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EVALUATION OF *NIGELLA SATIVA L.* CALLUS EXTRACTS UNDER ELICITATION FOR PHYTOCHEMICAL AND ANTIBACTERIAL ACTIVITY

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ABSTRACT

The indiscriminate use of commercial antimicrobial drugs has resulted in multiple drug resistance and adverse effects on the host. The present study was aimed to elucidate the effect of different biotic/abiotic elicitors on the growth as well as to enhance the metabolite production in leaf and epicotyl calli of *Nigella sativa.* Further, antibacterial activity of these callus extracts was evaluated against five Gram positive and Gram negative bacteria. TLC data confirmed the presence of alkaloids, phenols, flavonoids, glycosides and importantly terpenoids in control and elicitated callus extracts. Calculated MIC values showed that maximum inhibition was caused by $MnCl_2$ (1.08±0.3 µg ml^{-1}), pectin (1.28±0.5 µg ml^{-1}) and control epicotyl callus (2.02±0.3 µg ml^{-1}) and minimum by $CoCl_2$ and cellulose leaf callus where *E. coli* being most sensitive followed by *S. typhi, B. cereus, S. aureus K. pneumoniae* towards the extracts. Results showed that the extracts were better antibacterial agents when compared with commercially available antibiotics.

KEY WORDS: Callus, elicitors, phytochemicals, TLC, antibiotics

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INTRODUCTION

Nigella sativa L. (Family-Ranunculaceae) an annual herbaceous plant commonly known as Kalounji or Black cumin has been used from centuries for treatment of various ailments, including infectious diseases which make it one of the important medicines of Tibbe Nabawi (Prophetic Medicine). Its seeds have been extensively studied in the last 4-5 decades and these studies have reported it to possess a number of medicinal properties[1, 2]. Their crude extracts [3, 4] and essential oil [5] have been reported to possess antibacterial activity against several bacteria. These activities have been predominantly attributed to the presence of active compounds in the fixed oils, volatile oils and different extracts studied [6]. The antibacterial principle compounds present in *Nigella sativa* seed extracts were isolated and characterized found to be thymoquinone, dithymoquinone and thymol [7]. In two different studies, one study showed that diethyl ether extract caused concentration-dependent inhibition of *Staphylococcus aureus, Pseudomonas aeruginosa*, *Escherichia coli* and *Candida albicans* [8] and the other reported high inhibition caused by methanol and chloroform extracts against *S. aureus*, *P. aeruginosa* and *C. albicans* [9]. In the search for alternatives to produce desirable medicinal compounds from plants, plant tissue cultures are found to have potential as a supplement to traditional agriculture in the industrial production of bioactive plant metabolites [10]. Different plant tissue culture strategies have been extensively studied to improve the production of plant chemicals. Elicitation being one of them is done to enhance the production of secondary metabolites in plant cultures by using biotic/abiotic molecules. They are considered as signaling molecules as their incorporation in the cultures generates signal-transduction cascade and leads to activation and expression of the related genes with the biosynthesis of metabolites [11] and also stimulates plant's antioxidant defense system [12]. Area of *N. sativa* tissue culture has been over looked by the researchers except for few studies by Banerjee and Gupta [13] and Schmauder and Doebel [14] attempting primarily with morphogenesis and embryogenesis of *N. sativa* cultures. Banerjee and Gupta [13] studied shoot differentiation to occur upon omission of coconut milk first, then the auxin from the medium or upon supplementation with indol acetic acid (2.0 mg/L) and coconut milk (15%). Production of Thymol from the leaf callus cultures of *N. sativa* under different hormonal effects has also been reported [15]. Schmauder and Doebel [14] indicated the difficulty of inducing the synthesis of secondary products in suspension cultures of this species. The newly reported antibiotics by pharmacological industries have failed to discourage the growth of many bacteria that have genetic ability to transmit and acquire resistance to drugs [16]. Therefore, the present study was undertaken to investigate the presence and enhancement of different phytochemicals of *N. sativa* callus extracts grown under elicitation. The antibacterial activity of these extracts was also evaluated to show their bioactivity against five pathogenic bacterial strains.

