International Journal of Pharmacy and Biological Sciences
ISSN: 2321-3272 (Print), ISSN: 2230-7605 (Online)
IJPBS | Volume 9 | Special Issue 2- ICESMT| 2019 | 107-115
International Conference on "Empowering Society with Microbial Technology"
Held at Tuljaram Chaturchand College of Arts, Science and Commerce, Baramati - 413 102,
Dist. Pune, Maharashtra, India, 7-9 February 2019
| Research Article | Biological Sciences |Open Access |MCI Approved|
|UGC Approved Journal|

Analytical Determination of Major Constituent of *Nigella Sativa* and it's Antibacterial, Antifungal and Antimycetoma Activities

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Received: 30 Jan 2019 / Accepted: 20 Feb 2019 / Published online: 01 Apr 2019
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Abstract
There are many plants which are having therapeutic potentials. *Nigella sativa,* known as 'black cumin' or 'Kalonji', is a herbaceous plant which grows in Mediterranean countries but has been cultivated into other parts of the world. In Islam, it is considered as one of the significant forms of healing medicine available. Prophet Muhammad (PBUH) once stated that the Kalonji can treat every disease -- except death (Sahih Bukhari 71:592). There are many biological activities of *Nigella sativa which* are reported in recent years including antioxidant, anti-inflammatory, anticancer and antibacterial, anti-diabetic, antifungal and many more. So, in this research, different seed extracts from *Nigella sativa* have been tested for antibacterial, antifungal, anti-mycetoma activities. *Madurella mycetomatis* is the commonest causative organism causing Eumycetoma and *Streptomyces somaliensis* is the commonest organism causing actinomycetoma[1]. In general, the present treatment for mycetoma is expensive and unsatisfactory. It needs a long duration and has many side effects [2]. If drugs not effective and bone is infected, amputation of the limb or debride tissue is required. In the present study, anti-bacterial, anti-fungal activity of the methanol extracts and antimycetoma activity of methanol and chloroform extracts of *Nigella sativa seed* were studied in vitro against bacterial strains of *Staphylococcus aureus*, *Proteus vulgaris*, Fungal strains of *Candida albicans, Aspergillus flavus* and the organism *Nocardia brasiliensis* of mycetoma. Data obtained from this study suggest that *Nigella sativa* may be used for treatment of infections, alone or in combination with some antibiotics especially in case of the bacteria *P. vulgaris* and fungi *C. albicans.* And it has been used for a long time as food without any serious toxicity reported, it can be considered as a safe and affordable treatment for mycetoma.

Keywords
Nigella sativa, black cumin, Kalonji, Sahih bukhari, Mycetoma

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INTRODUCTION:
Nigella sativa (Family Ranunculaceae) is an extensively used medicinal plant throughout the world. It is very popular in various traditional systems of medicine like Ayurveda, Unani and Tib. Seeds and oil of black cumin have a long history of folklore usage in various systems

of medicines and food. It has been suggested for using on regular basis in Tibb-e-Nabwi (Prophetic Medicine). Studies on *N.* have been carried out by various researchers and a broad spectrum of its pharmacological actions have been explored which may include antidiabetic, anticancer, immunomodulator, analgesic, antimicrobial, anti-inflammatory, spasmolytic, bronchodilator, hepato-protective, renal protective, gastro-protective, antioxidant properties, etc. This is also revealed that most of the therapeutic properties of this plant are due to the presence of Thymoquinone which is major bioactive component of the essential oil[3]. Due to its marvelous power of healing, *N.* has got the place among the top ranked evidence based herbal medicines. However, we have a lot of diseases that have no treatment like Mycetoma (Madura foot) which is endemic in tropical and subtropical regions like Sudan, Somalia, Senegal, India, Yemen, Mexico, Venezuela, Columbia, Argentina and others [1]. Mycetoma is a chronic progressive localized infection of the skin and subcutaneous tissue which is believed to originate following the implantation of the causative agents through minor trauma to the skin, usually from vegetative material such as thorns or splinters[4,5].Worldwide, 60% of mycetomas are bacterial (actinomycetoma) and 40% are fungal (eumycetoma)[6,7].The disease is characterized by triad of tumefaction, discharging sinuses, and grains.(fig1)

MATERIALS AND METHODS:

Preparation of plant extracts:

For Antibacterial and Antifungal activities:

The seeds were purchased from Totawala Hakeem located in Pune, Maharashtra. Methanolic extract of *Nigella sativa* seeds were prepared by soaking 100gm finely grounded powder of seeds in 100ml of methanol for few days. After few days filtration was done using Whatmanns filter paper no.1 and evaporate till dryness.

