

Full Length Article

Antifungal Activity of **Vegetative** Methanolic Extracts of *Nigella sativa* L. against *Fusarium oxysporum* and *Macrophomina phaseolina* and its Phytochemical Profiling by GC-MS Analysis

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Abstract

This study was conducted to evaluate the antifungal activity of **vegetative parts** of *Nigella sativa* L. against soil-borne fungal species *Fusarium oxysporum* and *Macrophomina phaseolina*. *N. sativa* seeds were sown under field conditions. The plants at their vegetative stage were collected and dried at room temperature. The plant material was crushed, sieved and extracted in methanol through maceration. After rotary evaporation of methanol the extract was partitioned by using different solvents as chloroform, n-hexane, ethyl acetate and n-butanol. The extracted fractions were assayed for their antifungal activity against two soil-borne fungal species namely *F. oxysporum* and *M. phaseolina* collected from First Fungal Culture Bank, IAGS, University of the Punjab, Lahore. Different concentrations viz. 1.56, 3.13, 6.25, 12.5, 25, 50, 100 and 200 mg mL⁻¹ were used for antifungal bioassays. Ethyl acetate extract completely inhibited the fungal biomass production of both fungal strains at 50 mg mL⁻¹ concentration followed by n-butanol extract and n-hexane extract. Fungicidal behavior of all extracts was directly proportional to concentrations. Aqueous extract could reduce fungal biomass up to 21%. Phytochemical profile of ethyl acetate, n-butanol and n-hexane fractions was generated through GCMS analysis. The presence of Octadecadienoic acid, Pentadecanoic acid, 1,2,3,4, butanetetrol, octadecanoic acid and Linoleic acid in phytochemical profile is the confirmation for antifungal activity of *N. sativa*. In conclusion, *N. sativa* has significant fungicidal potential and can be used as natural environment friendly fungi-toxicant against *F. oxysporum* and *M. phaseolina*. © 2019 Friends Science Publishers

Keywords: Black seed; Fungal biomass; GCMS analysis; Octadecadienoic acid; Phytochemical compounds

Introduction

Plants have always been an ironic source of medicaments either in traditional decoction methods or isolated pure compounds as potent drugs (Ambikapathy *et al.*, 2011). The use of medicinal plants has been very frequent for extraction of medicinally active ingredients to use as antibiotics, laxatives, blood purifiers and antimalarial agents (Mustafa *et al.*, 2018). Plants are rich source of secondary metabolites with strong defensive properties (Arif, 2009). These secondary metabolites could be classified as alkaloids, terpenoids, saponins, cardiac glycosides, alkaloids, cyanogenic glycosides, monoterpenes, diterpenes, triterpenes, tetraterpenes, sesquiterpenes and coumarins (Hussain *et al.*, 2015). In spite of having therapeutic properties, phytochemicals show great tendency towards microbial growth inhibition (Abushaala *et al.*, 2017). The possible modes of action of secondary metabolites could be inhibition of fungal cell wall formation, disruption of cell wall, malfunctioning of fungal mitochondrion and inhibition

of fungal protein synthesis (Freiesleben and Jager, 2014). A large number of plant species have been reported to possess natural substances that are lethal to fungal pathogens and have caused severe losses to agroforestry from centuries (Chohan and Perveen, 2015).

N. sativa of family ranunculaceae is commonly known as black seed. It is native to Middle East, South East Asia and Egypt and historically in use for medicinal and preservative purposes (Ali and Blunden, 2003). Phytochemical profile of *N. sativa* includes vitamins, minerals, fats, carbohydrates, thymol, carvacrol, alpha and beta pinenes, hexadecanoic acids, stearic acid, oleic acid, transanethole, apiole, thymoquinone, terpenoids, tannins, flavonoids and phloba tannins (Aftab *et al.*, 2018). Traditionally it has been used to treat asthma, headache, dermal problems, cancer, malarial infection, hypertensive and hypoglycemic states (Aftab *et al.*, 2018). The plant is used to dispel parasites and worms from intestinal tract and is useful for bronchitis soothing and coughs (Chakravarty, 1993).

