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# Comparative Antifungal Screening of Three Nigella sativa Seed Extract Preparations Against Medically Relevant Fungi

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**ABSTRACT:** Extracts from plant sources have been found to display significant variability in terms of their spectrum of antifungal activities because of the influence of extraction processes on the types of secondary metabolites obtained. In this case, three coded extracts of Nigella sativa seeds (ES, ESM, and CS) were tested at a screening concentration on various fungi (Candida spp., Cryptococcus spp., Aspergillus spp., Penicillium spp., and Coccidioides spp.) using agar diffusion. ES preparation displayed inhibition against Candida spp. (27 mm) and Cryptococcus spp. (41 mm), whereas the ESM preparation inhibited Candida spp. (39 mm), Penicillium spp. (50 mm), and Coccidioides spp. (49 mm). On the other hand, CS preparation inhibited Aspergillus spp. (24 mm), Coccidioides spp. (40 mm), and Cryptococcus spp. (42 mm). Solvent control did not display any inhibition. The response table showed that there is selectivity of preparation on fungi rather than broad spectrum activity by each preparation. Descriptive average inhibition zones are 13.6 mm for ES, 27.6 mm for ESM, and 21.2 mm for CS. These averages indicate the taxonomic coverage and not replicated treatment effect. These results highlight extract-fungus pairs for confirmation and further characterization by broth microdilution tests and compound identification. Since the data was non-replicated and compounds' identity was not available, inferential statistical tests, MIC, and molecular docking were not carried out. The study recommends coded extracts screening as an initial step in the process but highlights the necessity of isolate authentication, independent extraction batches, antifungal control, MIC/MFC test, and identification of active compounds.

## INTRODUCTION

Fungal infections have emerged as one of the pressing problems for medical practice and public health, especially among people with compromised immune systems and in situations where diagnostics and treatment possibilities are limited. The relatively small number of drug classes, variable innate susceptibility among fungi, and the development of resistance to them have led to increased interest in exploring natural products for novel chemically different antifungal leads. In this context, Nigella sativa has also been investigated for antifungal essential oils, peptides, quinones, and chemically prepared fractions, with demonstrated activity that differs significantly depending on fungal strain and assay (Shokri, 2016; Barashkova et al., 2021; Jahantiq et al., 2022; Zanjani et al., 2024).

Extracts of plants have a complex chemical composition and their perceived antiseptic properties are determined by a variety of factors including the origin of the plant, size of particles, composition of the solvent, ratio of the plant material to the

solvent, temperature and duration of extraction, concentration, storage and assay conditions. Solvent polarity leads to partitioning of phenolics, flavonoids, quinones, terpenoids, fatty acids, and peptides into preparations with different diffusion capabilities and biological targets (Tiji et al., 2021; Shafodino et al., 2022; Zouirech et al., 2024). Preparation-based selectivity is also corroborated by our previous findings on aqueous and ethanol extracts of plants, botanical mixtures, marsh plants contaminated with microorganisms, and antimicrobial nanoparticles in which biological responses varied depending on the preparation and the microorganism itself (Hussein et al., 2020; Allami et al., 2020; Mouhamad et al., 2020; Mahdi et al., 2022; Al-Dharob et al., 2022; Mouhamad et al., 2022).

Nigella sativa L. seeds contain volatile and non-volatile constituents, including thymoquinone-related quinones, fatty acids, phenolics, peptides, and other metabolites with reported antimicrobial activity. Essential oils, standardized fractions, seed extracts, and isolated seed peptides have shown different spectra against yeasts and filamentous fungi (Barashkova et al., 2021; Jahantiq et al., 2022; Shafodino et al., 2022; Neel et al., 2023; Bhavikatti et al., 2024). Activity may arise from a dominant compound or from additive and synergistic interactions among several constituents; it therefore cannot be assigned to thymoquinone or any other molecule without chemical confirmation of the tested preparation (Tiji et al., 2021; Zouirech et al., 2024).

The data were obtained from the source set consisting of three extract codes (ES, ESM, and CS), five fungi species, and one solvent control. In the first version of this essay, only ES and CS had been considered, whereas some of the largest inhibition zones were found for ESM. Furthermore, the inferential statistics were used without any replicate values in the results presented. Therefore, the current version represents the experiment as exploratory screening at a single concentration level and includes all three extracts.

The aim is to characterize the specificity of antifungal activity of the extract and to select the extract-fungi pairs for further validation experiments.

## 2. MATERIALS AND METHODS

### 2.1 Study design

The current experiment was performed as an exploratory in vitro screening test using two crossed factors: extract/fraction type (ES, ESM, CS, and solvent controls) and fungus species (with five different types). In this case, there was only one value of inhibition zone measured for each combination of extracts and fungi. In other words, the same fungi were not considered replicates from a statistical point of view.