Bacterial and fungal strains were obtained from Sanjeevan hospital, Pune. The strains used in the study are *Staphylococcus aureus, Proteus vulgaris, Candida albicans and Aspergillus flavus.* In addition, positive controls antibiotic discs such as Tetracycline and Clotrimazole were used for comparision.

For Antimycetoma activity:

100g of *N.* powder was extracted by using Methanol as a solvent.

The solvent was evaporated, and the oil was collected. The oil obtained from both extracts was serially diluted (0.05%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%) using methanol respectively.

Nocardia brasiliensis was used for antimycetoma activities.

Preparation of Specimens for antimycetoma activity:

Specimens of exudates, pus, and drainage should be examined for granules by using a dissecting microscope. If granules are present, the colour is noted, and then a portion of the specimen is teased apart gently, crushed between two glass slides, and examined microscopically; the remainder is washed several times in sterile distilled water, crushed with a sterile glass rod, and inoculated onto appropriate media (granules may be bacterial and should be plated accordingly). If no granules are present, the specimen is examined microscopically for hyphae and other fungal elements and directly inoculated onto isolation medium.

Inoculation on media:

Inoculation was performed in a sterile advanced safety cabinet using Sabouroud dexterose agar and blood agar media.

The plates were inverted, and the tubes were put in test tube rack and incubated aerobically at 35-37˚C for 7 days insure the growth of mycetoma was begin and clearly change was shown up to 21 -23 days to reach the complete growth [8].

Determination of Antibacterial and Antifungal activities:

Disc Diffusion Method:

i) Mueller Hinton agar is prepared as bacterial media and Sabarouds dextrose agar plates as fungal media were prepared.
ii) All glasswares, petriplates were sterilized in autoclave.
iii) In aseptic technique, using sterile swab a bacterial lawn is made on sterile petri plates from microbial inoculum suspension.
iv) Swab is made in one direction by rotating plate at 90º
v) Filter paper discs of 6mm diameter were impregnated with about 0.1ml/disc of extract dilution solution (140, 160, 120, 100, 80, 60, 40, 20 in ug/ml) and placed on agar plate in aseptic condition.
vi) MH agar Plates were incubated at 37ºc for 24 hours and Sabarouds dextrose agar plates at 28ºc for 2 days. DMSO is kept as negative control and for antibacterial activity Tetracycline and for antifungal activity clotrimazole were used as positive controls. After 24 hours zone of inhibition was measured and MIC of *Nigella sativa* methanolic extract against bacterial and fungal strains were determined.

Screening for Antimycetoma activity:

Different concentrations of methanolic extracts were screened for their antimycetoma activity against Actinomycetoma (***Nocardia brasiliensis)*** using blood agar as culture media. The tubes and plates were examined for inhibition of the growth of the above

species from subcultures of the organism and the results were obtained.

Anlytical Determination of major Constituent (Thymoquinone) of *Nigella sativa:*

HPLC Analysis:

Isolation of Thymoquinone:

Thymoquinone (TQ), is also known as 2-methyl, 5-isopropyl 1,4-benzoquinone is an important constituent of oil obtained from seeds of *Nigella sativa.* Ghosheh and co-workers developed method for analyzing oil of *Nigella sativa* seeds through high performance liquid chromatography (HPLC). The constituents from the oil were extracted by using C18 PrepSep mini columns and quantification of these recovered constituents by HPLC was completed on a reversed phase µBondapak C18 analytical column. Isocraticmobile phase of water: methanol:2-propanol (50:45:5% v: v) at flow rate of 2 ml/min and 254 nm radiation was used for detection of TQ.

Previous studies reported isolation of TQ from seeds of *Nigella sativa* by subjecting 20g of finely powdered seeds to Soxhlet extractor with hexane and solvent was removed under vacuum followed by stream of nitrogen. The extract was loaded on silica gel column and eluted with hexane, 15%diethylether in hexane, diethyl ether, and methanol 500mL each and analyzed on HPLC after evaporation and reconstitution in methanol. HPLC analysis showed 368mg/g of thymoquinone in hexane fraction and 658mg/g in 15%diethylether in hexane fraction. Supercritical fluid carbondioxide extraction (SCFE-CO_2) of *Nigella sativa* oil at 150 bar, 40°C, 120 min produced 4.09mg of thymoquinone per ml of CO_2 extract as reported by Solati.