The fungi are mostly intracellular pathogens and survive inside the host cell. They make an interaction between the host and the assaulting species. The pathogens infect both plant and animal cells in apparently inimitable ways and the survival of host cells is quite challenging (Gladieux and Giraud, 2017). There are some undeniable benefits of using chemicals under modern agriculture as yields increase, pest control and crop production within no time (Dayan *et al.*, 2009) with some hazardous health effects. Due to the continuous advancement in agronomy, it is needed to introduce natural fungicides and pesticides to compete resistant pathogenic varieties (Nascimento *et al.*, 2000; Stevic *et al.*, 2017). Ascomycetes *F. oxysporum* of nectriaceae is one of the most deteriorating disparaging fungi. It is soil borne fungus with wide distribution in temperate and tropical regions. The pathogenic isolates of *F. oxysporum* cause wilt in various agricultural crops. It has saprophytic characteristics and degrades lignin. It is also opportunistic fungus to human beings and arthropods at some extent (Fourie *et al.*, 2009). *M. phaseolina* commonly known as Charcoal rot fungus belongs to family sphaerospidaceae. It mostly affects roots and lower stem portions. This type of fungal infection spreads through seed and soil. It causes major economic loss by reducing oil contents at seed maturity stage (Syed *et al.*, 2015). In Pakistan 67 susceptible crops of *M. phaseolina* have been reported (Khan, 2007). It is non-host specific fungus and causes various diseases like root rot, seedling blight, stem rot, damping off and basal stem rot diseases (Khan, 2007).

Phytopathogenic fungal infections are generally controlled by synthetic fungicides. However, the extensive utilization of synthetic fungicides showed hazardous effects on environment and human health (Harris *et al.*, 2001). There is always increasing production and regulation demand on the use of agro-chemicals resultantly the more resistant pathogens emerge against the employed products. Hence, there is an urge to explore novel natural fungicides with lesser side effects (Dellavalle *et al.*, 2011). Currently, a huge amount of data is being reported on the pharmaceutical properties of plants as Taxol is synthesized from *Taxus brevifolia* (antitumor), Vinblastine from *Catharanthus roseus* (anticancer), Silymarin from *Silybum marianum* (liver tonic, anticancer and anti-inflammatory) (Cowan, 1999; Shakya, 2016). Seed and oil contents of *N. sativa* are being consumed for healing skin infections (Shokri, 2016) however, researchers yet to explore the vegetative parts of *N. sativa* for antifungal activity.

It is a climate sensitive and harsh crop to cultivate, therefore production of seed according to the consumer need is hard to achieve. Thus present study focused on exploration of its vegetative part as an alternative of seed and oil to overcome and compete with soil-borne fungal pathogens *F. oxysporum* and *M. phaseolina*. Their GCMS analysis was also done to identify natural environment friendly fungicides present in it.

Materials and Methods

Collection of Plant Material

N. sativa, seeds were collected from gene bank of Plant Genetic Resource Institute (PGRI), National Agricultural Research Centre (NARC) Islamabad, Pakistan. Germplasm was planted in the month of November at Botanic Garden, Lahore College for Women University, Lahore (31.45° N and 74.39° E). Soil was mixed with farm yard manure and prepared within pH range 5-8. The watering of soil was done on daily basis to maintain the normal growth of the plant in the green house. The temperature of soil ranged from 20-25°C. The plant to plant distance was kept 20cm whereas row distance was maintained 30 cm (Ahmad and Ghafoor, 2003). The plant material was collected in April. Later on it was washed, dried at room temperature, converted into powder form and sieved for extraction.