### 2.2 Plant material and extract preparation

The Nigella sativa seeds were obtained from Baghdad, Iraq, followed by cleaning, air drying, and milling. The ethanol extract (ES) and chloroform extract (CS) were obtained as per the procedures recorded in the laboratory protocol. The third preparation is kept as the coded extract, ESM, since there is no clear record about the solvent used to obtain it in the surviving lab record. Thus, it is considered as a coded preparation and not a chemical one based on the assumption. To make sure the reproducibility of the preparation, the laboratory record needs to be reconciled with the final submission and must report botanical identification, wet/dry seeds, particle size, plant to solvent ratio, time and temperature for extraction, agitation, filtration, concentration procedure, dry yield, reconstitution fluid, storage conditions, and batch number. All these factors affect the composition and antimicrobial activity of the Nigella sativa extracts (Tiji et al., 2021; Shafodino et al., 2022; Neel et al., 2023; Zouirech et al., 2024). Extraction yield is the weight of dry extract divided by the weight of dry seeds and multiplied by 100.

$$\text{Extraction yield (\%)} = (\text{mass of dried extract} / \text{mass of dry seed powder}) \times 100$$

In a confirmation experiment, at least three separate batches need to be set up for each extraction process. These batches must be analyzed on separate days, and technical replicates in one batch must be averaged together, not treated as separate entities. This is important because batch-to-batch variation and plate variation are different sources of error in experiments. (Balouiri et al., 2016; Tiji et al., 2021; Neel et al., 2023).

### 2.3 Fungal isolates and biosafety

Listed in the sources used is Candida spp., Cryptococcus spp., Aspergillus spp., Penicillium spp. and Coccidioides spp. The

strain level identification, codes, source, and confirmation method have not been mentioned and need to be included. Studies on Coccidioides require an approved high containment protocol and training of researchers as the organism is pathogenic; the paper needs to identify the biosafety approval and facility rather than routine handling like a regular teaching laboratory organism.

In order to confirm, yeast inoculates should be made from fresh cultures with appropriate adjustment of their concentration to those recommended by CLSI method while for conidial inoculates, they should be counted or spectrophotometrically measured and verified microscopically whether there is contamination of hyphae. Identification of species will involve phenotyping and confirmation using MALDI-TOF MS or sequence analysis wherever possible. This is critical since genus level identification may conceal significant variations among species in terms of antifungal susceptibility. (CLSI M27; CLSI M38; Shokri, 2016).

**2.4 Agar-diffusion screening**

The source research used an agar well diffusion assay approach. Inoculum standardization needs to be done separately for both yeast and filamentous fungi, maintaining a consistent agar thickness and well diameter, while explicitly noting the deposited amount of volume and extract concentration (A-Rubaye et al., 2022). The zone diameter must be measured in millimeters with a calibrated micrometer ruler by two blind observers, with any discrepancies settled before unveiling. A negative control with only solvent and an appropriate reference antifungal should always be run on all test days. The proposed method is consistent with the antimicrobial screen assay used in previous research from the same authors on botanic and nanomaterial testing, but the diffusion zones need to be considered a screening value since factors such as viscosity, solubility, precipitation, and diffusion are known to affect them irrespective of antifungal efficacy. (Balouiri et al., 2016; Hussein et al., 2020; Allami et al., 2020; Al-Dharob et al., 2022; Mouhamad et al., 2022; CLSI M44).

**2.5 Confirmatory MIC and MFC design**

The MIC and MFC data could not be obtained since there were no dilution series in the provided data set. The confirmatory testing protocol should follow CLSI recommendations for yeasts (CLSI M27) and filamentous fungi (CLSI M38) and include quality control strains, separate extracts, and certain dilution series. The MFC should be determined by plating out colonies from well plates that do not have growth. It is not allowed to calculate MICs from diffusion zone diameters. (CLSI M27; CLSI M38).

**2.6 Statistical analysis**

The present data were described in terms of means, standard deviation of the sample, median, range, and number of taxa responding to extracts coded in each row. The taxon-extract response matrix was depicted in the form of grouped bar charts and a heat map. An ANOVA, Kruskal-Wallis test, post hoc pairwise comparisons and p-values, and effect sizes were not performed since only one observation per cell prevents estimation of within-cell experimental variation.

For confirmatory analysis, either zones of inhibition or log2(MIC) values should be analyzed with factorial mixed-effects modeling with fixed effects being extract, fungus, and interaction between them, and independent extraction batch and assay date as random effects. The estimated marginal means should be corrected for multiple comparisons. In the case when there are too many zeros in data, the preferred approach would be to use two-part modeling.