MATERIALS AND METHODS

I. Plant Material

Seeds of *N. sativa* were procured from a local grocery store in Lucknow, India and authenticated by a botanist at National Botanical Research Institute, Lucknow. Seeds were surface sterilized by washing thoroughly under tap water containing few drops of Tween-20 then rinsed with 70% ethanol for 30 seconds followed by washing with sterile water twice. They were then immersed in 0.2% mercuric chloride solution for 5min after which they were rinsed with sterile water twice were allowed to grow in glass petri plates having three folds of damp blotting paper in distilled water at room temperature of about 25±2°C under aseptic condition. They were kept in dark for three days till sprouting initiated after then exposed to light (photoperiod of 14/10 h and light intensity of 100 µmol m^{-2} s^{-1}) where complete germination with leaf, epicotyl, hypocotyl and root took eleven days.

II. Induction and maintenance of callus under biotic/abiotic elicitors

Explants including leaves (40-50), segments (0.5 cm) of epicotyl, hypocotyl and root were taken from the 11th day seedling and were surface sterilized by washing with autoclaved distilled water followed by immersing them in 70% ethanol for 4-5min and then again washing with autoclaved distilled. They were cultured in triplet on solidified MS medium supplemented with the combination of Kinetin (Kn) 2mg/L + Naphthalene acetic acid (NAA) 1mg/L as this combination gave best callusing which is reported in our earlier study [17]. Cultures were kept under a photoperiod of 16/8 with 3000 lux of white fluorescent light at 25±2°C temperature. Explant that responded towards callusing were then inoculated in triplets on solidified MS media supplemented with Kn 2mg/L + NAA 1mg/L growth regulators and 10 ppm of biotic and abiotic elicitors (Pectin, Cellulose, $MnCl_2$ and $CoCl_2$) added from the stock solution (1mg/ml) of the elicitors stored at 4^0C for callus induction whereas, control cultures lacking elicitor. Growth pattern was observed under different stress conditions. Fresh weight of all the calli was recorded and the percentage induction of callus was calculated by the following formula.

$$\text{Callus induction \%} = \frac{\text{Number of calli}}{\text{Number of explants inoculated}} \times 100$$

III. Extraction method

Calli of leaf and epicotyl were taken and weighed. Crude extracts used in the study were prepared by grinding different calli in 100% of methanol using mortar and pestle at room temperature with periodical mixing on shaker for overnight, then filtrated and concentrated at 45 °C to get 5 mL of sample. Extracts were stored at -20°C for further use.

IV. Study of phytochemicals in N. sativa calli under different biotic and abiotic elicitation by thin layer chromatography

(i) Preparation of TLC plate

Silica gel slurry was prepared by macerating silica gel powder (TLC grade, HIMEDIA) with distilled water and was poured on glass plates which served as matrix. TLC plates were kept in an oven at a temperature of 100-110°C for 2-3 hours for activation. The TLC tank was equilibrated with the developing solvent system for one hour by using 200ml (approximately) of solvent mixtures. The samples were loaded with a capillary tube evenly along the line about 2cm above the bottom end of the plates. Loaded samples ran with the solvent and the plates were removed when they reached the solvent front line.

(ii) Analysis of the data

The data were analyzed by spraying with color reagent. Non-radioactive samples were sprayed with appropriate stain and visualized as directed in the protocol. The compounds which give fluorescence in the UV range were visualized on a transilluminator whereas other components were easily seen as bands in visible light. The solute front and the solvent front were recorded and the R_f value was calculated from this data.

a. TLC for alkaloids

The alkaloid spots were separated using the solvent mixture chloroform and methanol (15:1). The color and R_f values of the separated alkaloids were recorded both under ultraviolet (UV-254nm) and visible light after spraying with Dragendorff's reagent.

b. TLC for phenols

Phenols were separated using chloroform and methanol (27:0.3) solvent mixture. The color and R_f values of these phenols were recorded under visible light after spraying the plates with Folin-Ciocalteu's reagent heating at 80°C for 10min.

c. TLC for flavonoids

The flavonoid spots were separated using chloroform and methanol (19:1) solvent mixture. The color and R_f values of these spots

were recorded under ultraviolet (UV-254nm) light.

d. TLC for glycosides

The glycosides were separated using ethyl acetate:methanol:water (80:10:10) solvent mixture. The color and R_f values of these spots were recorded by observing under ultraviolet (UV-254nm) light.

e. TLC for Terpenoids

Terpenoids were separated using toulene:chloroform:ethanol (40:10:10). Plates were sprayed with freshly prepared anisaldehyde-sulphuric acid reagent and heated to 100°C for 10 min. R_f values of the spots were measured.