Experimental Aspects

Solvents and reagents were procured from SD Fine Chemicals Limited and Standard Sample of TQ was supplied by Aldrich, India. Oil of *Nigella sativa* was obtained fromTotawala hakeem, Pune, India. All the solvents used were purified by procedures described in Vogel's Textbook of Practical Organic Chemistry. TLC was checked on Pre-Coated Silica Gel 60 G254 plates from Merck India Limited. HPLC grade methanol was used for HPLC experiment without further purification. Column chromatography was used for purification of compounds with petroleum ether and ethyl acetate as solvents.

NMR spectrscopy:

^{1}HNMR and ^{13}CNMR spectrum were determined by using NMR spectroscopy.

RESULTS:

Antibacterial and Antifungal activities:

This study reports the antibacterial and antifungal activities of 7 concentrations of *Nigella sativa* against *S. aureus and P. vulgaris, C.albicans, A. flavus*. The results of the antibacterial and antifungal activities of the investigated extract are shown in tables below. (table 1, 2, 3, 4).

Table 1*:* Zone of inhibition for *Staphylococcus aureus* in mm of diameter.

Extract Concentration (µg/ml)	Diameter of Zone of Inhibition(mm)
140	17
120	15
100	12
80	11
60	9
40	No zone of inhibition
20	No zone of inhibition

Table 2: Zone of inhibition for *Proteus vulgaris* in mm of diameter.

Extract Concentration (µg/ml)	Diameter of Zone of Inhibition(mm)
140	19
120	16
100	14
80	13
60	10
40	No zone of inhibition
20	No zone of inhibition

Table 3: Zone of inhibition for *Aspergillus flavus* in mm of diameter.

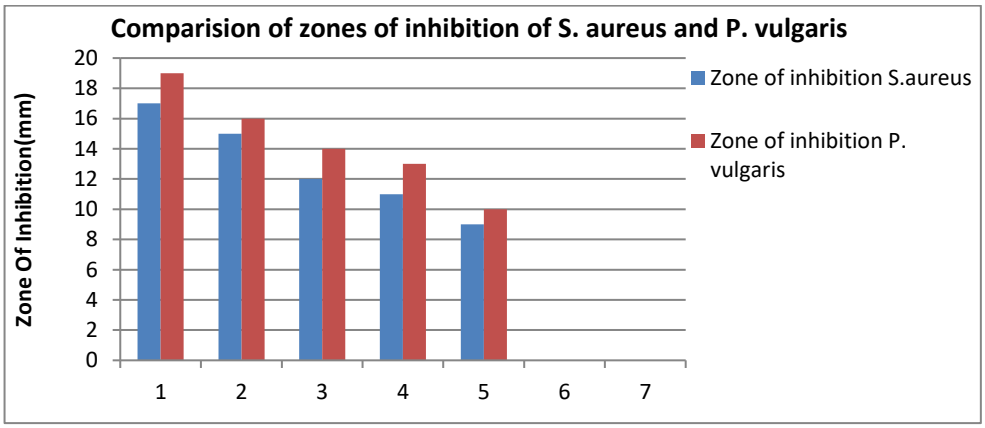
Extract Concentration (µg/ml)	Diameter of Zone of Inhibition(mm)
140	22
120	19
100	17
80	16
60	15
40	10
20	No zone of inhibition

Table 4: Zone of inhibition for *Candida albicans* in mm of diameter.