Plant Extraction

The powdered plant material (2.5 kg) was soaked in methanol and filtered after two weeks. The extract was filtered and evaporated on a rotary evaporator under vacuum (350 Psi⁻¹) at 45°C. The crude methanolic extract was collected. This extract was thoroughly dissolved in 500 mL distilled water. The dissolution was later on mixed with *n*-hexane (500 mL) and complete separation of aqueous and *n*-hexane fractions was done by separating funnel thrice. Approximately 6.4 g of *n*-hexane was collected after complete evaporation of the filtrate. The partitioning of chloroform, ethyl acetate and *n*-butanol was done serially through aqueous phase by using 500 mL of each of chloroform, ethyl acetate and *n*-butanol. The collected yield by evaporating all the fractions was 2.7 g chloroform, 3.2 g ethyl acetate, and 3.8 g *n*-butanol. Finally, the aqueous fraction was evaporated to get gummy mass (1.9 g) under reduced pressure (350 Psi⁻¹) (Javaid and Rauf, 2015).

Culture Collection

The fungal strains *F. oxysporum* f. sp. *cepae* (ACC# FCBP-PTF0021) and *M. phaseolina* (ACC# FCBP-PTF814) were collected from Fungal Culture Bank of Pakistan (FCBP), Institute of Agricultural Sciences, University of the Punjab Lahore, Pakistan.

Laboratory Bio Assays

Antifungal activity of all the collected fractions from methanolic extract was evaluated against the freshly revived targeted fungal species *F. oxysporum* and *M. phaseolina* as follows. Measured quantity (1.2 g) of all the fractioned and dried extracts were dissolved per mL of dimethyl sulphoxide (DMSO) and 5 mL of malt extract broth was added to it for preparing 200 mg mL⁻¹ solution. The stock

solution and malt extracts were mixed proportionally to prepare 6 mL of each of the lower concentrations of 100, 50, 25, 12.5, 6.25, 3.125 and 1.56 mg mL⁻¹. The same procedure was opted for control treatments (1 mL DMSO in 5 mL malt extract broth) through serial double dilution against all the experimental ones. Bioassays were accompanied in 10 mL volume glass test tubes each having 1 mL medium. The inoculation was done by adding one drop of *F. oxysporum* and *M. phaseolina* spores aseptically under separate treatment. Each treatment was prepared in triplicate fashion. The whole setup was incubated for 7 days at 37°C. After 7 days there was very clear fungal mycelial growth in the test tubes. The fungal biomass in each test tube was collected by filtration on filter papers and dried at 60°C. The fungal biomass production was measured by weighing filter paper and growth reduction according to concentrations was recorded (Sattar *et al.*, 2018).

Statistical Analysis

All the collected data were analyzed using analysis of variance (ANOVA) technique to evaluate overall significance followed by LSD test at 5% probability level to compare treatment means using computer software Statistix 8.1.

GC-MS Analysis

GC-MS analysis of ethyl acetate and *n*-butanol fractions isolated from vegetative parts of methanol extract was done by following the methodology of Derwich *et al.* (2010). For ethyl acetate fraction trace GC (ULTRA S/N 20062969, Thermo Fischer) equipped with HP-5MS non polar fused silica capillary column (30 m x 0.32 mm, film thickness 0.25 µm) was used. The operating conditions were maintained like oven temperature program from 50°C (2 min) to 260°C at 4°C/min heating rate and held for 2 min, split injection ratio 1:20, carrier gas helium, flow rate 1 mL min⁻¹, temperature of injector and detector (FID) fixed at 260°C and 280°C, respectively. The constituents were identified depending upon their Kovats Index, calculated in relation to the retention time as reference products, in comparison with those of the chemical compounds gathered by the similarity of their mass spectra with those gathered in the NIST-MS library, or reported in the literature.