V. Determination of in vitro antimicrobial effect

(i) Pathogenic bacterial strains used for the study

The pathogenic bacterial isolates used in study are listed in the Table 1. These isolates were procured from NCIM Pune, India.

Table 1
Pathogenic bacterial strains

BACTERIA	STRAIN
Escherichia coli	NCIM 2065
Staphylococcus aureus	NCIM 2099
Klebsiella pneumoniae	NCIM 2957
Salmonella typhi	NCIM 2501
Bacillus cereus	NCIM 2156

(ii) Inoculum preparation

Active cultures for each bacterial species were prepared by transferring a loopful of cells from the stock cultures to nutrient broth. The inoculated tubes were incubated without agitation for 24 hrs at 37°C. The cultures were diluted with fresh nutrient broth to achieve optical densities corresponding to 10^6 cfu mL^{-1} [18].

(iii) Broth dilution assay

A modified macro-broth dilution technique was used to determine the minimum inhibitory concentration (MIC) values. Log phase cultures of bacteria were diluted 100 folds in NB (100µl bacterial cultures in 10ml NB which contained 10^5 cfu of bacteria). Gradually increasing concentrations of the extracts were added to test tubes containing the bacterial cultures to know the inhibitory concentration inhibiting the bacterial growth. The tubes were incubated at 37°C for 18-24 hours. Visible turbidity and optical density of cultures was determined at 620nm using NB as control. The lowest concentration that inhibited visible growth of the tested organisms was recorded as the MIC.

(iv) Agar well diffusion assay

Agar well diffusion method was used to test the antibacterial effect of different callus crude extracts [19, 20]. Media plates (11 cm in diameter) were prepared with nutrient agar. After solidification a total of four wells (7 mm in diameter) per agar plate were cut. For test, three doses of extract (25, 50, 75µl/well) were used. Standard antibiotics streptomycin (30 µg) (HIMEDIA), ciprofloxacin (10 µg) (HIMEDIA), doxycycline (30 µg) (HIMEDIA), ampicillin (10 µg) (HIMEDIA) and ofloxacin (5 µg) (HIMEDIA) were used as positive control and Dimethyl sulphoxide i.e. DMSO (75µl/well) as negative control. 100 µl (10^5 cfu) of each diluted microbial suspension were inoculated on nutrient agar plates and spread over using sterile cotton swab. The extracts and positive control (streptomycin, ciprofloxacin, deoxycycline, ampicillin and ofloxacin) were added separately to each well of agar plate and allowed to diffuse at room temperature for 15-20 min. Plates were incubated at 37°C for 24h after which they were examined for zones of growth inhibition and the diameter of these zones was measured. The assay was repeated three times for each extract. The antimicrobial effects were recorded as the

mean diameter of the resulting inhibition zones of growth in millimeter.

RESULTS

I. Response and effect of biotic/abiotic elicitors on callus induction from different parts of the seedling of N. sativa L.

Explants inoculated (leaf, 0.5cm long segments of epicotyl, hypocotyl and root taken from 11th day seedling) on solidified MS media (Kn 2mg/l and NAA 1mg/l) showed that epicotyl segment (0.5 cm long) gave best and fast response with creamy white to greenish friable callus followed by leaf disc segment (0.5 cm long) which gave compact, green callus but callusing here was delayed when compared to epicotyl whereas, root and hypocotyl segment did not responded towards callusing. Therefore, only leaf and epicotyl explants were further studied for elicitation.

