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# *In Vitro* Callus Induction and Antimicrobial Activities of Callus and Seeds Extracts of Nigella Sativa L

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## Research Article

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**ABSTRACT**

In this study, extracts of Nigella sativa seeds and its induced callus were investigated for their antimicrobial activities against four standard bacteria (Bacillus subtilus, Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa) and two fungi (Candida albicans and Aspergillus niger) by using agar diffusion method.

To induce callus, hypocotyls and cotyledons explants from N. sativa were cultured in MS medium supplemented with different types and different concentrations of growth regulators. Explants of N. sativa showed a rapid rate of initiation of callus after two weeks when grown in MS media supplemented with NAA at 1.0 mg/l and 5.0 mg/l of NAA respectively, while a slow rate of induction of callus observed when the hypocotyls grown in MS media supplemented with 5.0 mg/l 2, 4-D and 0.5 mg/l 2, 4-D, when the explants were cotyledons. The NAA in this study was found to be the suitable hormone regulator for N. sativa for both types of explants used.

Methanolic extracts of seeds and callus of N. sativa showed activity against Escherichia coli with inhibition zone (21 mm) and (23 mm) respectively and no antifungal activity was observed for both seeds and callus extracts.

The antibacterial activity of Penicillin and Gentamicin were determined against the tested bacteria and compared with the antibacterial activity of the tested extracts of N. sativa seeds and callus. Methanolic extracts show antimicrobial activity against E. coli higher than that of Gentamicin and Penicillin at 10 µg/disc.

Phytochemical screening for the seeds and callus extracts indicated the presence of secondary metabolites such as alkaloids, flavonoids and tannins which may be responsible for the antimicrobial activity of the tested extracts.

## INTRODUCTION

Nigella sativa L. belongs to the family Ranunculaceae. It is an herbaceous plant, used for centuries for the treatment of various ailments including infectious diseases [1].

Researchers are increasingly turning their attention to natural products looking for new leads to develop better drugs against viral and microbial infections [2]. N. sativa seed and/or its constituent have been reported to demonstrate many pharmacological activities. The antioxidant, antibacterial and antifungal activities have been investigated by many researchers [3-9]. Seeds of N. sativa have a long history of use for food and medicinal purposes, no adverse or side effects have been reported when used within the recommended dosage [10]. Researchers believe that one of its constituent 'nigellone' shown to be an effective prophylactic agent in asthma and bronchitis with higher efficacy in children than in adults [11]. N. sativa seeds extracts could have a therapeutic effect against cerebral ischemia [12].

Yasni et al. tested the antimicrobial activity of black cumin (N. sativa) extracts in inhibiting the growth of pathogenic and spoilage bacteria, ethanol extract was the best extract in inhibiting the growth of bacteria while both aqueous and hexane extracts were less effective as antimicrobial agents [13].

Kamal et al. studied antibacterial activity of N. sativa seed, in various germinating stages, against five pathogenic bacteria resistant to a number of available antibiotics, his results showed that the activity depend on the growth stage and not on the dose , they concluded that N. sativa seed has moderate antibacterial activity [14].

Acharyya et al. screened the phytochemical of the crude methanol extracts and found that it was contained phenolics and flavonoids,these compounds have previously been reported to possess antimicrobial activities. Zahra et al. investigated the crude extracted phyto-constituents of N. sativa seeds against two G+ve (Bacillus subtilus, Staphylococcus aureus) and two G-ve strains of bacteria (Escherichia coli, Pasturella multocida) and one strain of fungi (Aspergillus niger). Phytoconstituents showed varying degree of inhibition against all the four bacteria and fungi. Flavonoids showed inhibition against the four tested bacteria with maximum inhibition (29 mm) against B. subtilus. Alkaloids showed inhibition against G+ve, while tannic acid showed inhibition against G-ve, against fungal tannic acids showed considerable inhibition value (18 mm) [15, 16].

