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Screening of *Nigella Sativa* Seeds for antifungal activity

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Abstract

In present investigation, antifungal activity of methanolic and ethanolic extracts of the seeds of *Nigella sativa* was investigated on different pathogenic fungal strain such as *Aspergillus*, *Candida*, *Cryptococcus* and *Issatchenkia* species. The extracts were prepared by the cold maceration technique, and its antifungal activity was assessed by 'The National Committee of Clinical Laboratory Science (NCCLS)' method. We found that methanolic extract of plant exhibits potent inhibition of fungus growth against *Candida Parapsilosis*, and *Issatchenkia Orientalis* with IC_{50}Value 4.846 µg/ml, and 6.795 µg/ml, respectively and ethanolic extract also shows significant anti-fungal activity against fungus strain *Issatchenkia Orientali* with IC_{50} value 5.805 µg/ml.

Key Words: *Nigella Sativa*, NCCLS, Anti-fungal.

Introduction

Nigella sativa (Family: *Ranunculaceae*) is an annual flowering plant, also known as Kalijiri in Hindi. It has a pungent bitter taste and faint smell of strawberries. It is mainly used as a spice and also in the preparation of candy and liquor. *Nigella sativa* has a potential anti-inflammatory, anti-microbial, anti-fungal, anti-parasitic, and anti-cancer activity. [1]

Seeds of the *Nigella sativa* contain 37% oil and 4.1 % ash (calcium salts), protein (16-19.9%), carbohydrates (33-34%), fibre (4.5-6.5%), saponins (0.013%), moisture (5-7%). The oil has chemical constituents like thymoquinone, dihydrothymoquinone, thymodihydroxyquinone, alpha-pinene, 4-terpineol. Other constituents are also present in the seeds like Linoleic acid,

lonolenic acid, Arachidic acid. Most of the biological activity of *Nigella* sativa plant showed by the oil constituents like, Thymoquinone. [1]

The antifungal activity of Thymoquinone from *Nigella sativa* had been evaluated by the standard agar plate method. [2]

The standardization of the vitro antifungal susceptibility testing has advanced greatly in recent years. The National Committee for clinical Laboratory Standards (NCCLS) has set benchmark methodology by providing laboratory tested reproducible, consensus peer-reviewed standards. NCCLS M27 –A2 standard for yeasts provides a broth microdilution test which could be a good screening method for plant extract with its high through-put potential, considerable savings in media usage and requirement of small quantity of sample [3] and hence we decided for anti-fungal screening of various extracts of the *Nigella sativa* on the various fungal strains by the standard broth dilution method (NCCLS) as well as some modification in NCCLS method which used sphectrophotometric determination of the end point.

Materials and Methods

Chemicals

Methanol (Finar Chemicals,), Ethanol (Finar Chemical), RPMI-1640 medium supplemented with Glutamine and Phenol red without bicarbonate (Himedia, lot no.-0000026654), 3-(N-morpholino) propanesulfonic acid (MOPS) (Himedia, lot no.-0000028915), Resazurin (Himedia, lot no.-0000002880), Czapek yeast extract agar, Amphotericin B, Dimethyl sulphoxide (DMSO) (Himedia, lot no.-0000027905), Water for Injection.

Fungal strain

The fungal strains used for the study were obtained from the Microbial Type Culture Collection (MTCC, India). The fungus strains used in the study are *Aspergillus fumigates*-MTCC-2550, *Aspergillus flavus*-871, *Candida albicans*-183, *Candida tropicalis*-184, *Candida parapsilosis*-1744, *Issatchenkia orientalis*-3020, *Cryptococcus albidus Var. albidus*-2661, *Cryptococcus layrentii var. layrentii*-2898.

Collection of plant material

The plant material (*Nigella sativa* seeds) was collected from the Ms Lallubhai Vrajlal Gandhi and sons, (Ahmedabad, Gujarat, India) and authenticated by Dr. Ritesh Vaidya, Bio-science department of Ganpat University.

Extraction Preparation

Methanolic extract

Methanolic extract of the *Nigella sativa* seeds was prepared by socking 150 gm finely grounded powder of *Nigella sativa* seeds in 150 ml of methanol for 7 days. After 7 days, the extract was filtered through Whatman filter paper and evaporated till dryness. [4]

Ethanolic extract

Ethanolic extract of the *Nigella sativa* seeds was prepared by simple socking 50 gm of finely grounded powder of *Nigella sativa* seeds in sufficient amount of ethanol for 24 hours. After 24

hours, the extract was filtered through what Mann filter paper and the residual matter was again soaked with sufficient amount of ethanol for 24 hours. After 24 hours, the extract was filtered through Whatman filter paper and then was combined of two extract and evaporated till dryness.