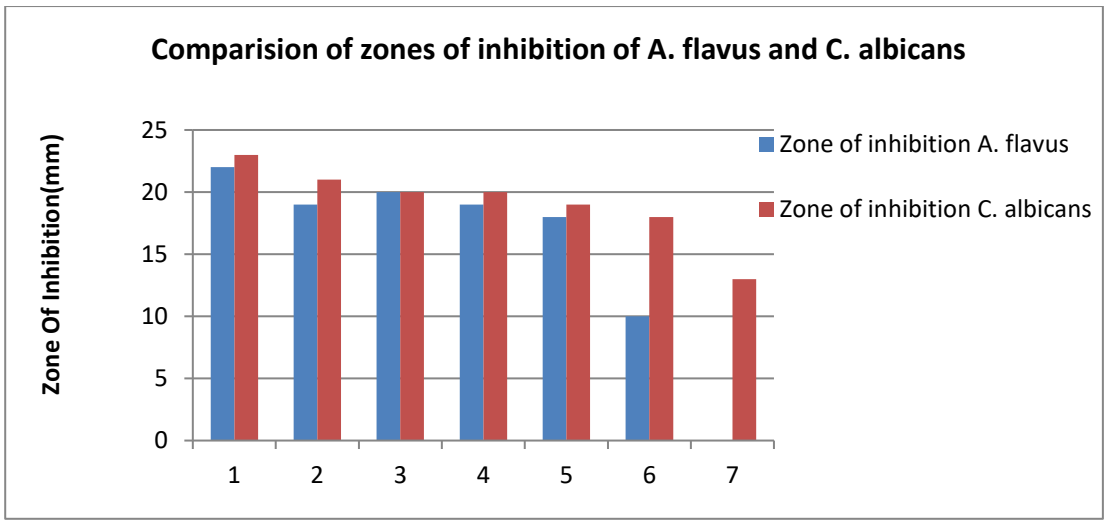
Extract Concentration (µg/ml)	Diameter of Zone of Inhibition(mm)
140	23
120	21
100	20
80	20
60	19
40	18
20	13

Table 5: The methanol extract was tested separately against *Nocardia brasiliensis* and the results are shown below

Organism	Extract	Concentrations%										
		0.05	1	2	3	4	5	6	7	8	9	10
Nocardia brasiliensis	Methanol (After 8 days)	+	+	-	-	-	-	-	-	-	-	-
	Methanol after 30 days	+	+	+	+	-	-	-	-	-	-	-



Graph 1: Effect of different concentrations of *Nigella sativa* extract on *S.aureus and P. vulgaris*



Graph 2: Effect of different concentrations of *Nigella sativa* extract on *A. flavus and C. albicans*

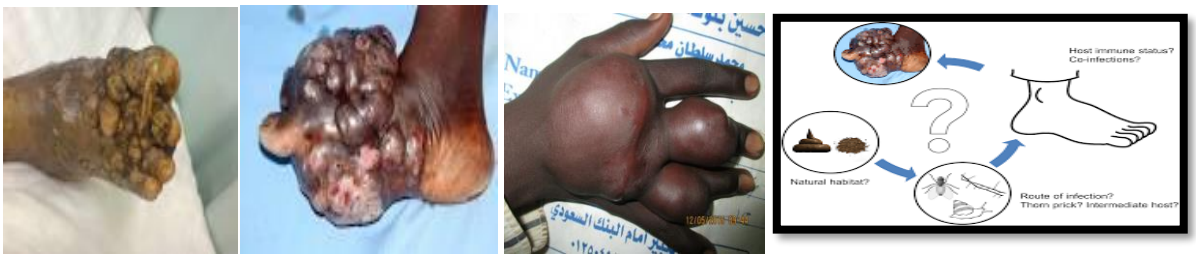


Fig 1: Mycetoma infections and their routes

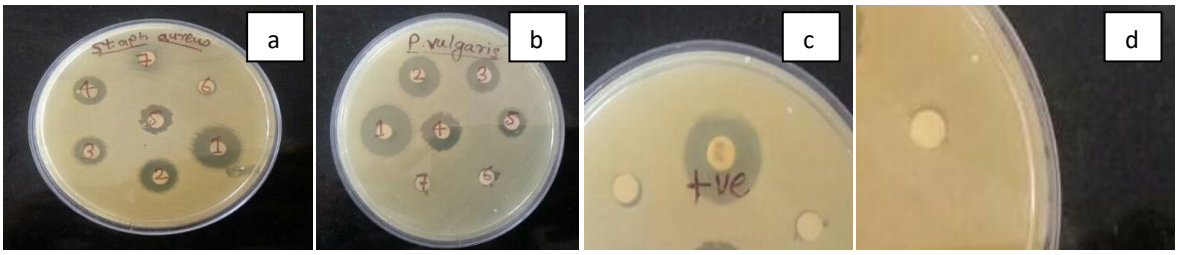


Fig 2: Antibacterial activity of methanol extract of *N. sativa* seeds. Different concentrations of extract were tested for antibacterial activity against*(a) S.aureus ,(b) P. vulgaris,* (c) positive control(Tetracycline), (d) negative control(DMSO)