Landa et al. investigated the crude methanol extracts from callus culture of some Nigella species (N. arvensis, N. damascena, N. hispanica, N. integrifolia and N. sativa) for their antimicrobial activity. Growth inhibition was determined in G+ve and G-ve bacterial strains as well as yeast. The result showed that the extracts of all calli tested exhibited significant antimicrobial activity, especially against B. cereus, S. aureus and S. epidermidis. Compared with other Nigella species, a callus culture of N. hispanica was the most effective against the microorganisms used in their study. Purkayastha et al. found that preliminary phytochemical analysis demonstrated the presence of most of the phytochemicals including saponins, cardiac glycoside, steroids, terpenoids, flavonoids and tannins from Foeniculum vulgare, Juniperus osteosperma and Nigella sativa [17,18].

Hasan et al. (2013) revealed that methanol extract at the concentration of 100 mg/mL of Nigella sativa seeds had a remarkable sensitivity against some pathogenic bacterial strains (Streptococcus pyogene, Pseudomonas aeruginosa, Klebseilla pneumoniae and Proteus vulgaris) [19].

## MATERIALS AND METHODS

**Source Of Nigella Sativa, Microorganisms And Reference Drugs:**

Nigella sativa seeds were purchased from different local market in Khartoum State. The standard microorganisms used in this study were the following: *Bacillus subtilus* (NCTC 8236), Staphylococcus aureus (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), Aspergillus niger (ATCC 9763) and *Candida albicans* (ATCC 7596).

The test organisms were obtained from the Department of Microbiology, Medicinal and Aromatic Plants Research Institute, Khartoum.

Reference drugs used in this study were Ampicillin (10 µg/disc) and Gentamicin (10 µg/disc) sensitivity discs from Himedia, and Gentamicin 50 µg/ml from SPIC, China.

**Seed Surface Sterilization And Germination:**

Seeds of *N. sativa* were surface sterilized by soaking in 50 % Clorox (0.5 % free chlorine) with 2 drops of Tween-20 for 5 min, and rinsed 3-5 times in sterile distilled water.

Surface sterilized seeds of *N. sativa* were directly cultured in the germination medium MS [20] . Basal medium. *N. sativa* seeds were incubated at 25 ± 20°C under cool white fluorescent light and 16 photoperiods for (4-5 weeks).

**Callus Induction:**

The hypocotyls and cotyledons were used as explants for Nigella sativa in this study. MS medium was used. Two types of auxin (2, 4-D and NAA) were used separately at different concentrations (0.0 as control, 0.05, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, and 7.0) mg/l, to assess their effects on callus induction for explants of *Nigella sativa*.

Each of the sterilized explants was cut into 2-3 mm pieces using sterile scalpel. Four pieces were inoculated in each vial containing sterile culture medium (MS medium) with different concentrations of growth regulators. Cultures were incubated for 8 weeks in the dark at 25±20C and data were recorded every two weeks.

**Preparation Of Plant Crude Extract:**

The coarsely powdered plant material was exhaustively extracted for 4 hours with petroleum ether in Soxhlet apparatus. The petroleum ether extract was filtered with a filter paper and evaporated under reduced pressure at 30°C using a rotatory evaporator apparatus (Rota-vap). The extracted plant material was air dried and repacked again and extracted with methyl alcohol. The methanolic extract was filtered with a filter paper and evaporated under reduced pressure at 65°C using Rot-evap.

**Preparation Of Callus Crude Extract:**

This is done in a fashion similar to that of plant extraction except the callus was dried at first by freeze drying using freeze dryer and then powdered and extracted with two different solvents, petroleum ether and methanol in Soxhlet apparatus.

**Preparation Of Bacterial Suspension:**

One ml aliquots of 24 hours broth culture of the test organisms were aseptically distributed onto nutrient agar slopes and incubated at 37°C for 24 hours. The bacterial growth was harvested and washed off with sterile normal saline, and finally suspended in 100 ml of normal saline to produce a suspension containing about (108 -109 ) colony forming units per ml, the average number of viable organisms per ml of the stock suspension was determined by means of the surface viable counting technique [21] . The suspension was stored in the refrigerator at 40°C until used.