**3. RESULTS**

**3.1 Extract-by-taxon inhibition pattern**

The inhibition matrix exhibited selective inhibition but not broad spectrum inhibition. ES was inhibitory to two out of the five organisms; Candida spp. (27mm) and Cryptococcus spp. (41mm). ESM was inhibitory to three organisms and showed the two biggest zone diameters; Penicillium spp. (50mm) and Coccidioides spp. (49mm), as well as Candida spp. (39mm). CS was inhibitory to Aspergillus spp. (24mm), Coccidioides spp. (40mm) and Cryptococcus spp. (42mm). The control did not show any inhibition of the tested organisms.

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**Table 1.** Reported inhibition-zone diameters for the complete extract-by-fungus matrix

| Fungal taxon | ES (mm) | ESM (mm) | CS (mm) | Control (mm) |
|---|---|---|---|---|
| Candida spp. | 27 | 39 | 0 | 0 |
| Aspergillus spp. | 0 | 0 | 24 | 0 |
| Penicillium spp. | 0 | 50 | 0 | 0 |
| Coccidioides spp. | 0 | 49 | 40 | 0 |
| Cryptococcus spp. | 41 | 0 | 42 | 0 |

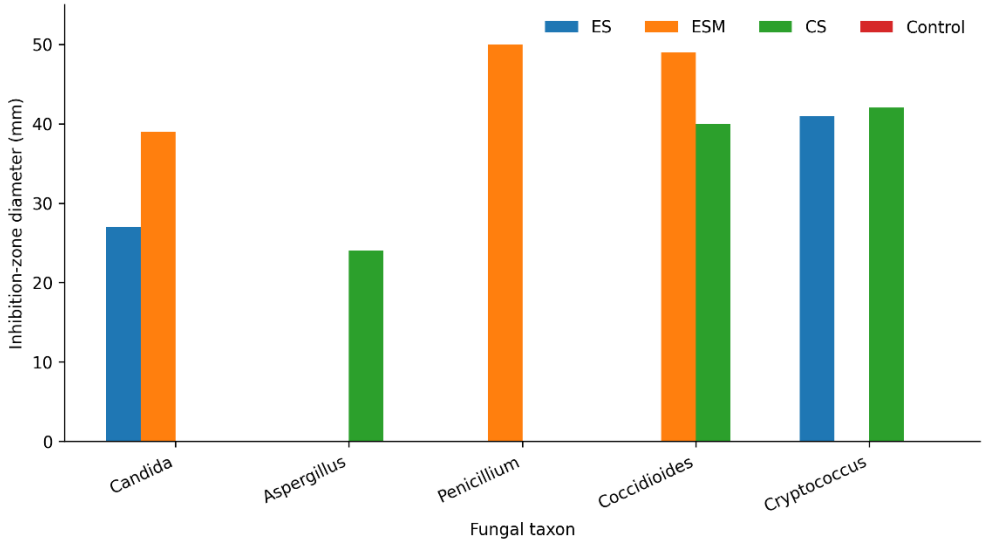


**Figure 1**. Reported inhibition-zone diameters by fungal taxon and extract/fraction code. Values are descriptive single-cell records, not replicate means.

### 3.2 Descriptive coverage of the three extract codes

The descriptive mean was largest (27.6 mm) for ESM, which inhibited three different taxa. CS also inhibited three different taxa, but the mean obtained for CS was smaller (21.2 mm). ES inhibited two different taxa, and the mean obtained was smallest among all treatments other than the control (13.6 mm). These means were calculated on coverage across different fungal taxa, not as estimates of treatment effectiveness.

**Table 2.** Descriptive summaries across the five fungal taxa.

| Group | Mean | SD | Median | Range | Responsive taxa | Coverage (%) |
|---|---|---|---|---|---|---|
| ES | 13.6 | 19.3 | 0.0 | 0–41 | 2/5 | 40 |
| ESM | 27.6 | 25.6 | 39.0 | 0–50 | 3/5 | 60 |
| CS | 21.2 | 20.6 | 24.0 | 0–42 | 3/5 | 60 |
| Control | 0.0 | 0.0 | 0.0 | 0–0 | 0/5 | 0 |