Assessment of anti-fungal activity:
Preparation of broth medium:
10.4 gm of RPMI-1640 medium supplemented with glutamine and phenol red and 34.53gm 3-(N-morpholino) propanesulfonic acid (MOPS) was dissolved in 400 ml of distilled water. pH was adjusted to 7.0 at 25 °C with 1 mol/L sodium hydroxide. The volume was made up to 0.5 L with water and was filtered, sterilized and was stored at 4°C until required.

Preparation of Inocula:
Fungal strains were sub-cultured on to their respective growth medium and incubated for 48 hrs at 25-30°c. From cultured plates, several colonies were transferred to 5 ml of sterile distilled water. The suspensions were mixed for 15 second to ensure homogeneity and subsequently diluted to match the turbidity of a 0.5 McFarland standard (i.e. OD = 0.12–0.15 at λ = 530 nm, corresponding to 1–5 X 10^6CFU/ml). Then the working suspensions were prepared by 1 in 30 further dilution of the stock suspension in sterile distilled water to yield 1–5 X 10^3 CFU/ml. 0.1ml sterilized solution of resazurin (20 mg/ml in water) was supplemented to the working suspension.

Preparation of sample:
Stock solutions of the plant extracts and the positive control drug Amphotericin B were prepared in dimethyl sulphoxide (DMSO) at the concentrations of 100 mg/ml. Further it was diluted to 1:50 in broth.

Preparation of Plates:
Microdilution susceptibility testing was performed in flat-bottom 96-well clear plates containing broth medium (50µl) in each well. Sample solutions (50µl) were subsequently serially diluted two-fold in the plates with the broth, starting with the final concentration of 5000 mg/L. The working inoculums suspension (50µl) was added to give final inoculums concentration of 0.5–2.5 X 10^3CFU/ml. Amphotericin B was used as the standard antifungal drug. Sterility and growth controls in the presence of DMSO were also included. The plates were then incubated at 37 °C for 48 h. The amount of growth was measured using plate reader at λ=450nm. [3]

Percentage inhibition of the extract against all cell line was calculated using the following formula.

$$\% \text{ cell survival} = \frac{(At - Ab)}{(Ac - Ab)} \times 100$$

At = Absorbance of Test,
Ab= Absorbance of Blank (Media),
Ac= Absorbance of control (cells)

% cell inhibition = 100 - % cell survival

The effects of extracts were expressed by IC50 values calculated from dose response curves.

Results and Discussion

NCCLS test replicates for anti-fungal activity were analyzed every 24hrs for the three days to determine the percentage growth inhibition in the presence of the extracts. The percentage growth inhibition of extracts against each fungus strains was calculated for the 24 hours, 48 hours, and 72 hours. The IC_{50} values obtained by the NCCLS method are shown in the table 1. the most potent activity was exerted by methanolic extract which in terms of calculated IC_{50} value , against *Candida parapsilosis*-1744 (4.846 µg/ml), *Candida albicans*-183 (6.484 µg/ml), and *Issatchenkia orientalis*-3020 (6.795 µg/ml). Ethanolic extract displayed potent activity, IC50 value against *Issatchenkia orientalis*-3020 (5.805 µg/ml), *Candida parapsilosis*-1744 (7.093 µg/ml).

With the NCCLS method, IC_{50} values for all the fungus strains were obtained. The control drug Amphotericin B was found to be effective against all species and activities are mentioned in Table 1.

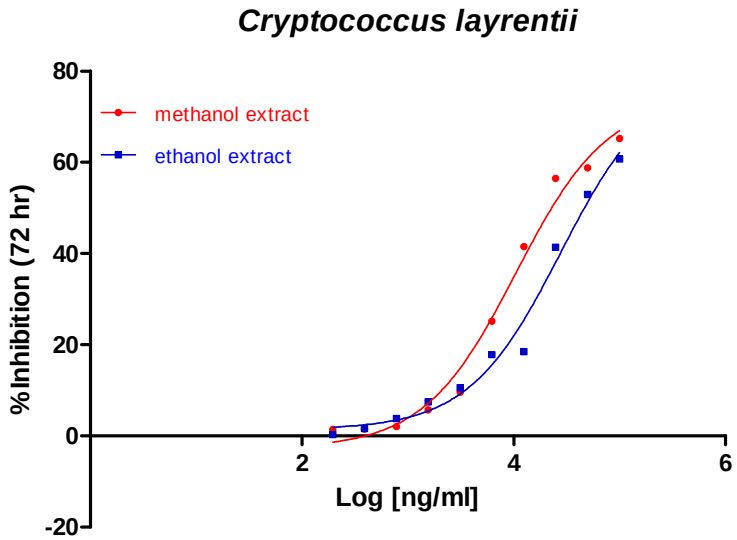

Figure 1: Effect of methanolic and ethanolic extract of *Nigella sativa* against *Cryptococcus layrentii*.