**Preparation Of Fungal Suspension:**

Fungal cultures were maintained on Sabouraud dextrose agar, incubated at 25°C for 4 days. The fungal growth was harvested and washed with sterile normal saline and suspended in 100 ml of sterile normal saline. The suspension was stored in the refrigerator until used.

**In Vitro Testing Of Extracts For Antimicrobial Activity:**

The cup-plate-agar diffusion method [22] was adopted. Negative controls involving the addition of the respective solvents instead of extracts were carried out separately. After incubation the diameters of the resultant growth inhibition zones were measured. Mean values were tabulated. For fungal organisms instead of nutrient agar, Sabouraud dextrose agar was used.

**Preliminary Phytochemical Screening:**

General phytochemical screening for all extracts was carried out using the methods described by Wall et al., Sofowora, Harborne, and Martinez and Valencia with some minor modifications [23-26].

## RESULTS AND DISCUSSION:

**Callus Induction:**

Results in **(Table 1)** showed that among all concentrations of NAA, 1 mg/l gave the highest callus induction from hypocotyls explants, while 5.0 mg/l showed the highest callus induction from cotyledons explants. The auxin 2, 4-D at 0.05 mg/l had no activity in callus induction from hypocotyls explants. The highest callus initiation from hypocotyls explants obtained from 5.0 mg/l 2, 4-D, while 0.5 mg/l 2,4-D showed the highest callus initiation from cotyledons explants. Al-Said et al. obtained callus from different varieties of N. sativa by using MS and B5 media supplemented with 0.1 mg/l kinetin and either 2,4-D or NAA (1.0 or 3.0 mg/l),the callus induction showed variation between the varieties. Al-Ani found that the best callus production from N. sativa leaf explants was obtained in MS medium supplemented with 1.0 mg/l 2, 4-D and 1.5 mg/l kinetin. **(Tables 2, 3)** represented that the auxin NAA had more influence in callus induction from N. sativa hypocotyls and cotyledons explants than the auxin 2,4-D [27, 28]

**Table 1:** Effect of NAA and 2, 4-D on callus induction of Nigella sativa hypocotyls and cotyledons explants.

**Notes:** B= Brown, DB= Dark Brown

- = no callus, + =slow rate of callus formation, ++ = medium rate of callus formation, +++ = fast rate of callus formation

| Growth regulators | Hormonal Conc. mg/ l | Hypocotyls | | | Cotyledons | | |
|---|---|---|---|---|---|---|---|
| | | % of callus formation | Rate of callus formation | color | % of callus formation | Rate of callus formation | Color |
| NAA | 0.0 | - | - | - | - | - | - |
| | 0.05 | 95 | + + | B | 95 | + + | DB |
| | 0.5 | 100 | + + + | B | 95 | + + | DB |
| | 1.0 | 100 | + + + | B | 90 | + + | DB |
| | 2.0 | 100 | + + + | B | 95 | + + | DB |
| | 3.0 | 90 | + + | B | 90 | + + | DB |
| | 4.0 | 95 | + + | B | 100 | + + + | DB |
| | 5.0 | 100 | + ++ | B | 95 | + + | DB |
| | 6.0 | 95 | + + | B | 94 | + + | DB |
| | 7.0 | 100 | + + + | B | 100 | + + + | DB |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 2,4-D | 0.0 | - | - | - | - | - | - |
| | 0.05 | - | - | - | 67 | + | DB |
| | 0.5 | 55 | + | B | 100 | + + + | DB |
| | 1.0 | 88 | + + | B | 25 | + | DB |
| | 2.0 | 88 | + + | B | 50 | + | DB |
| | 3.0 | 88 | + + | B | 25 | + | DB |
| | 4.0 | 90 | + + | B | 75 | + | DB |
| | 5.0 | 100 | + + + | B | 60 | + | DB |
| | 6.0 | 100 | + + + | B | 60 | + | DB |
| | 7.0 | 94 | + + | B | 56 | + | DB |

**Table 2.** Effect of NAA different concentrations on callus induction of *Nigella sativa* hypocotyls and cotyledons explants.