Results

The analysis of variance showed the significant results for antifungal potential of vegetative parts of *N. sativa* against *F. oxysporum* and *M. phaseolina*. Ethyl acetate extract completely inhibited fungal biomass production at 50 mg mL⁻¹ in both fungal strains, whereas chloroform *n*-hexane and *n*-butanol extract inhibited fungal biomass production up to 88%, 78% and 76%, respectively at 50 mg mL⁻¹ concentration in case of *F. oxysporum* (Fig. 1). Both chloroform and ethyl acetate extract showed complete

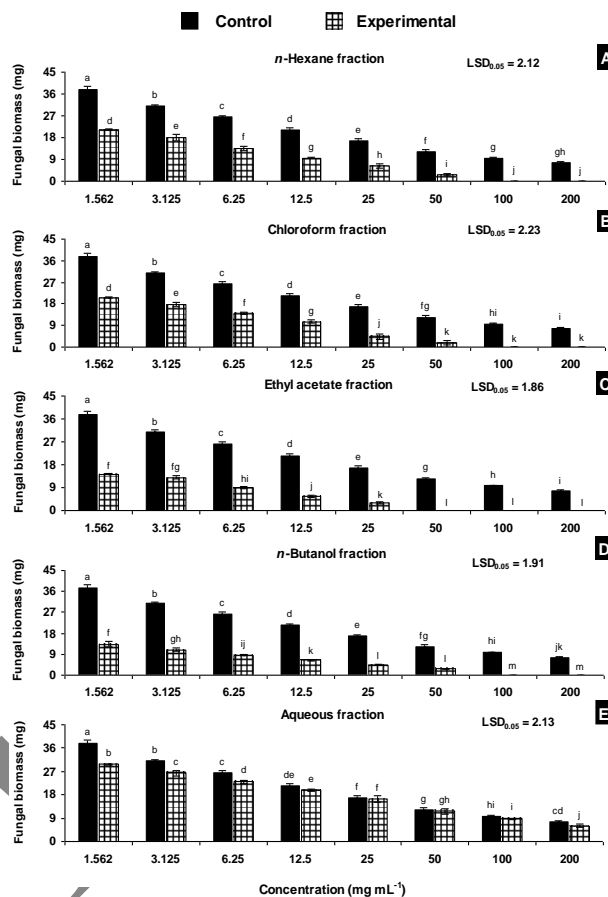


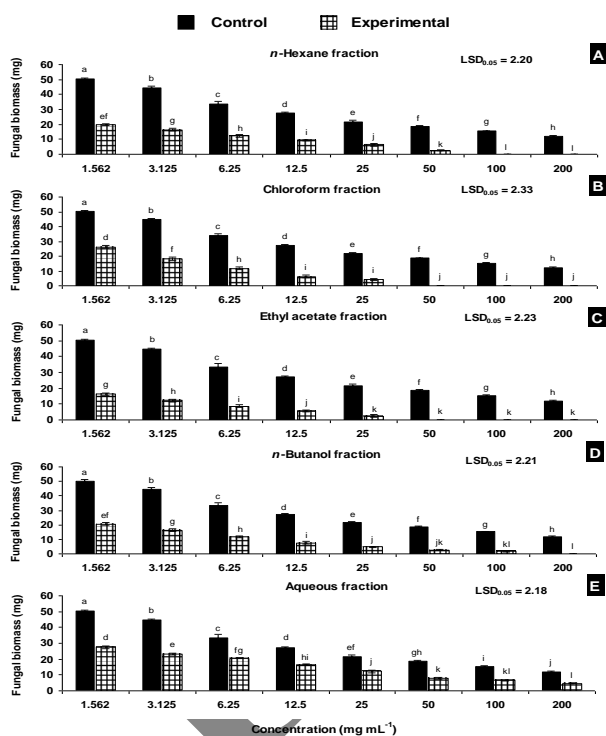
Fig. 1A, B, C, D and E: Effect of different concentrations of sub-fractions of methanolic extract of vegetative parts of *Nigella sativa* on growth of *Fusarium oxysporum* f. sp. *cepae*. Vertical bars show standard errors of means of three replicates. Values with different letters at their top show significant difference ($P \leq 0.05$) as determined by LSD Test