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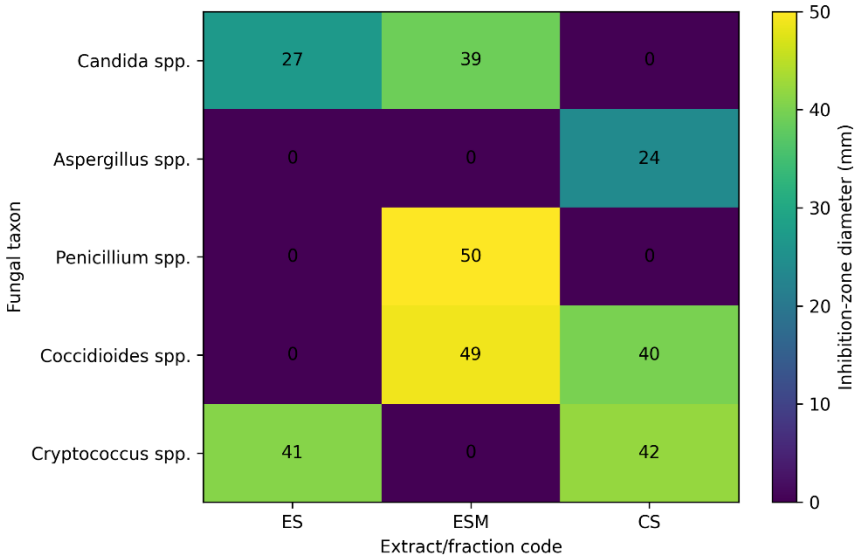


**Figure 2.** Heatmap of the complete antifungal response matrix. Darker intensity denotes larger reported inhibition zones.

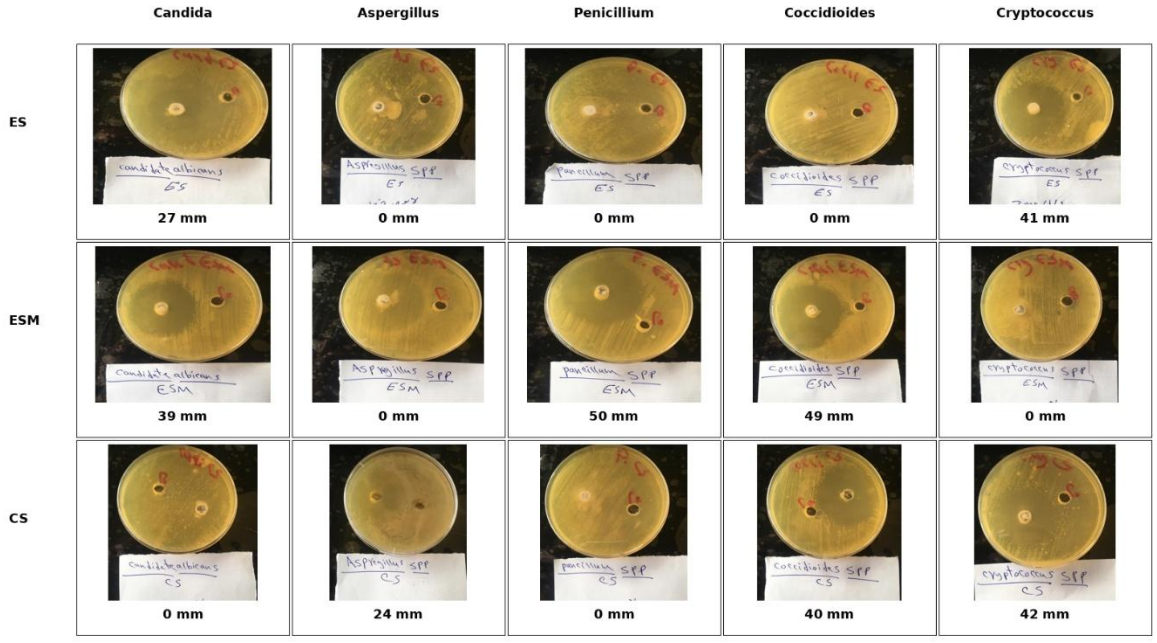


**Figure 3.** Integrated table of the original agar diffusion pictures for ES, ESM and CS tested against the five fungal isolates. Numbers correspond to inhibition zone sizes measured. Pictures are meant to illustrate the experiment conducted but not serve as replacements for numbers.

### 3 Taxon-specific selectivity

**Candida spp.** showed susceptibility to ES (27 mm) and ESM (39 mm), while there was no effect on CS, which suggests that there were not equal amounts of active ingredients in CS.

**Aspergillus spp.** Responded only to CS (24 mm), which was the smallest non-zero zone in the matrix. This pair requires a second round of testing using the broth dilution method because diffusion differs for chemical dissimilar substances.

**Penicillium spp.** The results were only seen for the ESM (50 mm), contrary to the claim made in the initial draft that Penicillium showed resistance to all the extracts. The nature of the ESM, as well as the information present in the initial plate,

should be verified..