| NAA Concentration Mg/l | Hypocotyls callus formation % | | | | Cotyledons callus formation % | | | |
|---|---|---|---|---|---|---|---|---|
| | 2week | 4week | 6week | 8week | 2week | 4week | 6week | 8week |
| 0.0 | - | - | - | - | - | - | - | - |
| 0.05 | 80 | 85 | 90 | 95 | 20 | 25 | 70 | 95 |
| 0.5 | 20 | 45 | 100 | 100 | 20 | 20 | 80 | 95 |
| 1.0 | 75 | 85 | 100 | 100 | 30 | 60 | 70 | 90 |
| 2.0 | 65 | 80 | 95 | 100 | 45 | 50 | 90 | 95 |
| 3.0 | 35 | 50 | 90 | 90 | 20 | 25 | 80 | 90 |
| 4.0 | 25 | 60 | 80 | 95 | 25 | 35 | 100 | 100 |
| 5.0 | 50 | 56 | 94 | 100 | 10 | 75 | 95 | 95 |
| 6.0 | 30 | 45 | 80 | 95 | - | 40 | 65 | 94 |
| 7.0 | 40 | 70 | 100 | 100 | - | 20 | 65 | 100 |

**Table 3.** Effect of 2,4-D different concentrations on callus induction of *Nigella sativa* hypocotyls and cotyledons explants.

| 2,4-D Concentration Mg/l | Hypocotyls callus formation % | | | | Cotyledons callus formation % | | | |
|---|---|---|---|---|---|---|---|---|
| | 2week | 4week | 6week | 8week | 2week | 4week | 6week | 8week |
| 0.0 | - | - | - | - | - | - | - | - |
| 0.05 | - | - | - | - | 8 | 25 | 58 | 67 |
| 0.5 | - | 44 | 55 | 55 | 8 | 33 | 100 | 100 |
| 1.0 | 22 | 44 | 88 | 88 | - | 25 | 33 | 25 |
| 2.0 | - | 44 | 88 | 88 | 8 | 33 | 58 | 58 |
| 3.0 | 11 | 44 | 88 | 88 | 17 | 25 | 25 | 25 |
| 4.0 | 45 | 65 | 90 | 90 | - | 13 | 44 | 75 |
| 5.0 | 25 | 75 | 90 | 100 | - | 20 | 40 | 60 |
| 6.0 | 15 | 85 | 100 | 100 | - | 25 | 50 | 60 |
| 7.0 | 25 | 65 | 94 | 94 | - | 19 | 13 | 56 |

**Antimicrobial Activity:**

Antimicrobial activities of six extracts obtained from seeds and callus of Nigella sativa against four bacteria and two different fungi, measured by the diameter of the zone of inhibition by using agar diffusion method were shown in **(Table 4).** The petroleum ether extracts of *N. sativa* seeds show no activity against the tested microorganisms while methanolic extracts of *N. sativa* seeds and callus have been found to possess remarkable antibacterial activity. Thymol and Thymoquinon are present in the methanol soluble portion of *N. sativa* seeds oil so they will be extracted in methanol solvent [29], this may explain the reason for the ineffectiveness of petroleum ether extracts. **(Figures 1-4)** show that all the methanolic extracts of both seeds and callus exhibited antibacterial activity against *E. coli* with maximum inhibition zone (23 mm) in methanolic extracts of hypocotyls (NAA) callus. Little inhibition was observed against *S. aureus* and no inhibition observed against *P. aeruginosa* and only methanolic extracts of cotyledons callus (2, 4-D) showed inhibition against *B. subtilus*. Contrary to Zuridah et al. and Khalid et al. whom found that methanolic extracts of N. sativa seeds showed maximum inhibition against *B. subtilus* and *S. aureus* but *E. coli* and *P. aeruginosa* were weakly sensitive, our result found that *E. coli* show the maximum sensitivity to extract [30,31].

