon encompassing the complete Mds3 gene in fluconazole resistant strains of C. albicans before and after subjected to antifungal effect of Nigella sativa.*

Keys: Positive control (+ve)
Negative control (-ve)
C. albicans C1 before treatment (*C1*)
C. albicans C2 before treatment (*C2*)
C. albicans CAF2 before treatment (*CAF2*)
C. albicans CAI4 before treatment (*CAI4*)
C. albicans C1 after treatment with aqueous extract of *Nigella sativa* (A*C1*)
C. albicans C1 after treatment with methanolic extract of *Nigella sativa* (M*C1*)
C. albicans C1 after treatment with *Nigella sativa* Oil (O*C1*)
C. albicans CAI4 after treatment with *Nigella sativa* Oil (O*CAI4*)
C. albicans CAF2 after treatment with *Nigella sativa* Oil (O*CAF2*)
C. albicans C2 after treatment with aqueous extract of *Nigella sativa* (A*C2*)
C. albicans C2 after treatment with *Nigella sativa* Oil (O*C2*)
C. albicans C2 after treatment with methanolic extract of *Nigella sativa* (M*C2*)

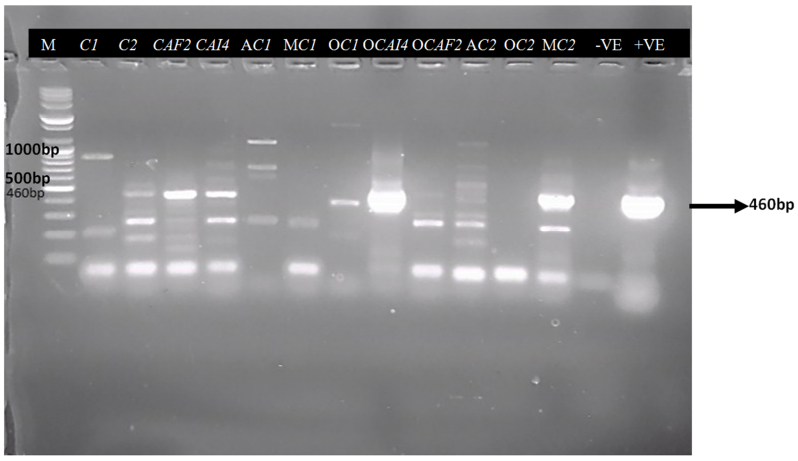

Figure 2. *Gel electrophoresis result showing the ~460bp amplicon encompassing the complete MDR1 gene in fluconazole resistant strains of C. albicans before and after subjected to antifungal effect of Nigella sativa.*

Keys: Positive control (+ve)
Negative control (-ve)
C. albicans C1 before treatment (*C1*)
C. albicans C2 before treatment (*C2*)
C. albicans CAF2 before treatment (*CAF2*)
C. albicans CAI4 before treatment (*CAI4*)
C. albicans C1 after treatment with aqueous extract of *Nigella sativa* (A*C1*)
C. albicans C1 after treatment with methanolic extract of *Nigella sativa* (M*C1*)
C. albicans C1 after treatment with *Nigella sativa* Oil (O*C1*)
C. albicans CAI4 after treatment with *Nigella sativa* Oil (O*CAI4*)
C. albicans CAF2 after treatment with *Nigella sativa* Oil (O*CAF2*)
C. albicans C2 after treatment with aqueous extract of *Nigella sativa* (A*C2*)
C. albicans C2 after treatment with *Nigella sativa* Oil (O*C2*)
C. albicans C2 after treatment with methanolic extract of *Nigella sativa* (M*C2*)

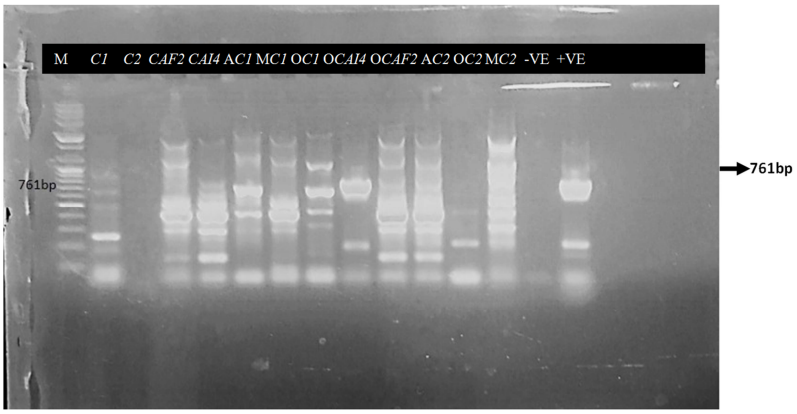

Figure 3. *Gel electrophoresis result showing the ~761bp amplicon encompassing the complete EGR11 gene in fluconazole resistant strains of C. albicans before and after subjected to antifungal effect of Nigella sativa.*