**Coccidioides spp.** responded to ESM (49 mm) and CS (40 mm) but not to ES. Because this organism requires stringent biosafety controls, isolate identity and containment documentation are essential.

**Cryptococcus spp.** responded strongly to ES (41 mm) and CS (42 mm) but not to ESM, showing that a high response to one fraction does not predict response to another fraction from the same seed material.

## 4. DISCUSSION

Interaction between preparations and taxa is significant. No one preparation inhibited all tested taxa, and no one taxon was susceptible to all three preparations. Such selectivity can be attributed to the differences in partitioning of chemicals, as well as in the architecture of cell wall and membrane, their permeability, efflux activity and metabolism in yeasts versus filamentous fungi. Preparation-dependent responses have been reported in the authors' antimicrobial studies involving botanical preparations, combined herb formulation, Ag, ZnO and TiO2-based systems, where no universal activity was achieved (Hussein et al., 2020; Allami et al., 2020; Mahdi et al., 2022; Al-Dharob et al., 2022; Mouhamad et al., 2022).

Escherichia coli? Actually, no – ES and CS were very active against Cryptococcus spp., while ESM had no inhibitory action on it. On the other hand, ESM and CS inhibited Coccidioides spp. Therefore, several chemically dissimilar antifungal activities are presumably present in Nigella sativa seeds. Previous reports on the activity of standardized fractions, seed peptides, essential oil and phytochemical extracts indicate the existence of diverse antifungal spectrums for each chemical fraction (Barashkova et al., 2021; Jahantiq et al., 2022; Neel et al., 2023; Bhavikatti et al., 2024). Accordingly, chemical profiling is required before attributing the observed effect to thymoquinone, phenolics, fatty acids, peptides and other compounds.

EMM is critical for interpretation because it produces the largest zones and the only response towards Penicillium spp. If ESM is excluded, an erroneous conclusion about total resistance of Penicillium to EMM would be drawn. It is scientific to keep ESM as coded preparation rather than to attribute an unconfirmed solvent name to it; however, the original extraction protocol should be retrieved before journal submission, since reproducibility depends on the preparation chemistry.

Agar diffusion is suitable for preliminary screening, but zone diameter is jointly determined by antifungal activity, solubility, viscosity, molecular size, precipitation, and diffusion through agar. A large zone therefore does not necessarily correspond to a low MIC, and a zero zone does not prove inactivity. The present matrix should be used to prioritize ES-Cryptococcus, CS-Cryptococcus, ESM-Penicillium, ESM-Coccidioides, CS-Coccidioides, ESM-Candida, ES-Candida, and CS-Aspergillus for standardized broth microdilution rather than to rank therapeutic potency (Balouiri et al., 2016; Shokri, 2016; Jahantiq et al., 2022; Zanjani et al., 2024; CLSI M27; CLSI M38).

The current dataset cannot support the previously reported Kruskal–Wallis p-value, pairwise comparisons, or $\eta^2$. Treating five fungal taxa as replicate observations confounds treatment effect with biological differences among taxa. A valid inferential analysis requires repeated independent observations within every extract–taxon cell.

Translation toward medical application requires species-level isolate confirmation, cytotoxicity testing in mammalian cells, selectivity-index calculation, MIC and MFC determination, time-kill analysis, chemical fingerprinting, fraction purification, stability testing, and comparison with reference antifungals. Molecular docking should be added only after GC-MS or LC-MS identifies credible ligands and a biologically relevant fungal target is selected. The authors' previous in silico work on medicinal-herbal antimicrobial candidates illustrates how computational analysis can complement, but not replace, experimental screening (Hussein et al., 2021). To dock thymoquinone or any other potential compound without proving that they have been identified in ES, ESM, or CS is only decorative and not supportive of the research claim. An appropriate virtual screening approach should include ligand identification, target identification and justification, validated protein structure, positive control or redocking validation, identification of binding site, and interaction details regardless of docking scores as proof of antifungal activity.

## 5. CONCLUSIONS

From the full dataset, there is evidence of selective antifungal activity among the three codes of Nigella sativa extracts/fractions. ESM shows the highest level of descriptive information and inhibition zones, while CS shows inhibition

activity against three genera including Aspergillus spp. ES, on the other hand, selectively inhibits Candida spp. and Cryptococcus spp. While these results can inform targeted confirmatory tests, they do not lend themselves to inferential statements. The manuscript becomes scientifically defensible only with a clear definition of ESM and inclusion of replicate-level data, isolate identifications, biosafety information, MIC/MFC and chemical characterization data.

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