**Table 4.** Preliminary screening for antimicrobial activity of *Nigella sativa* seeds and callus extracts against standard microorganisms.

| Sr. No. | Part used (Extracted) | Solvents used | Zone of inhibition(mm) ± SE | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | $G^{+VE}$ | | $G^{-VE}$ | | Fungi | |
| | | | B.s | S.a | E.c | Ps.a | As.n | C.alb |
| 1. | seed | Methanol | 0.0 | * | 21.3 ± 1.89 | 0.0 | 0.0 | 0.0 |
| | | P.E. | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 2. | Hypocotyls (NAA) callus | Methanol | 0.0 | * | 23 ± 3.00 | 0.0 | 0.0 | 0.0 |
| | | P.E. | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 3. | Cotyledons (NAA) callus | Methanol | 0.0 | 0.0 | 12.5 ± 1.76 | 0.0 | 0.0 | 0.0 |
| | | P.E. | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 4. | Hypocotyls (2,4-D) callus | Methanol | 0.0 | * | 13.3 ± 1.20 | 0.0 | 0.0 | 0.0 |
| | | P.E. | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 5. | Cotyledons (2,4-D) callus | Methanol | 12 ± 0.0 | 11.5 ± 0.5 | 22.5 ± 2.50 | 0.0 | 0.0 | 0.0 |
| | | P.E. | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |

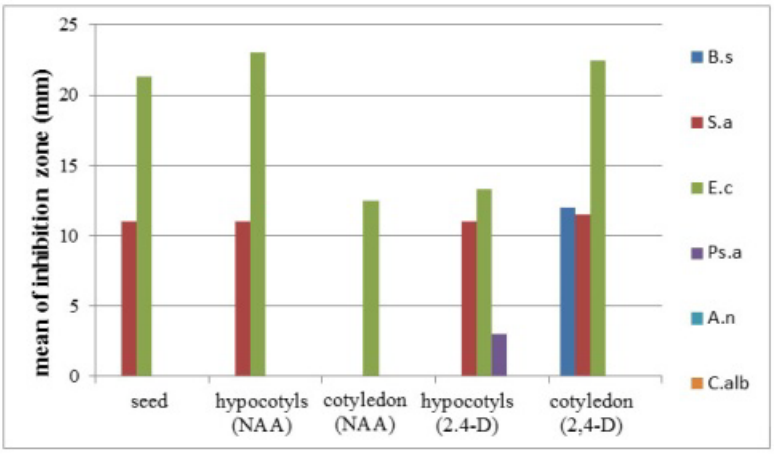


**Figure 1:** Average of inhibition zone (mm) of Nigella sativa seeds and callus methanolic extracts against standard microorganisms
B.s = Bacillus subtilus; S.a = Staphylococcus aureus; E.c = Escherichia coli
Ps.a = Pseudomonas aeruginosa; As.n = Aspergillus niger;
C. alb= Candida albicans m.e = methanolic extract