Keys: Positive control (+ve)
Negative control (-ve)
C. albicans C1 before treatment (*C1*)
C. albicans C2 before treatment (*C2*)
C. albicans CAF2 before treatment (*CAF2*)
C. albicans CAI4 before treatment (*CAI4*)
C. albicans C1 after treatment with aqueous extract of *Nigella sativa* (A*C1*)
C. albicans C1 after treatment with methanolic extract of *Nigella sativa* (M*C1*)
C. albicans C1 after treatment with *Nigella sativa* Oil (O*C1*)
C. albicans CAI4 after treatment with *Nigella sativa* Oil (O*CAI4*)
C. albicans CAF2 after treatment with *Nigella sativa* Oil (O*CAF2*)
C. albicans C2 after treatment with aqueous extract of *Nigella sativa* (A*C2*)
C. albicans C2 after treatment with *Nigella sativa* Oil (O*C2*)
C. albicans C2 after treatment with methanolic extract of *Nigella sativa* (M*C2*)

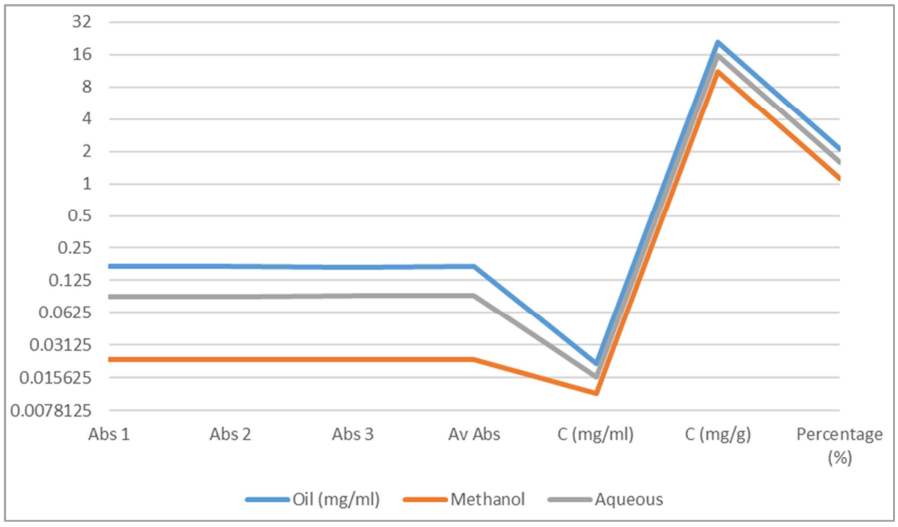

Figure 4: *Quantitative Determination of Thymoquinones.*

5. Discussion

This study showed 28 out of 50 (56%) *Candida* isolated from HVS of suspected PID patients and three Laboratory strains were resistant to fluconazole with the median Zone of inhibition of 8-11mm is within the resistant range. This agrees with the work of Oxmann (2010) which shows that *C. albicans* account for over 41% of infections caused by eight *Candida spp* isolated from blood. The work also disagrees with the report of Pfiler *et al*., (2010), which reported that *C. albicans* have 0-5% resistance among other *Candida spp*. Though the report showed that resistance to fluconazole varies with sample type, however, development of resistance to fluconazole has been well reported in *C. albicans* in recent times [1, 2, 5]. This justifies the significant level of resistance exhibited by the strains of *C. albicans* against fluconazole recorded in this study. This is in compliance with the work of Victor Kuete, (2012) which showed that out of 28 clinical isolates (50%l/. C *albicans* in vulvovaginalis sample) out of the 14 *C. albicans*, 12 (85.7%) were susceptible, 1 highly resistant and the other one (1) showed dose dependent susceptibility [24]. All these could be attributed to the fact that fluconazole has been reported to only have fungistatic and not fungicidal effect hence will encourage the development of acquired resistance. According to Berkow and Lockhart (2017) [1, 2] drug misuse and self-medication is another leading cause of development of resistance.