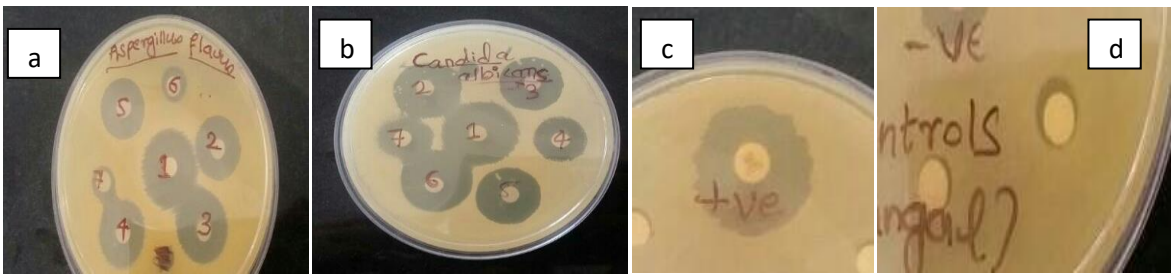


Fig 3: Antifungal activity of methanol extract of *N. sativa* seeds. Different concentrations of extract were tested for antifungal activity against*(a)C. albicans,(b)A. flavus*, (c)Positive control(clotrimazole), (d) negative control(DMSO)

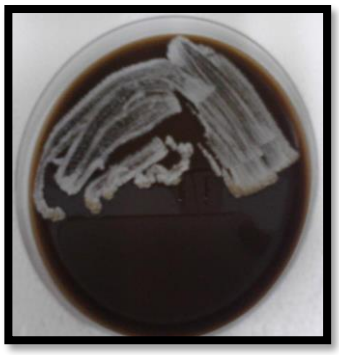


Fig 4: *Nocardia brasiliensis* subculture after 14 days

Fig 5: *Nocardia brasiliensis* subculture after 23 days

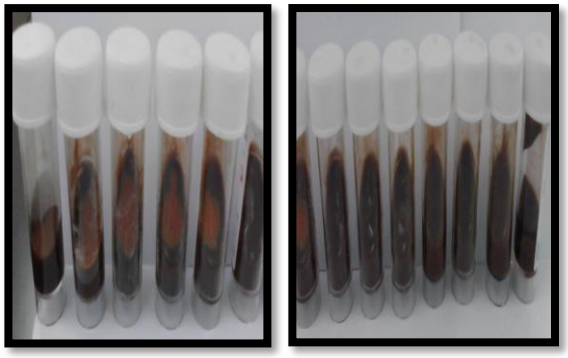


Fig 6: Culture of methanol extracts tested against *Nocardia brasiliensis*

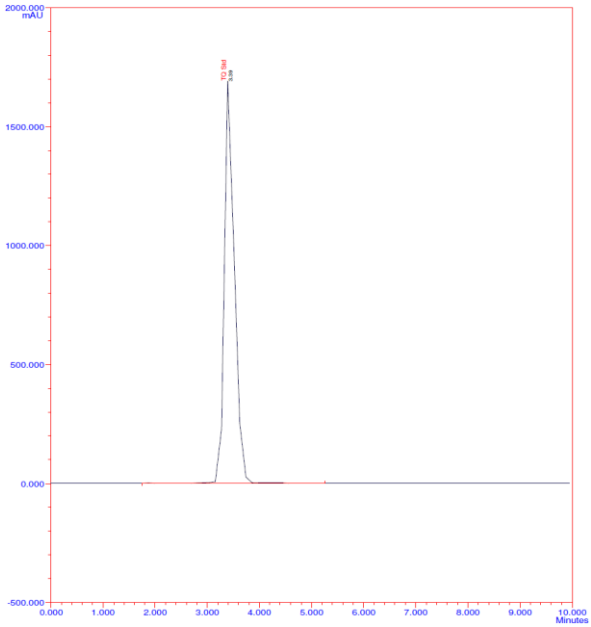


Fig 7: HPLC of Standard TQ Sample (Aldrich)