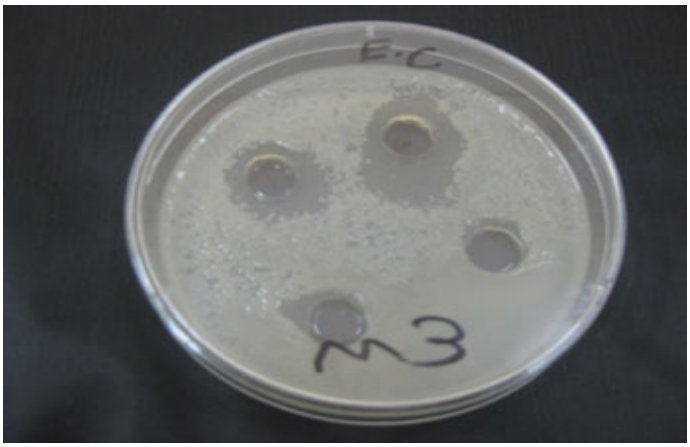


**Figure 2.** Inhibition zone (mm) of methanolic extract of *Nigella sativa* cotyledon callus (2,4-D) against *E. coli.*

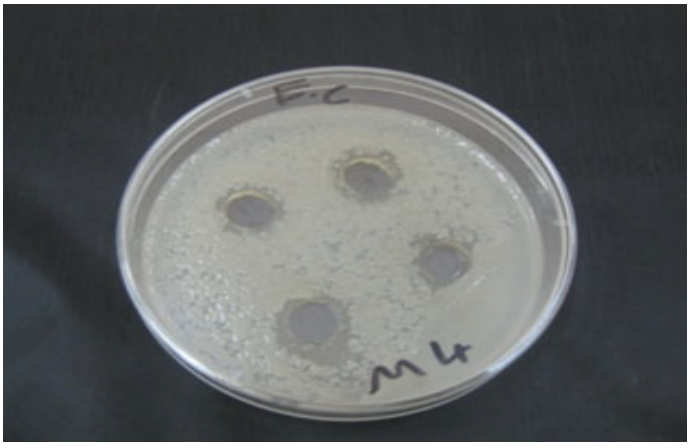


**Figure 3.** Inhibition zone (mm) of ethanolic extract of *Nigella sativa* cotyledon callus (NAA) against *E. coli.*

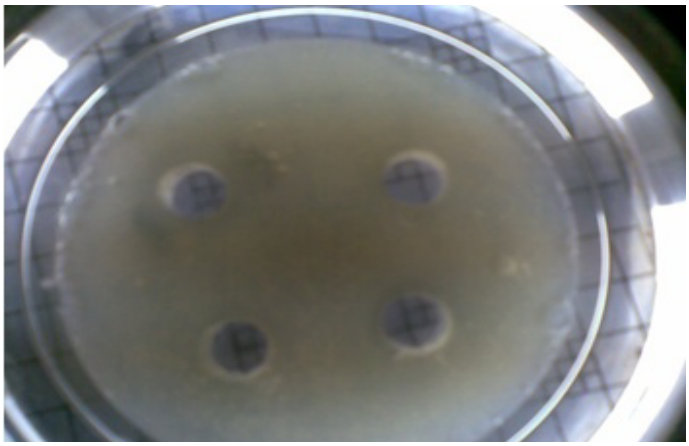

**Table 4.** Preliminary screening for antimicrobial activity of *Nigella sativa* seeds and callus extracts against standard microorganisms.

Our results agree with that obtained by Landa et al., their results showed that the extracts of all calli tested exhibited significant antimicrobial activity [32].

Comparison of the results given in **(Tables 4, 5)** showed that methanolic extract of *N. sativa* seeds, hypocotyls (NAA) and cotyledons (2,4-D) were more effective than Gentamicin at 10 µg/disc, while Ampicillin at 10 µ/disc showed clear resistant by *E. coli*. These results confirm the efficiency of the methanolic extracts of N. sativa and induced callus against *E.coli*. The investigation of antifungal activity revealed that none of all extract showed activity against the tested fungi.

**Table 5.** Antibacterial activity of reference drugs against standard microorganisms.
tested with Ampicillin and Gentamycin at 10 µg/disc.
B.s = *Bacillus subtilus*; S.a = *Staphylococcus aureus*; E.c = *Escherichia coli*
Ps.a = *Pseudomonas aeruginosa*
** M. D. I. Z. (mm) = Mean diameter of growth inhibition zone (mm).

| Drugs | M. D. I. Z. (mm)** | | | |
|---|---|---|---|---|
| | B.s | S. a | E. c | Ps. a |
| Ampicillin 10µg/disc | * | 0.0 | 0.0 | 0.0 |
| Gentamicin 10µg/disc | * | 14 | 15 | 13 |
| Genamycin 50 µg/ml | 25 | 32 | 28 | 30 |

**Phytochemical Screening:**

Results in **(Table 6)** were represented the results of preliminary phytochemical screening of N. sativa seeds and callus methanolic extracts, alkaloids, flavonoids and tannins are identified. Seeds and callus showed positive results for the presence of falvonoids. The methanolic extracts of the seeds and cotyledon (NAA) callus showed the presence of alkaloids. Only the seeds methanolic extract showed positive results for the presence of tannins. Our findings agree with Kamal et al. who found that the extracts of seeds of *N. sativa* in different germination stages have revealed the presence of alkaloids, tannins and flavonoids. Many other studies found that the seeds of *N. sativa* contain active chemical compounds like: fixed and essential oils, proteins, alkaloids, as well as rich amount of flavonoids, tannins and saponins [18,14].

