In Vitro Callus Induction and Antimicrobial Activities of Callus and Seeds Extracts of *Nigella sativa* L.

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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 (21mm)&(23mm) 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 10µg/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.

Keywords: Antimicrobial activity, Callus induction, *Nigella sativa* ,Phytochemical analysis

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 , 4 , 5, 6 , 7 , 8 , 9 , 10) .

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 (11) . 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 (12) . *N. sativa* seeds extracts could have a therapeutic effect against cerebral ischemia (13) .

(14) 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.

(15) 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.

(16) 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. (17) 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 (29mm) 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 (18mm).

(18) 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 results 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. (19) 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.*

(20) 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).*

The objectives of this study are to induce callus from *Nigella sativa* , using different explants and different auxins and to determine the efficiency of secondary metabolites extracted from the callus compared to that extracted from seeds of *N. sativa* 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)* .

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 (21) basal medium. *N. sativa* seeds were incubated at 25±2^{0}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±2^{0}C 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 ^{0}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^{0}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 37^0 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 (10^8 -10^9) 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 (22). The suspension was stored in the refrigerator at 4^0 C until used.

Preparation of fungal suspension:

Fungal cultures were maintained on Sabouraud dextrose agar, incubated at 25^0 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 (23) 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 (24), (25) (Sofowora (1982), (26) and (27) , with some minor modifications.

Statistical analysis:

Data were analyzed by SPSS package software. The results were presented as mean ± SE of three replicates and analyzed with Duncan LSD. The data were considered significant when P value was <0.05.

Results

Callus induction:

Results in table (1) showed that among all concentrations of NAA, 0.5 mg/l gave the highest callus induction from hypocotyls explants, while 4.0 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.

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

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

B= Brown, DB= Dark Brown, - = no callus, + =slow rate of callus formation, ++ = medium rate of callus formation, +++ = fast rate of callus formation.

Table (2) showed that the effect of auxin NAA . 100 % of callus induction of hypocotyls explants *N. sativa* was obtained at eighth week with concentration of 0.5 Mg/l NAA, whereas 4.0 Mg/l of NAA at eighth week gave 100% of callus induction of cotyledons explants.

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	95	10	75	95	95
6.0	30	45	80	95	-	40	65	94
7.0	40	70	100	100	-	20	65	100

Table (3) reveal that 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 at the same period. These results explain that 2,4-D auxin induced highest callus by low concentration when cotyledons explants was used.

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 standard 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.

Figures (1, 2, 3 and 4) show that all the methanolic extracts of both seeds and callus exhibited antibacterial activity against *E.coli* with maximum inhibition zone (23mm) 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.*

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

* = small inhibition

B.s = Bacillus subtilus; S.a = Staphylococcus aureus; E.c = Escherichia coli

Ps.a = Pseudomonas aeruginosa; As.n = Aspergillus niger

C. alb= Candida albicans P.E. =Petroleum Ether

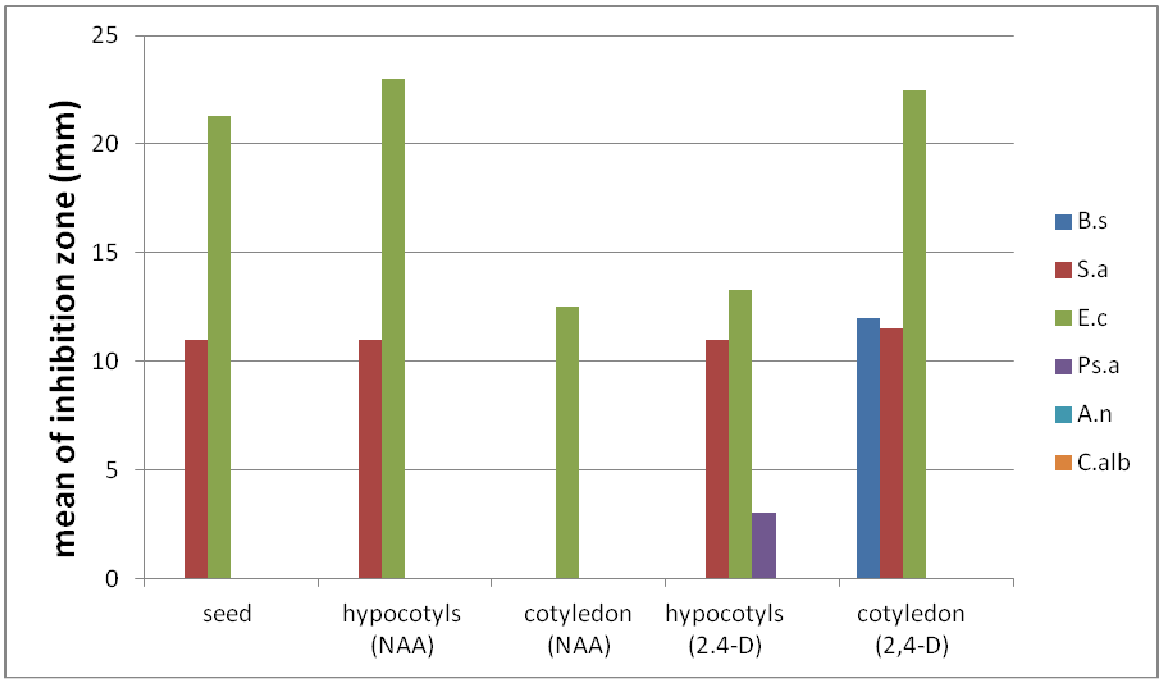

Fig. (1) Histogram showing the average of inhibition zone (mm) of *Nigella sativa* seeds and callus methanolic extracts against standard microorganisms