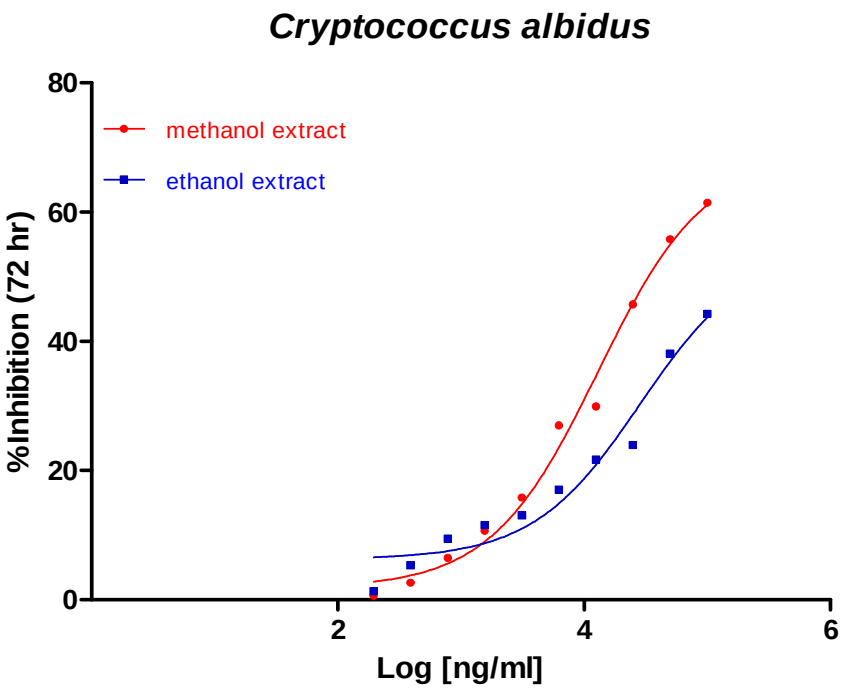

Figure 2: Effect of methanolic and ethanolic extract of *Nigella sativa* against *Cryptococcus albidus*.

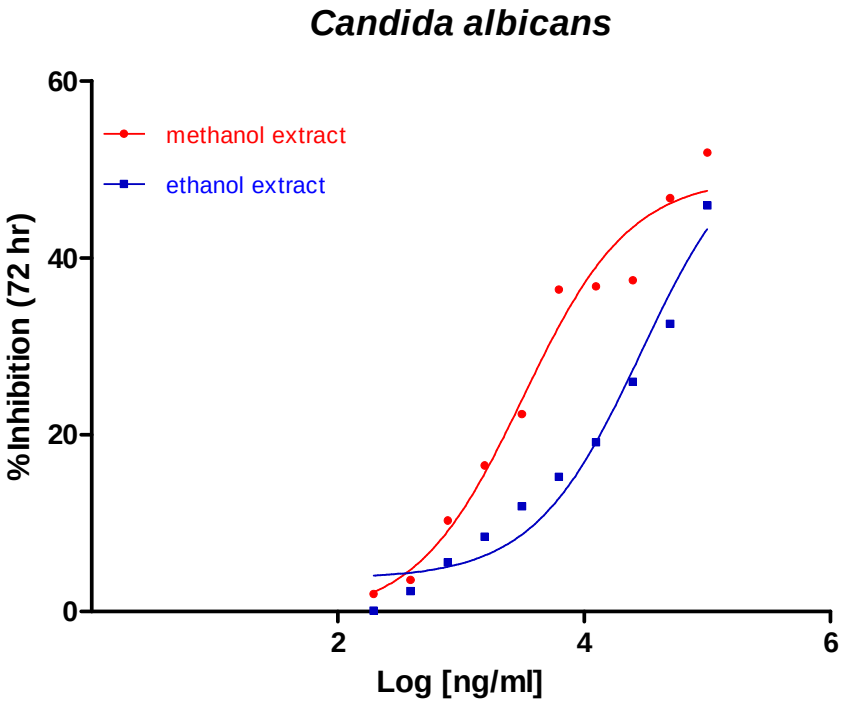

Figure 3: Effect of methanolic and ethanolic extract of *Nigella sativa* against *Candida albicans*.