inhibition of *M. phaseolina* at 50 mg mL⁻¹ whereas *n*-butanol and *n*-hexane extract showed 86% inhibition at 50 mg mL⁻¹, whereas aqueous extract worked very poorly for fungal biomass reduction against *F. oxysporum* i.e., 5% at 50 mg mL⁻¹. The fungal biomass reduction showed variable trend from 25-1.56 mg mL⁻¹ for all the extracts i.e., *n*-hexane showed 62-43% reduction, chloroform 76-45%, ethyl acetate 82-62%, *n*-butanol 74-65% and aqueous being very mild as 5-12% as indicated in Table 1. In case of *M. phaseolina* the fungal biomass reduction from 25-1.65 mg mL⁻¹ was noted as *n*-hexane 71-60%, chloroform 82-48%, ethyl acetate 89-67%, *n*-butanol 78-59% and at last aqueous with 43-35% as presented in Fig. 2.

The mass spectrometer analyzes the compounds eluted at different times to identify the nature and structure of the compounds. GCMS analysis of *n*-butanol fraction indicated the presence of five compounds as Pentadecanoic acid, Octadecadienoic acid, 6, Pentadecen-1-ol, 9, 12-Octadecadiene-1-ol and Di-*n*-octyl phthalate as mentioned

Table 1: Fungal biomass reduction in different concentrations of various fractions of methanolic extract of vegetative parts of *N. sativa*

Concentration (mg mL ⁻¹)	Reduction in fungal biomass over control (%)				
	<i>n</i> -Hexane	Chloroform	Ethyl acetate	<i>n</i> -Butanol	Aqueous
<i>Fusarium oxysporum</i> f. sp. <i>Cepae</i>					
1.562	43	45	62	65	12
3.125	42	43	58	65	15
6.25	49	47	66	67	13
12.50	58	50	73	70	8
25	62	76	82	74	2
50	78	88	100	76	5
100	100	100	100	100	10
200	100	100	100	100	22
<i>Macrophomina phaseolina</i>					
1.562	60	48	67	59	35
3.125	63	59	72	63	38
6.25	63	65	74	64	38
12.50	66	77	79	72	39
25	71	82	89	78	43
50	86	100	100	86	57
100	100	100	100	89	57
200	100	100	100	100	61

**Fig. 2A, B, C, D and E:** Effect of different concentrations of fractions of methanolic extract of vegetative parts of *Nigella sativa* on growth of *Macrophomina phaseolina*. Vertical bars show standard errors of means of three replicates. Values with different letters at their top show significant difference ($P \leq 0.05$) as determined by LSD Test

in Table 2, Fig. 3. GCMS analysis of ethyl acetate extract showed the presence of 1,2,3,4-Butanetetrol, 2-Dodecenal, Pentadecanoic acid, 10-Octadecanoic acid and Stearic acid (Table 3, Fig. 4). The *n*-hexane fraction indicated the

presence of nine compounds named Palmitic acid, Hexadecanoic acid, Octadecadienoic acid, Linolenic acid, Octadecenoic acid, Octadecanoic acid, Eicosadienoic acid, Glycerol linoleate and Phthalic acid (Table 4, Fig. 5).