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

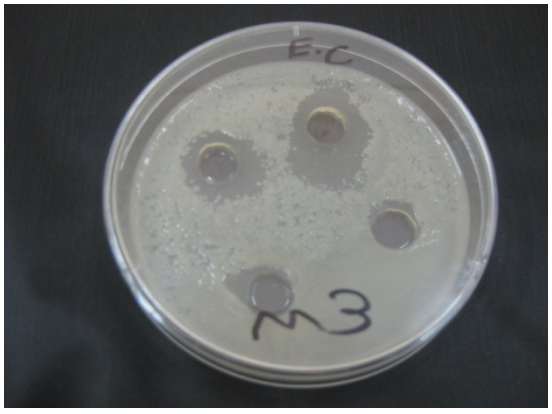

Fig.(2) *I. Z. of methanolic extract of *Nigella sativa* cotyledon callus (2,4-D) against *E.coli*

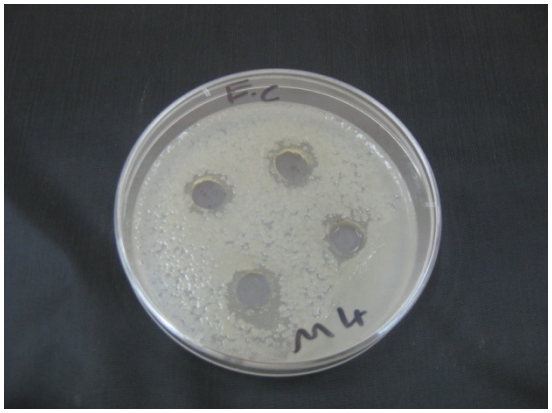

Fig. (3) *I. Z. of methanolic extract of *Nigella sativa* cotyledon callus (NAA) against *E. coli.*

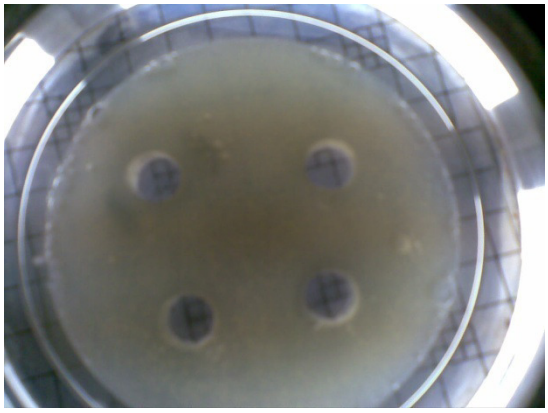

Fig. (4) * I. Z. of methanolic extract of *Nigella sativa* seeds against *E.coli*

* I. Z. = Inhibition zone

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 10µ/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

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

* = not tested with Ampicillin and Gentamycin at 10µg/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).

Phytochemical screening:

Results in table (6) were represented the preliminary phytochemical screening of *N. sativa* seeds and callus methanolic extracts. The alkaloids, flavonoids and tannins are identified. Seeds and callus showed positive results for the presence of falvonoids. The methanolic extracts of the seeds and cotyledons (NAA) callus showed the presence of alkaloids. Only the seeds methanolic extract showed positive results for the presence of tannins.

Table (6) Preliminary Phytochemical screening of *Nigella sativa* seeds and callus methanolic extracts

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

M= Seeds

M1= hypocotyls (NAA) callus

M2= hypocotyls (2,4-D) callus

M3= cotyledons (2,4-D) callus

M4= cotyledons (NAA) callus

Discussion

100 % of callus induction of hypocotyls explants *N. sativa* was obtained at eighth week with concentration of 0.5 Mg/l NAA with MS medium, whereas 4.0 Mg/l of NAA with MS medium at eighth week gave 100% of callus induction of *N. sativa* when cotyledons explants was used. These results explain that the hypocotyls explants more suitable than cotyledons explants when auxin NAA was used. Opposite results were obtained from *N. sativa,* when 2,4-D auxin was used with MS medium . 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 at the same period. These results revealed that 2,4-D auxin induced highest callus by low concentration when cotyledons explants was used.

The researcher (28) obtained callus from different varieties of *N. sativa* by using MS &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. (29) 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, these findings were in the same line with the present study.

The antimicrobial activity of petroleum ether extracts of *N. sativa* seeds show no activity against the tested microorganisms used in this study, 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 (30,31) , this may explain the reason for the ineffectiveness of petroleum ether extracts.

Contrary to (32) and(33) , 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 results found that *E. coli* show the maximum sensitivity to this extract. Our findings agree with that obtained by (18), where their results showed that the extracts of all calli tested exhibited significant antimicrobial activity.

Comparison between the activity of the methanolic extracts of *N. sativa* seeds and the callus and that of antibacterial activity of some reference drugs against standard microorganisms, revealed 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 10µ/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 secondary metabolites of seeds and callus of *N. sativa* showed positive results for the presence of falvonoids. The methanolic extracts of the seeds and cotyledons (NAA) callus showed the presence of alkaloids. Only the seeds methanolic extract showed positive results for the presence of tannins. These results agree with (15), 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 ((34,19) .

Conclusion:

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. 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.

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