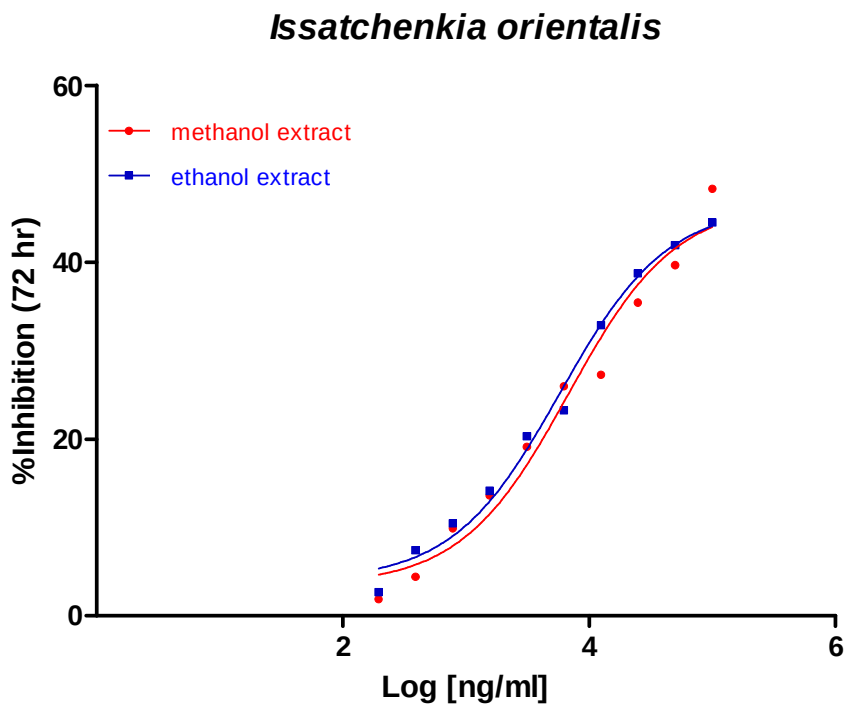

Figure 4: Effect of methanolic and ethanolic extract of *Nigella sativa* against *Issatchenkia orientalis*.

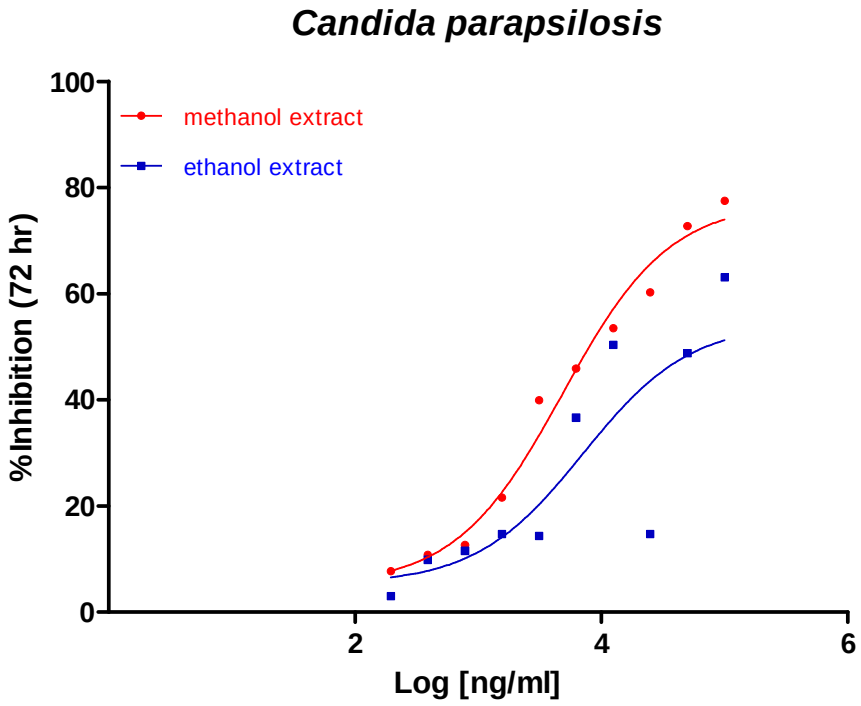

Figure 5: Effect of methanolic and ethanolic extract of *Nigella sativa* against *Candida parapsilosis*.