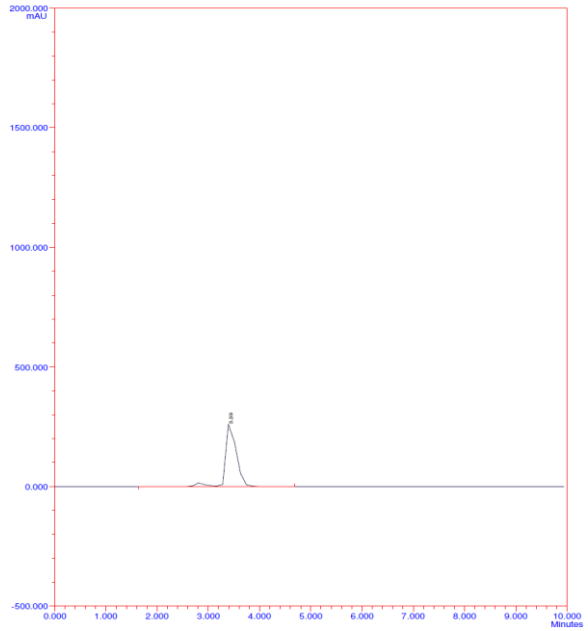


Fig 8: HPLC of TQ Fraction after Isolation and Purification

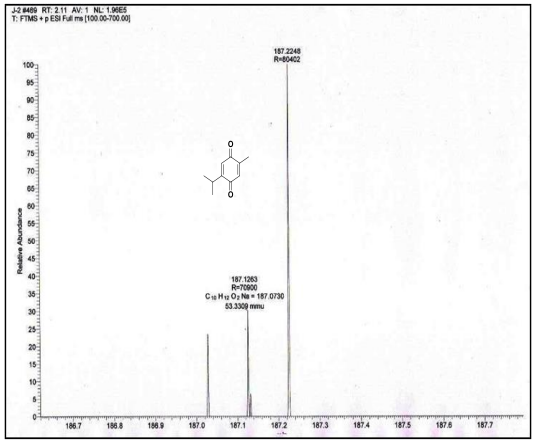


Fig 9: High Resolution Mass Spectrum of Isolated TQ

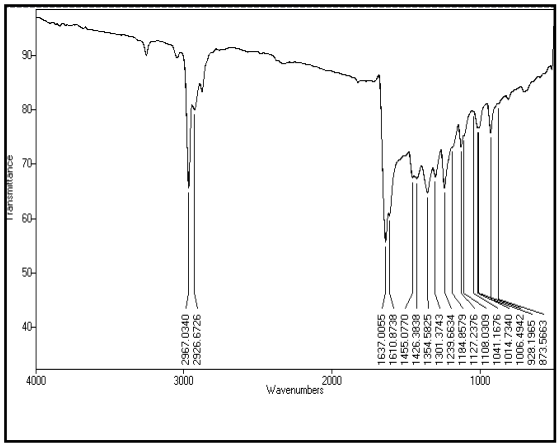


Fig 10: Infra Red Spectrum of TQ

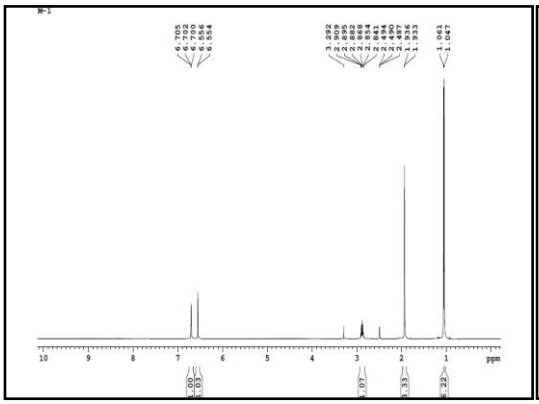


Fig 11: ^{1}HNMR Spectrum of TQ

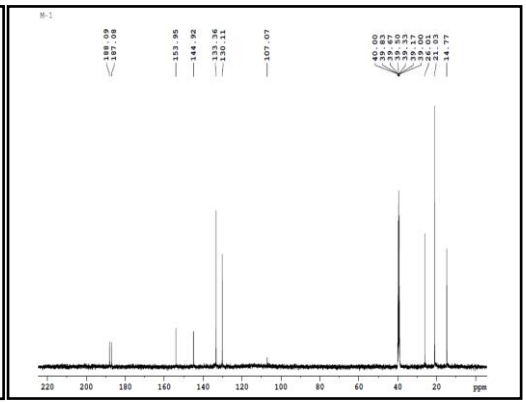


Fig 12: ^{13}CNMR Spectrum of TQ

Cultures of ***Nocardia brasiliensis*** treated with methanolic extract showed inhibition of growth with oil concentrations of 4%. (table 5)

HPLC analysis:

Retention time of 3.39 minutes shown by purified column fraction matched with that of standard TQ sample (Figure). The weight of TQ obtained from column was 1.03g after drying. Thus, the w/w percentage of TQ obtained from *Nigella sativa* oil sample was found to be 20.6%.