**Table 6:** Preliminary Phytochemical screening of Nigella sativa seeds and callus methanolic extracts.
M= Seeds
M1= hypocotyls (NAA) callus
M2= hypocotyls (2, 4-D) callus
M3= cotyledons (2, 4-D) callus
M4= cotyledons (NAA) callus

| Test | Reagents | M | M1 | M2 | M3 | M4 | Observation |
|---|---|---|---|---|---|---|---|
| Flavonoids | $ALCL_3$ | +++ | ++ | + | ++ | ++++ | Yellow color |
| | KOH | +++ | ++ | + | ++ | ++++ | Yellow color |
| Alkaloids | Valser's | ++ | - | - | - | ++ | Turbidity |
| | Mayer's | ++ | - | - | - | ++ | Turbidity |
| Tannins | Gelatin salt | + | - | - | - | - | Turbidity |
| | $Fe\ Cl_3$ | + | - | - | - | - | Black color |

The present study concluded that *N. sativa* and their derived callus have a potential to produce active compounds with antimicrobial activities, when compared with some reference drugs. The methanolic extracts of N. sativa seeds, hypocotyls (NAA) callus and cotyledons ( 2,4-D) callus was more active than Gentamycin at 10 µg/disc against *E. coli*.

## CONCLUSION

Although many studies have appeared on the antimicrobial activity of plants and their secondary metabolites, very few studies are on in vitro derived callus. In this study, the main emphasis was on the ability to use the in vitro callus for antimicrobial activity.

1. MS media supplemented with auxin NAA was more suitable for inducing callus of Nigella sativa than 2,4-D.2
2. Hypocotyls are the suitable explants for callus formation of N. sativa.
3. Methanol is the suitable solvent to extract the active compound of both N. sativa and its induced callus.
4. The results of the present study indicated that N. sativa and its derived callus have a potential to produce active compounds with antimicrobial activities, when compared with some reference drugs.
5. Findings obtained in this study indicated the ability to utilize plant biotechnology technique towards development of desired bioactive metabolites extracted from callus culture instead of using intact plants for pharmaceutical purposes.
6. N. sativa callus extracts show higher activity than seeds methanolic extract against E. coli. Thus it can emphasize biotechnological method in development of new antibiotic.
7. Further work on the isolation and purification of the active principle from mentioned plant and its callus would throw new light in the development of new herbal drugs for local use as broad spectrum antimicrobial agent.