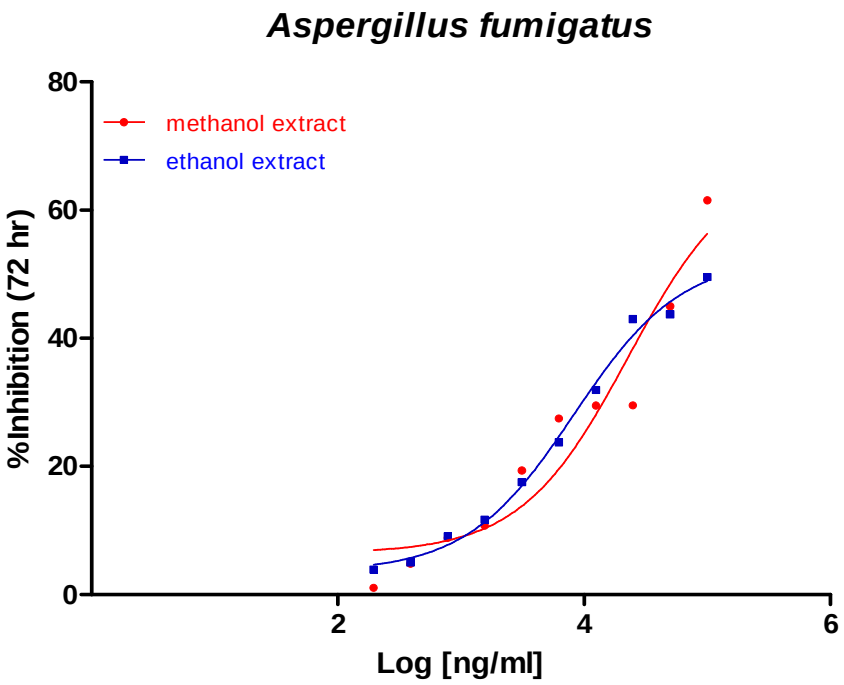

Figure 6: Effect of methanolic and ethanolic extract of *Nigella sativa* against *Aspergillus Fumigates*

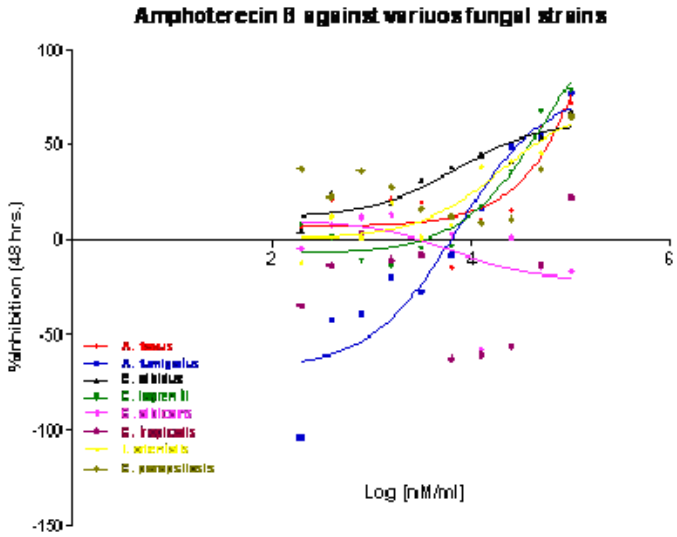

Figure 7: Effect of Amphotericin B against Various fungus strains.

Table I: IC_{50} value against different pathogenic fungal strains by NCCLS method.

IC_{50}	*A. flavus*	*Cryptococcus layrentii*	*I. orientalis*	*Cryptococcus albidus*	*C. parapsilosis*	*C. albicans*	*C. tropicalis*	*A. fumigatus*
Methanolic extract	13.941	10.498	6.795	12.986	4.846	6.484	65.585	22.799
Ethanolic extract	>100	27.797	5.805	28.504	7.093	28.758	32.317	8.282
Amphotericin	43.29	44.58	20.71	23.18	61.25	>100	>100	27.36

Conclusion

Plant flora has been a great source of therapeutic agents. [5, 6] Both the extracts of *Nigella Sativa* seeds exhibited potent inhibition of almost all the fungal strains. Both extracts were found to be more potent than standard drug Amphotericin. The plant can be a source of an important pharmacophore in future.

Acknowledgement

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