Table 2

Morphology and growth pattern of the epicotyl callus under biotic/abiotic elicitation

Days	Control callus	+Pectin callus	+Cellulose callus	+$MnCl_2$ callus	+$CoCl_2$ callus
1	No response, green explant	No response, green explant	No response, green explant	No response, green explant	No response, green explant
10	Swelling of explant	Swelling of explant	Swelling of explant	Swelling of explant	Swelling of explant
20	Curling of explant	Curling of explant	Curling of explant	Curling of explant	Curling of explant
30	Initiation of green callus	Curling proceeds	Curling proceeds	Curling proceeds	Curling proceeds
40	Callusing over few parts of explant	Callusing over entire explant	Callusing over few parts of explant	Callusing over entire explant	Callusing over few parts of explant
50	Callusing over entire explant	Callusing continues	Callusing increases	Callus size increases	Callusing continues
60	Green callus, size increases	Light green callus	Green callus, size increases	Green callus, size constant	Light green callus, size constant

Table 3

Morphology and growth pattern of the leaf callus under biotic/abiotic elicitation

Days	Control callus	+Pectin callus	+Cellulose callus	+$MnCl_2$ callus	+$CoCl_2$ callus
1	No response, green explant	No response, green explant	No response, green explant	No response, green explant	No response, green explant
10	Swelling of explant	Swelling of explant	Swelling of explant	Swelling of explant	Swelling of explant
20	Curling of explant	Curling of explant	Curling of explant	Curling of explant	Curling of explant
30	Initiation of green callus	Initiation of green callus	Curling proceeds	Initiation of green callus	Curling proceeds
40	Callusing over few parts of explant	Callusing over entire explant	Callusing over few parts of explant	Callusing over entire explant	Callusing over few parts of explant
50	Callusing over entire explant	Callus size increases	Callusing proceeds	Callus size increases	Callusing proceeds
60	Green callus, size constant	Green callus, size increses	Green callus, size constant	Green callus, size increses	Green callus, size constant

Results shown in Table 2 and Table 3 clearly showed that the morphology and growth of the calli were greatly influenced by the exogenously supplied elicitors (biotic and abiotic) as compared to the control callus in the case of both epicotyl and leaf. Results demonstrated that Pectin and $MnCl_2$ (Fig 1) induced cell division which resulted in initiation of callus induction earlier than the control but retarded biomass accumulation and percentage induction than the control callus (267±0.2 and 82.6±0.7 respectively) in the case of epicotyl explant (Table 4). Whereas, in the case of leaf explant, control explant marked earlier callus initiation. Callusing was delayed under elicitation and here again Cellulose and $CoCl_2$ showed delayed initiation as compared to Pectin and $MnCl_2$. Fresh weight and percentage induction was again higher in control callus (213±0.4 and 77.3±0.3respectively).

Figure 1

Callusing response of epicotyl and leaf explants under different biotic/abiotic elicitors.

A: Epicotyl explants

B: Leaf explants

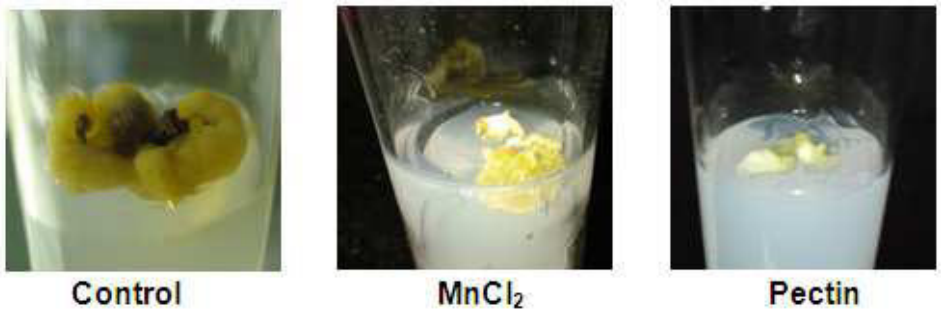

Table 4

Percentage callus induction and weight of the N. sativa calli under elicitation.