NMR spectroscopy:

Thymoquinone

2-isopropyl-5-methylcyclohexa-2,5-diene-1,4-dione

FTMS of **TQ** shows two peaks of m/z 187.07 and 187.22. m/z peak of 187.22 with 100% relative abundance appears as peak of sodiated adduct of TQ $[TQ\text{-}Na]^{+}$ where expected m/z peak of 164.20 appears with added ^{23}Na at 187.22.

^{1}HNMR spectrumof TQ shows first peakas adoublet at 1.06δ which belongs to two methyl groups of iso-propyl moeity present at second position of quinone ring, with splitting constant of 7Hz (fig 11). One hydrogen of the same moeity appears as multiplet due to neighbouring methyl groups at 2.86 δ and 7 Hz splitting constant. Methyl group at fifth position of quione ring appears as a singlet at 1.9δ. Hydrogen at third position appear at 6.5 δ and hydrogen at sixth position appears slightly downfield with shift of 6.7 δ as singlet which appear to be in agreement with the reported value of ^{1}HNMR by earlier group, which used advance NMR techniques like Two-Dimensional HeteronuclearSingle Quantum Coherence Transfer Spectra (2D HSQCT) on Bruker Avance AQS 500 MHz[21]. ^{13}C NMR of TQ showed aliphatic carbon atoms at shielded positions. Methyl group at fifth position of quionone ring appears at 26.01 δ, methyl groups of iso-propyl moeity appear at 14.77 δ with –CH appearing at 21.03 δ(fig 12). Doubly bonded carbon atoms of quinone ring appear in deshielded region with carbon bearing methyl group appearing at 144.92 δ and its neighbouring –CH appearing at 133.36 δ. Carbon bearing iso-propyl group appearing at 153.95 δ and its neighbouring -CH at 130.1 δ .Carbonyl carbon at fourth position appears 188.09 δ (188.3 δ reported) and carbonyl carbon at first position appears at 187.08 δ. The chemical shifts of two carbon pair can not be atributed with certanity , IR spectrum shows C=O peak

at 1637cm^{-1} with a shoulder and C-H streching of methyl groups at 2967cm^{-1}.

DISCUSSION:

The study shows a better alternative of common antibiotic drugs may be folk medicines. *Nigella sativa* is traditional medicine used from a very long time. Due to overuse of common antibiotics various cases of antibiotic resistance in microbes occurred. A large number of populations depends on traditional medicines for cure from various microbial diseases. In our study we found *Nigella sativa* possess a very good antibacterial and antifungal response as compared to various common antibiotics. It will be better for the mankind to rely on these traditional plants as they have very least side effect as compared to various antibiotics and chances of antibiotic resistance due to overuse of antibiotics may be diminished.

From disc diffusion method it is observed that the plant extract shows significant inhibition of bacteria and fungi. Among them *Nigella sativa* shows better activity against *P. vulgais and Candida albicance*.

Nigella sativa has more bacterial activity against *P. vulgaris* than *S. aureus* and more fungal activity against *Candida albicance* than *A. flavus.*This finding seems to be in agreement with previously reported studies[9].

Nigella sativa essential oil have stronger antibacterial properties compared to Tetracycline (graph 1) and antifungal properties compared to Clotrimazol. (graph 2). These results are similar to previous study on kalonji [10]

The antibacterial activity is mainly due to the methanol part of *N.* as thymol and thymoquinone [11]. These are present in the soluble portion of the methanol essential oil of N. sativa [12].

Agarwal *et al*[13] who reported that the oil inhibited one strain of S. aureus even upto 1:100 dilution, the least concentration tested. However, the zones of inhibition observed in our study were small at least concentration which may be due to difference of the strains tested. The authors also reported activity of oil against E. coli. This may be because of the reason that *N. sativa* obtained from different commercial sources or isolated by different methods from the same seeds have been shown to vary significantly in their content of Thymoquinone, which has antibacterial activity and various storage conditions are expected to make a difference in the amounts of the quinone constituents of the oil, especially if the seed oil samples are exposed to heat and light. Secondly, variability in the performance of Mueller- Hinton agars from different manufacturers has been shown to be statistically significant. The size of inoculums used, depth of medium in the plates, inoculation technique and time period between inoculation and application of discs, incubation temperature and time of incubation will also cause differences in the results obtained.