Discussion

The antifungal property of *N. sativa* vegetative parts has not been reported prior to this study. *N. sativa* has been documented as a treasure house for bulk of phytoconstituents including quinones, phenols, alkaloids, acids, fats, glycosides, tannins, vitamins and fibres. Quinones and phenols have strong antimicrobial properties (Martines and Benito, 2005). Plant extracts and their essential oils have been reported as strong fungicidal against food and soil borne fungi (Zahirul *et al.*, 2011). In this study it had been noticed that *n*-butanol extract at 1.56 mg mL⁻¹ suppressed the growth of *F. oxysporum* at maximum in comparison to hexane, chloroform and distilled water extract. Isaac and Abu-Tahon (2014) made similar observations that butanolic extract of *Ocimum basilicum* reduced the growth of *F. oxysporum* maximum at the concentration of 1.5-2.0 mg mL⁻¹. Moreover, butanol fraction from methanolic extract showed its significant inhibitory efficiency and its GCMS analysis indicated the presence of five compounds as Pentadecanoic acid, Octadecadienoic acid, 6, Pentadecen-1-ol, 9,12-Octadecadiene-1-ol and Di-*n*-octyl phthalate (Table 2). Very similar and profound results were observed in work of Javaid and Akhtar (2015). They noticed the maximum growth suppression in *n*-hexane, chloroform and ethyl acetate fractions as 46–79%, 40–73% and 35–76%. Phenolic compounds are generally extracted in *n*-hexane. The most active phytochemicals of *N. sativa* are thymoquinone, thymohydroquinone and thymol are phenolic in nature. Previously *n*-hexane extraction of seeds has been proved effective fungicide because of the presence of these phenolic compounds (Shokri, 2016). Therefore, it is assumed that the antifungal activity of *n*-hexane extracts of vegetative parts is because of phenolic compounds (Martines and Benito, 2005).

Recently Waheed *et al.* (2016) studied the antifungal potential of *Calotropis procera* and noticed the maximum biomass reduction in hexane extract against *M. phaseolina*. It had been observed that the higher antifungal potential of any extract is due to the presence of phenols and flavonoids in that extract (Banaras *et al.*, 2017). Overall trend showed that ethyl acetate extract had the maximum inhibition against both fungal strains. Higher antifungal activity of ethyl acetate extract of *N. sativa* was linked with presence of 1,2,3,4-butanol, 2-Dodecenal, Pentadecanoic acid, 10-Octadecanoic acid and Stearic acid (Table 3, Fig. 4). In another study it has been noticed that Alpha and beta pinenes are soluble in Ethyl acetate and thus exhibit antifungal property (Asdadi *et al.*, 2014).

Table 2: Phyto-constituents identified from *n*-butanolic fraction of *N. sativa* through GCMS

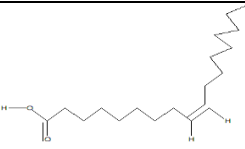
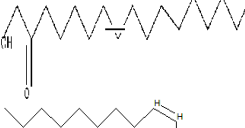
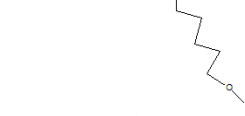
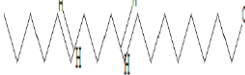
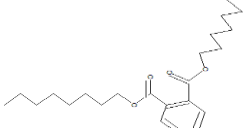
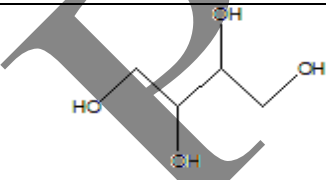
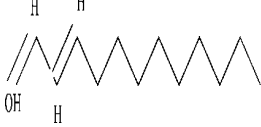
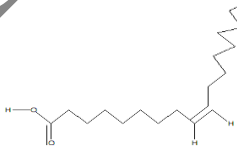
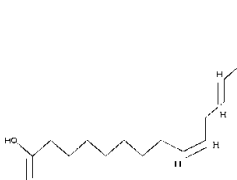
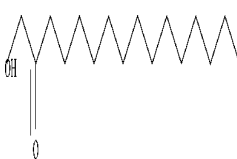
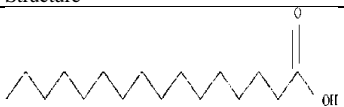
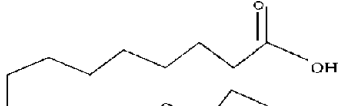
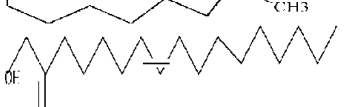