S. No.	Different Calli	% Induction	Fresh Weight (mg)
1.	Epicotyl Callus	82.6±0.7	267±0.2
2.	Leaf Callus	77.3±0.3	213±0.4
3.	Pectin Epicotyl Callus	60.4±0.4	167±0.5
4.	Pectin Leaf Callus	48.0±0.2	183±0.2
5.	Cellulose Epicotyl Callus	72.8±0.5	154±0.6
6.	Cellulose Leaf Callus	55.3±0.3	115±0.4
7.	$MnCl_2$ Epicotyl Callus	74.7±0.4	209±0.4
8.	$MnCl_2$ Leaf Callus	70.2±0.6	189±0.3
9.	$CoCl_2$ Epicotyl Callus	68.5±0.2	178±0.2
10.	$CoCl_2$ Leaf Callus	50.2±0.6	147±0.5

II. Elicitation effects on different phytochemical accumulation

Table 5
Qualitative analysis of phytochemicals of N. sativa calli under elicitation.

S.NO	COLOUR OF SPOT	R_f VALUE	DIFFERENT CALLI									
TLC results for alkaloids												
			Leaf	Epicotyl	$MnCl_2$ leaf	$MnCl_2$ epi	$CoCl_2$ leaf	$CoCl_2$ epi	Pec leaf	Pec epi	Cel leaf	Cel epi
1	Green	0.67	+	++	-	++	++	+	+	++	+	++
2	Orange	0.58	++	++	-	+++	++	+	-	++	+	++
3	Pale green	0.64	+	+	-	++	+	+	-	-	+	+
TLC results for phenols												
1	Blue	0.16	-	-	+	-	-	+++	+	++	-	+
2	Dark blue	0.08	+	+	+	+++	++	++	-	++	-	+
TLC results for flavonoids												
1	Green	0.16	-	+	-	+	-	-	+	+	-	+
2	Blue	0.23	+	-	-	+	+	+	-	+	-	-
3	Blue	0.33	-	+	-	+	-	-	-	+	+	+
4	Blue	0.56	-	+	-	+	-	-	-	-	-	+
5	Green	0.80	-	+	-	+	-	-	-	-	-	+
TLC results for glycosides												
1	Green	0.14	-	+++	++	++	+	++	-	+	-	+
2	Green	0.20	+	-	++	++	+	++	+	+	-	+
TLC results for terpenoides												
1	Thymol (pink)	0.76	++	+++	+	+++	+	+	++	+++	+	+
2	Thymoquinone (blue)	0.82	++	++	+	++	-	+	++	+++	+	+
3	Purple	0.97	-	-	+	+	+	+	+	+	-	+
4	Light blue	0.73	+	+	-	+	+	-	+	+	+	+
5	Light blue	0.6	+	++	+	++	+	+	+	++	+	++

*(*epi-Epicotyl, *Pec-pectin, *Cel- cellulose,* '-' symbolizes absence of the metabolite; *'+' symbolizes presence of the metabolite; *'++' symbolizes moderate presence; *+++ symbolizes good presence)*

The data of qualitative analysis of phytochemicals present in the methanolic extract of different elicited calli showed the presence of alkaloids, phenols, flavonoids, glycosides and terpenoids tabulated in Table 5. Three alkaloids were seen as distinct bands on the TLC plate (R_f =0.67, 0.588 and 0.64) which were visible in the visible range of light. Green spot (R_f =0.67) was reported in all the extracts except for $MnCl_2$ leaf callus whereby prominently seen in $MnCl_2$ epicotyl callus followed by pectin and $CoCl_2$ epicotyl callus. Second spot of orange color was again absent in $MnCl_2$ leaf callus and pectin leaf callus while present in all other and the third pale green color spot (R_f = 0.64) was again absent in $MnCl_2$ leaf, pectin leaf and pectin epicotyl callus thus TLC of alkaloid showed that $MnCl_2$ somehow inhibited the formation of alkaloids in leaf calli. Study of phenolic contents of *N. sativa* calli by thin layer chromatography gave two spots of phenols (R_f = 0.16 and 0.08). Blue spot (R_f = 0.16) was not so distinctively reported in all extracts but was present in considerable amount in $CoCl_2$ and pectin epicotyl callus although went absent in control leaf, control epicotyl, $MnCl_2$ epicotyl, $CoCl_2$ leaf and cellulose leaf callus extracts. While the second spot (dark blue color R_f = 0.08) was more dominantly present in most of the extracts with a prominent presence in $MnCl_2$ and $CoCl_2$ epicotyl callus extracts and went missing in pectin leaf and cellulose leaf callus extract. Results again showed the inhibition of phenol formation in leaf callus by cellulose. Five flavonoid spots were reported in diminished amounts in the different samples and they showed fluorescence of different colors under UV illumination on a transilluminator. There was no such occurrence pattern though $MnCl_2$ reported the presence of all flavonoids in diminished amount. The results of glycosides