Although plants have been traditionally used as local remedies against various infectious diseases, their activity against mycetoma causative agents have been hardly studied. Only one report appeared to literature which studied the in vitro Activity of plant extracts such as artisinin and tea tree oil on *Madurella mycetomatis* and another study was reported on In vitro antifungal activity of Sudanese Medicinal Plants against *Madurella mycetomatis*[1] but in our study organism is *Nocardia brasiliensis* which is done for the first time.

Data obtained from this study suggest that *Nigella sativa* may be used for treatment of infections alone or in combination with some antibiotics especially in case of the bacteria *P. vulgaris* and fungi *C. albicance* (graph 1, 2). And it has been used for a long time as food without any serious toxicity reported, it can be considered as a safe and affordable treatment for mycetoma.

CONCLUSION:

It may be concluded from this study that *N.* seed extract has antibacterial and antifungal and antimycetoma activities against *Staphylococcus aureus, Proteus vulgaris, Aspergillus flavus, Candida Albicans, Nocardia brasiliensis* and proved more active than standard antibacterial and antifungal drugs which are Tetracyclin and Clotrimazole and Sulphonamides which is used for mycetoma treatment. It is expected that using natural products as therapeutic agents will probably not elicit resistance in microorganisms. It is essential that research should continue to isolate and purify the active components of this natural herb and use in experimental animals. Finally, in vivo testing is necessary to complete this study.

ACKNOWLEDGEMENTS:

We would like to thank the department of Microbiology, Abeda Inmadar Senior College, Pune and acknowledge Sanjeevan hospital, Pune and also Mr. Mujahid Yusufi and Ms. Priti Singh (Chemistry Dept.) for their helpful and insightful discussions.

REFERENCES:

1. Fahal, AH. 2011 Review Mycetoma.Khartoum Medical Journal Vol. 04, No. 01, pp. 514 - 523 514.
2. Mahgoub, E. S., and S. A. Gumaa. (1984). Ketoconazole in the treatment of eumycetoma due to Madurella mycetomii.Trans R Soc Trop Med Hyg. , 78:376-9.
3. Ahmad A, Husain A, Mujeeb M, Alam SK, Najmi AK, Damanhouri ZA (2013) . A review on therapeutic potential of *Nigella sativa:* A miracle herb. Asian Pac J Trop Biomed, 3(5): 337-352**.**
4. Fahal, A. H. 2004. Mycetoma: a thorn in the flesh. Trans. R. Soc. Trop. Med. Hyg. 98:3-11.

5. Mahgoub E. S. 1994. Medical treatment of mycetoma in the Sudan. Sudan. Medical Journal.; 32 (supp): 88-97.
6. Kemper, C.A. (2000) Eumycetoma. Curr. Treatment Options Infect. Dis. Kinderlerer, J.L. 2, 533-538.
7. Pang KR, Subcutaneous fungal infections. Dermatol Ther 2004; 17:523
8. McGinnis, M. R. 1980.Laboratory Handbook of Medical Mycology.Academic Press, London, N.Y.
9. Rajashri R. Thigle, Sachin A. Nitave, (2014). Antifungal activity of *Nigella sativa and Lawsonia inermis* and its comparative study. World J. Pha. Res. 3(4), 1468-1472.
10. E. Haloci, S, Manfredini, V. Teska,(2012). Antibacterial and Antifungal Activity Assesment
11. Of *Nigella sativa* Essential Oils. International Science Index, Pharmacological and Pharmaceutical Sciences, waset, 6(6)14703.
12. Randhawa MA. Al Ghamdi MJ (2002). A review of pharmacotherapeutic effects of *Nigella sativa.* Pak. J. Med. Res. 41(2):77-83.
13. Abou Basha LI, Rashid MS, Aboul EHY (1995). TLC assay of thymoquinonem black seed oil (*Nigella sativa* Lim) and identification of dethymoquinon and thymol. J. Liq. Chromatogr. 18(1):105-115.
14. Agarwal R, Kharya MD, Shrivastava R. (1979) Antimicrobial and anthelminthic activities of
15. the essential oil of *Nigella sativa* Linn. Indian J Exp Biol 17:1264–5.