The presence of these phenolic compounds has however been reported to confer on plants, considerable antimicrobial properties, which is attributed to the redox properties of the compounds [30]. From the result obtained in this work, the oil of *Nigella sativa* demonstrated better inhibitory efficacy against the fluconazole resistant strains of *C. albicans*, for reason which is not farfetched; *CAI4* and *CAF2* were only sensitive to the oil extract and resistant to aqueous and methanolic extracts. This may be due to the absence of saponin and carbohydrate. Saponin is a glycoside that reduces surface tension, which affects cell wall integrity partially. Also, the absence of carbohydrate which could be used by the mutants to restore cell wall integrity must have played a role in combatting resistance. This could be due to the absence of saponin and carbohydrates in the oil. The oil could be more enriched with several bioactive chemicals. Furthermore, the mode of antimicrobial activities conferred on plants by tannins, alkaloids and flavonoid, including their ability to inactivate microbial adhesions, enzymes and cell envelope transport proteins (Omojate *et al*., 2014), are all indicative of the antimicrobial efficacy of the herb against the test strains in this report.

Following the report of Yessuf (2015) [26], the significant activities displayed by the oil and seed extracts of *Nigella sativa* also reinforces the hypothesis that the herb could be explored as potential antimicrobial drug. Moreover, 7also observed that the seed of the herb is a potential source for active ingredients with therapeutic modalities in different clinical settings. The least concentration (26mg/ml) that was recorded for the aqueous seed extract of *Nigella sativa* as the MIC against strain *C2* of *C. albicans* in this research is highly related to the concentration (27.7mg/ml) reported by Venkatachalam *et. al*., (2010) [29] as MIC50% against same fungal species. The least MFC of 100mg/ml documented for the aqueous extract of the herb against *C. albicans C1* and *C2* is however higher than the MFC (72.3mg/ml) reported in the study of Venkatachala5m *et al*. (2010) [29]. The contrasting result could be due to differences in the species of *Nigella sativa*, plant chemical compounds and/or methodologies employed. Remarkable result achieved for the inhibitory activity (MIC) of *Nigella sativa* oil in this research also corroborates the antifungal efficiency claimed by Al-Quarashi *et al*. (2007) [32] of the herb against *Aspergillus niger*. Karou *et al*. (2007) [31] also attributed many physiological activities such as stimulation of phagocytic cells and anti-infective actions in plants to tannins, as a result of its molecular property to form complexes with proteins [33].

Several studies have documented the resistance in *C. albicans* to fluconazole and other azole drugs as attributable

to different mechanisms including but not limited to increased drug efflux, target mutation, target expression deregulation and alteration in ergosterol biosynthesis pathway related to important genes such as *EGR11*, *MDR1*, *MDS3* (Pfaller *et al*. 2012; Whaley *et al*., 2017; Feng *et al*., 2018; Benedetti *et al*., 2019). The results obtained here proved the above statements and findings in the case of *C1*, *CAF2* and *CAI4*. This is also supported by the work of Victor Kuete (2012) [24], which showed that in all the fluconazole resistant strains, *EGR11* was amplified and even in the susceptible dose dependent C, *albicans*. *EGR11* was not expressed in *C2* in this research which disagrees with the work of [34, 35]. It also ceases to disagree because the statement issued by Somanon *et al*. (2020) [34], observed that "*EGR11* overexpression is linked to azole resistance in many fungi". Therefore, *C1* could be one of them.

MDR1 gene was amplified in *C2, CAF2* and *CAI4* prior to treatment with *Nigella sativa* oil and extracts, this is in line with the findings of Joachim M., 2002 [35] which states that in many fluconazole resistance *C*, *albicans*, the reduced drug accumulation was due to strong expression of *MDR1*gene. The amplifications of *MDR1* in these three strains is also in compliance with the findings of Lucia *et al.* (2020) which stated that forced *MDR1* over expression resulted in increased resistance to fluconazole [36]. Joachim (2002) observed that *MDR1* is over-expressed in Fluconazole-resistant *C. albicans* [35]. The result of a research by Lucia *et al*., (2020) also to an extent explained why *MDR1* was not expressed in *C1* [36], which implies that artificial expression of *MDR1* from ADH1 promoter did not or slightly enhanced resistance to fluconazole and that the degree of resistance depends on the *MDR1* promoter level. This can mean that *C1* has a very low *MDR1* promoter.