showed the presence of two different glycosides with similar green fluorescence color band (R_f = 0.14 and 0.20). A prominent spot (R_f = 0.14) was reported in all the extracts except for leaf, pectin leaf and cellulose leaf callus extracts whereas a spot (R_f = 0.20) was present in all the extracts except for epicotyl and cellulose leaf callus extracts. Results of glycosides thus stated that the elicitation of cellulose in leaf callus may have inhibited the formation of glycosides. Lastly TLC analysis showed the presence of six different terpenoids (R_f values 0.97, 0.82, 0.76, 0.73 and 0.6) where standard thymoquinone and thymol were used and their R_f values came out to be 0.82 and 0.76 respectively. Terpenoid did not followed any pattern of occurrence as thymol was reported in all extracts but prominently occurred in epicotyl, $MnCl_2$ and pectin epicotyl callus extracts same was the case with thymoquinone as it was prominently reported in control leaf, epicotyl, $MnCl_2$ epicotyl, pectin leaf and pectin epicotyl callus extracts but went absent in $CoCl_2$ leaf callus extract. Rest other three were unevenly present in all the samples with light blue spot (R_f = 0.6) was present in all the samples.

III. In vitro antibacterial effect

The antibacterial results of different methanol extracts of leaf and epicotyl calli under biotic and abiotic elicitation on five Gram-positive and Gram-negative pathogenic bacterial strains are presented in Table 7 and 8 indicated that different callus extracts showed different degrees of growth inhibition depending on the explant (leaf and epicotyl), type of elicitation, dose and bacterial strains. The results of the antibacterial assay revealed that all the methanolic extracts (leaf and epicotyl calli) were found to be more effective against *E. coli, S. typhi and B. cereus* while *S. aureus* and *K. pneumoniae* remained much resistive towards them and that the extract of epicotyl calli gave maximum degree of inhibition even at lower doses as compared to the leaf callus extracts. Amongst the different elicitated epicotyl extracts $MnCl_2$ elicitation was reported to cause highest degree of inhibition towards all the strains followed by the pectin epicotyl and control epicotyl extracts. $MnCl_2$ epicotyl callus (abiotic elicitation) gave the highest activity against *E. coli* (32±0.3mm) and at the MIC value 1.08±0.3µg ml^{-1} (Table 6) whereby being the lowest concentration amongst all the extract which gave maximum inhibition and even far more than the standard antibiotics used. This degree of inhibition may be attributed due to the presence of higher and almost all the phytochemicals determined in the study. Likewise in the case of pectin epicotyl (biotic elicitation) and control callus extracts highest zone of inhibition was recorded against *B. cereus* (32±0.3) *and E. coli* (26±0.2) with MIC values 1.28±0.5 and 2.02±0.3µg ml^{-1} (Table 8) respectively. In the case of other two elicitated extracts both cellulose and $CoCl_2$ extracts gave smaller zone of inhibition moreover cellulose being inefficient in inhibiting *K. pneumoniae* at lower dose (25 µl). In leaf callus extracts gave lower degree of inhibition moreover even some of the bacterial strains remained resistant towards the lowest dose of some extracts. Here control leaf extract was reported to cause highest degree of inhibition towards all the strains followed by pectin and cellulose elicitation. Control callus extract gave the highest activity against *E. coli* (20±0.2mm) at the MIC value 2.80±0.4µg ml^{-1} whereby being the lowest concentration amongst all the extract which gave maximum inhibition. Pectin elicitated leaf extract reported maximum and nearly similar zone of inhibition against *E. coli* (18±0.3mm) and *B. cereus* (18±0.5mm) with MIC 2.02±0.7 and 2.22±0.5 µg ml^{-1} respectively while cellulose recorded maximum activity against *B. cereus* (20±0.4mm) with MIC 2.26±0.4 µg ml^{-1}. Further results showed that *K. pneumoniae* was the most resistive strain amongst the five towards the leaf callus extracts as no zone of inhibition was recorded for control, cellulose, $MnCl_2$ and $CoCl_2$ leaf callus extracts at lower doses (25 µl).