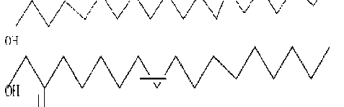


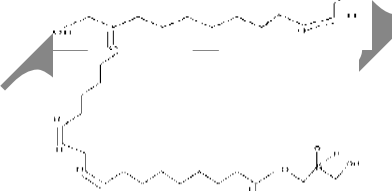
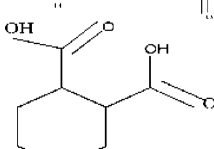
Sr. No.	Compound Name	Molecular Formula	Structure	Retention Time	Peak area (%)	Similarity Index	Mol. Mass
1	Penta decanoic acid	C ₁₇ H ₃₄ O ₂		10.9	11.52	95	270
2	Octadecadienoic acid	C ₁₉ H ₃₄ O ₂		11.8	68.47	96	294
3	6-Pentadecen-1-ol	C ₁₅ H ₃₀ O		12.0	7.73	88	226
4	9,12-Octadecadien-1-ol	C ₁₈ H ₃₁ ClO		13.1	8.65	91	298
5	Di-n-octyl phthalate	C ₂₄ H ₃₈ O ₄		13.9	3.64	92	390

Table 3: Phyto-constituents identified from ethyl acetate fraction of *N. sativa*

Sr. No.	Compound Name	Molecular Formula	Structure	Retention Time	Peak area (%)	Similarity Index	Mol. Mass
1	1,2,3,4-Butanetetrol	C ₄ H ₁₀ O ₄		3.11	34	89	122
2	2-Dodecenal	C ₁₂ H ₂₂ O		6.39	8.81	91	182
3	Pentadecanoic acid	C ₁₇ H ₃₄ O ₂		10.9	15.9	94	270
4	10-Octadecenoic acid	C ₁₉ H ₃₆ O ₂		11.8	30.5	92	296
5	Stearic Acid	C ₁₉ H ₃₈ O ₂		7.64	10	89	298

RT: Retention Time, SI: Similarity Index, MM: Molecular Mass

Table 4: Phyto-constituents identified from hexane fraction of *N. sativa* L

Sr. No.	Compound Name	Molecular Formula	Structure	Retention Time	%age Area	Similarity Index	Mol. Mass
1	Palmitic acid	C ₁₇ H ₃₄ O ₂		31.20	6.56	96	270
2	Hexadecanoic acid	C ₁₆ H ₃₂ O ₂		31.68	3.89	92	256
3	Octadecadienoic acid	C ₁₉ H ₃₄ O ₂		32.98	23.05	96	294
4	Linolenic acid	C ₁₉ H ₃₄ O ₂		33.04	14.65	96	294
5	Octadecenoic acid	C ₁₉ H ₃₆ O ₂		33.28	3.68	95	296
6	Octadecanoic acid	C ₁₉ H ₃₈ O ₂		33.58	35.61	95	298
7	Eicosadienoic acid	C ₂₁ H ₃₈ O ₂		35.44	1.29	92	322
8	Glyceryl linoleate	C ₂₁ H ₃₈ O ₄		35.68	8.96	89	354
9	Phthalic Acid	C ₁₆ H ₂₂ O ₄		40.08	2.05	95	278

More *et al.* (2016) observed the antifungal potential of *Syzygium cumini* against *F. oxysporum* and noticed that the ethyl acetate extract showed the maximum reduction in fungal growth similar to the findings of the present study. *N. sativa* is quite rich in saponins and it has been revealed that saponin containing plants and their extracts are potentially fungicidal because saponins are transient and hydrolysed by microbes to their corresponding sapogenin aglycones, which are aggressively toxic to microbes (Wallace, 2004).