MDS3 gene which is known for its role in pH regulated morphogenesis (virulence) and biofilm formation was amplified in *CAF2*. There is no work that support the involvement of *MDS3* in antimicrobial generally and also not in *C. albicans.* Morphogesis is induced by a variety of environmental conditions such as temperature 37°C, pH or presence of serum [38]. *CAF2* being a mutant with a loss of one copy of its URA3 gene might have found the condition favourable for mutagenesis. The absence of *Mds3* gene in the other strains could be supported by the findings of Neil et al (2012) [39], which states that *Candida* morphogenesis is not always needed in virulence, hence the absence in other strains.

Although, to the best of our knowledge, there is no report on the molecular mechanism of inhibition of fluconazole-resistant *C. albicans* by *Nigella sativa*. Differences observed in the genetic features of strains of *C. albicans* (Shown in Figure 1 through 3) exposed to the effect of oil and seed extracts of *Nigella sativa* compared with the same strains prior to exposure in this study could be an indication of anti-mutation or gene reversal effect exhibited by the herb.

In the case of *EGR11* gene which is vital for every cell, *EGR11* gene was virtually not clearly amplified in all strains prior to treatment with *Nigella sativa* extracts and oil, although it was observed that A*C1*, O*C1* and O*CAI4 EGR11* gene was amplified when compared with the positive control. All others revealed unclear moving-bands which can be as a result of frameshift mutation or pseudogenes or gel-band shifting. This could be supported by the findings of Norah (2020) who extracted DNA and PCR revealed several bands and the sequencing result showed that all bands were the same gene. *C2* sample was omitted.

PCR result of *MDR1* gene was amplified in *C2*, *CAF2* and *CAI4*, although the results of the treated strains which showed O*C1*, O*CAI4* and M*C2* was strongly improved, different band were seen at different ladders which can mean that the organisms were captured at different point of mutation. This can be supported by the findings of Davina *et al.* (2006) which states that, expression of *MDR1* gene is done by up-regulation of other genes, oil and metabolic extract may have promoter effect on *MDR1*in some strains. by, and/or limitation in time of running the gel. *C1* was absent in the original strain but amplified after treatment with oil, this could imply that the oil has the ability to expose hidden genes or act as promoter in expression of *MDR1* gene.

MDS3 gene amplified in only *CAF2* prior to treatment, but after treatment Mds3 gene was amplified in A*C1*, O*CAI4* and M*C2*. This can imply that aqueos, methanolic extracts and seed oil have exerted an expository effect on *C1*, *C2* and *CAI4*. It can be supported by the new finding on corona virus hidden gene or overlapping gene that led to the viral increased virulence and missed target in the production of vaccine (Colm Gorey 2020) so-also the story of bacteriophage hidden gene that destroyed bacteria Taxas A & M comm, Feb, 2021). The effect of the oil on *CAF2* was a knock out effect. Louis (2014) reported how genes could be knocked out using engineered endonucleases; hence, the oil may have played the same role in inhibiting the growth of *C2* [37].

6. Conclusion

The conclusion of the study reveals the following

1) *Nigella Sativa* is proven to contain the bioactive compounds that are necessary for pharmacological effects on oil, aqueous and methanolic extract, as well the genetic effect on the fluconazole resistant strains.
2) All *C. albicans* and mutant strain in the study have shown considerable resistant to fluconazole.
3) Nigelia Sativa extracts have proven to have considerable antifungal effects and mode of actions varied with extract type.
4) The MIC and MFC of the different extracts have been determined, with aqueous extract showing an explainable low MIC, this could be because the aqueous extract has the highest concentration of all active compounds, then the methanolic extract and seed oil respectively.
5) The extracts have shown concentration dependent inhibition. Resistance to fluconazole has also been shown to be concentration dependent.

6) The target genes have been successfully amplified in two or more study strains. Oil, aqueous and methanolic extracts collectively have shown promoter effect, expository and knock out or silencing gene effects.

7. Recommendations

From this study, it is recommended that Black cumin seed should be used in animal trial series to address genetic diseases. Also, antifungal sensitivity be done routinely to minimize developement of resistance as it was seen in the study that C2 (Clinical isolate) is more resistant to fluconazole than the *C1* (wild type). The Virulence of CAF2 and CAI4 due to URA3 gene deletion, as such its recommended that more substantive animal model research be carried out to determine the avirulence of the strains along side *C1*and other clinical isolates. CAF2 and CAI4 showed higher resistance to *N. sativa* than *C1* and C2, hence the need to address virulence and resistivity of propeertties of URA3 gene.

Future studies should be centered on RNA extraction instead of DNA. To enable the researcher have more room for research. Studies should be conducted on the fate of URA3 gene on exposure to other phytochemicals and possibility of the organism to restore the knocked-out gene.

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