## REFERENCES


1. Salman M T et al. Antimicrobial activity of black cumin seeds (Nigella sativa ) against multidrug resistant strains of Coagulase negative Staphylococci. Hippocartic Journal of Unani Medicine. (2008); 3: 107-115.
2. Srinivasan D et al. Antimicrobial activity of certain Indian medicinal plants used in folkloric medicine. Journal of Ethnopharamcology. (2001); 49: 217 – 222.
3. Akgul A. Antimicrobial activity of black cumin (Nigella sativa L.) essential oil. Gazi Journal of Faculty of Pharmacy. (1989); 6: 63-68.
4. Hanafy MSM and Hatem ME. Studies on the antimicrobial activity of (black cumin). Journal of Ethnopharmacology. (1991); 34: 275-278.
5. De M et al. Antimicrobial screening of some Indian spices. Phytotherapy Research. (1999); 13: 616–618.
6. Burits M and Bucar F. Antioxidant activity of Nigella sativa essential oil. Phytotherapy Research. (2000); 14: 323–328.
7. Farra HA et al. Effect of gamma radiation on the bacterial flora of Nigella sativa seeds and its oil constituents. Acta Pharmaceutica. (2000); 50: 195-207.
8. Sagdic O et al. Effects of Turkish spice extracts at various concentrations on the growth of Escherichia coli O157 H7. Food Microbiology. (2002); 19: 473-480.
9. Sagdic O. Sensitivity of four pathogenic bacteria to Turkish thyme and oregano hydrosols. Food Science Technology. (2003); 36: 467-473.
10. Khan MAU et al. The *in vivo* antifungal activity of the aqueous extract from Nigella sativa seeds. Phytotherapy Research. (2003); 17: 183–186.
11. Chakravarty N. Inhibition of histamine release from mast cell by nigellon. Annals of Allergy. (1993); 70: 237-242.
12. Hosseinzadeh H et al. Anti-ischemic effect of Nigella sativa L. seed in male rats. Iranian Journal of Pharmaceutical Research. (2006); 1: 53-58.
13. Yasni S et al. Antimicrobial activity of Black Cumin extracts (Nigella sativa) against food pathogenic and spoilage bacteria. Microbiology Indonesia. (2009); 3: 146-150.
14. Kamal A et al. Potential of Nigella sativa L. seed during different phases of germination on inhibition of bacterial growth. Journal of Biotechnology and Pharmaceutical Research. (2010); 1: 9-13.
15. Acharyya S et al. Evaluation of the antimicrobial activity of some medicinal plants against enteric bacteria with particular reference to multi-drug resistant Vibrio cholera. Tropical Journal of Pharmaceutical Research. (2009); 8: 231-237.
16. Zahra N et al. Antimicrobial activity of aqueous ethanolic extracts and crude extracted phytoconstituents of Nigella sativa seeds. Bioscience Research. (2011); 8: 19-25.
17. Landa P et al. In vitro anti-microbial activity of extracts from the callus cultures of some Nigella species. Biologia. (2006); 61: 285-288.
18. Purkayastha S et al. Evaluation of antimicrobial and phytochemical screening of Fennel Juniper and Kalonji essential oils against multi drug resistant clinical isolates. Asian Pacific Journal of Tropical Biomedicine. (2012); S1625-S1629.

19. Hasan NA et al. Antimicrobial activity of Nigella sativa seed extract. Sains Malaysiana. (2012); 42: 143-147.
20. Murashige T and Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue culture. Plant Physiology. (1962); 15: 467-497.
21. Miles AA and Misra SS. The estimation of the bactericidal power of the blood. Journal Hygiene. (1938); 38: 732-749.
22. Kavanagh F. Analytical Microbiology. Academic press New York and London. Vol II. (1972); Pp13-30.
23. Wall ME et al. Detection and estimation of steroid and sapogenins in plant tissue. Analytical chemistry. (1952); 24: 1337-1342.
24. Sofowora AE. Medicinal plants and traditional medicines in Africa.[ John Willey and Sons Ltd (eds.)]. New York. (1982); Pp. 274.
25. Harborne JB. Phytochemical methods 3rd edtn. Chapman and Hall (eds.) U.K. (1998); Pp. 74-203.
26. Martinez A and Valencia G. In Manual de prácticas de Farmacognosiay Fitoquímica 1st edition Medellin Universidad de Antioquia. Phytochemical screening methods. (2003); Pp: 59-65.
27. Al-Said MS et al. Biotechnological production of biologically- active metabolites by plant cell culture techniques. Press King Saud University. (2002); Pp. 218.
28. Al- Ani NK. Thymol production from callus culture of Nigella sativa L. Plant Tissue Culture and Biotechnology. (2008); 18: 181-185.
29. Basha AL I et al. TLC assay of thymoquinone in black seed oil (Nigella sativa L.) and identification of dithymoquinone and thymol. Journal of Liquid Chromatography. (1995); 18: 105-115.
30. Zuridah H et al. In vitro antibacterial activity of Nigella sativa against Staphylococcus aureus Psudomonas aeruginosa Klebsilla pneumonia Escherichia coli and Bacillus cereus. Asian Journal of Plant Science. (2008); 7: 331-333.
31. Khalid A et al. Antimicrobial activity analysis of extracts of Acacia modesta Artimisia absinthium Nigella sativa and Saussurea lappa against Gram positive and Gram negative microorganisms. African Journal of Biotechnology. (2011); 10: 4574-4580.
32. Miles AA and Misra SS. The estimation of the bactericidal power of the blood. Journal Hygiene. (1938); 38: 732-749.