The mass spectrometer analyzes the compounds eluted at different times to identify the nature and structure of the compounds. The larger compounds break into smaller ones by producing various peaks at variable retention times. Ethyl acetate and n-butanol extracts of vegetative parts showed the most significant results against both strains thus both extracts were checked for their chemical constituents

through GCMS analysis. *N. sativa* proteins have considerable effect on the fungal cell permeability (Shokri, 2016). The growing concern about food safety has recently led to the development of natural antimicrobials to control pathogens (Pundir and Jain, 2010). The phytochemicals derived from root, stem, leaves, fruits, flowers and seeds of medicinal plants include phenolic compounds, essential oils, proteins and antioxidants; that work together as biocontrol agents (Hussain *et al.*, 2014). The inhibition potential of plant extracts against the growth of microbes was attributed to the presence of antioxidants (Puupponen *et al.*, 2001).

Bioactive compounds are normally accumulated as secondary metabolites in the plant cells but their location, climate and growth phase affect it significantly (Karim *et al.*, 2017). These compounds are excessively found in leaves, thus preferred for therapeutic use.

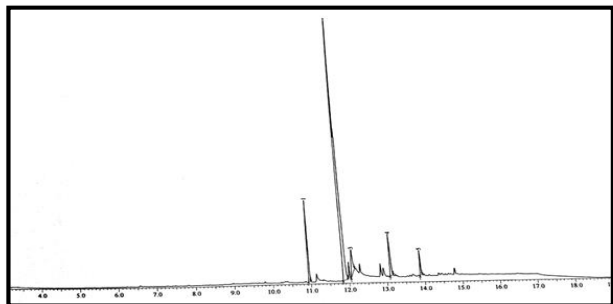


Fig. 3: GCMS spectrum of *n*-butanol fraction of *N. sativa*

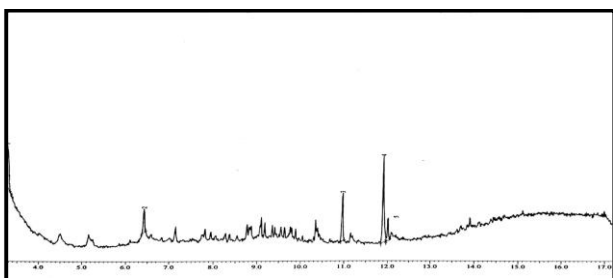


Fig. 4: GC-MS spectrum of Ethyl acetate fraction of *Nigella sativa*

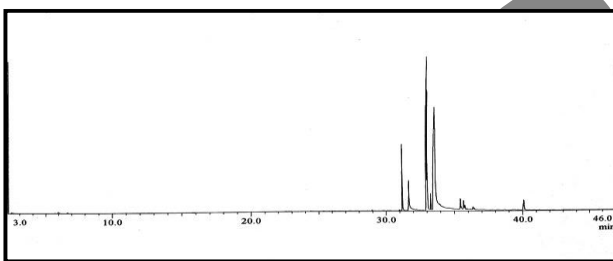


Fig. 5: GC-MS spectrum of Ethyl acetate fraction of *N. sativa*

The growth of disease causing microbes is inhibited by bioactive compounds, either singly or in combination (Jangra *et al.*, 2018). Both palmitic acid and linoleic acid exhibit antioxidant and antimicrobial properties (Mansour *et al.*, 2002). Furthermore, the minute quantities of phenolic acids, flavonoids, tannins and coumarins are reported to have antifungal properties (Mansour *et al.*, 2002). The plants could be source of novel bioactive principles, which can be exploited in the management of mycological disease problems. The natural bioactive compounds protect against fungal pathogens by affecting pathogens' physiology, morphology and ultrastructure or indirectly by promoting plant systemic resistance (Mohamed *et al.*, 2016).

Conclusion

In conclusion, *N. sativa* extracts had strong antifungal potential against pathogenic fungal strains in a concentration dependent manner. It possesses bioactive metabolites to use

as natural, cheap and environment friendly fungi toxicant. Therefore, it could be a potential source of natural nonhazardous antifungals.

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