Table 6
Minimum Inhibitory Concentration of different callus extracts of N. sativa under biotic/abiotic elicitation

Pathogenic bacterial strains	Minimum Inhibitory Concentration (µg ml^{-1}) Different Callus Extracts									
	Epi callus	Leaf callus	Pectin epi callus	Pectin leaf callus	Cel Epi callus	Cel leaf callus	$MnCl_2$ epi callus	$MnCl_2$ leaf callus	$CoCl_2$ epi callus	$CoCl_2$ leaf callus
E. coli	2.02±0.3	2.80±0.4	1.98±0.2	2.02±0.7	2.44±0.4	2.48±0.4	1.08±0.3	2.28±0.6	2.45±0.4	3.57±0.2
S typhi	2.52±0.8	3.18±0.5	2.08±0.4	2.37±0.2	2.15±0.7	2.57±0.6	1.85±0.3	2.49±0.8	2.67±0.3	3.44±0.2
B cereus	3.17±0.3	3.37±0.2	1.28±0.5	2.22±0.5	2.03±0.1	2.26±0.4	2.01±0.2	2.47±0.3	3.10±0.4	3.19±0.4
S aureus	3.25±0.4	3.09±0.3	2.39±0.5	4.53±0.2	2.63±0.2	2.43±0.1	2.32±0.1	2.39±0.3	2.83±0.3	3.02±0.3
K. pneumoniae	2.98±0.2	4.9±0.3	2.15±0.3	2.41±0.2	3.89±0.3	3.57±0.6	2.41±0.4	3.35±0.3	2.60±0.2	4.01±0.1

*(*epi-Epicotyl, *Cel- cellulose)*

Table 7
Antibacterial activity of methanolic extracts of N. sativa epicotyl callus under different biotic and abiotic elicitation.

S.No.	Organism	Diameter of Zone of Inhibition (mm)									
		Control callus	Pectin epi callus	Cellulose epi callus	$MnCl_2$ epi callus	$CoCl_2$ epi callus	Antibiotics				
							ST	CF	DO	AM	OF
1.	*E. coli*	26±0.2	24±0.4	18±0.4	32±0.3	22±0.2	10±0.2	12±0.4	14±0.4	10±0.3	9±0.2
2.	*S. typhi*	24±0.2	22±0.5	20±0.2	26±0.5	20±0.4	15±0.3	9±0.3	0	0	0
3.	*B. cereus*	20±0.4	32±0.3	24±0.3	24±0.4	16±0.4	14±0.2	18±0.4	10±0.2	11±0.4	11±0.4
4.	*S. aureus*	20±0.4	20±0.3	16±0.3	22±0.2	18±0.2	19±0.5	14±0.5	12±0.2	0	0
5.	*K.pneumoniae*	22±0.6	22±0.4	14±0.4	20±0.3	20±0.5	19±0.3	13±0.2	10±0.4	0	15±0.5

*(*epi-Epicotyl, * ST-streptomycin, *CF-ciprofloxacin, *DO-doxycycline, *AM-ampicillin and *OF-ofloxacin)*

Table 8
Antibacterial activity of methanolic extracts of N. sativa leaf callus under different biotic and abiotic elicitation.

S.No.	Organism	Diameter of Zone of Inhibition (mm)									
		Control Callus	Pectin leaf callus	Cellulose leaf callus	$MnCl_2$ leaf callus	$CoCl_2$ leaf callus	Antibiotics				
							ST	CF	DO	AM	OF
1.	*E. coli*	20±0.2	18±0.3	16±0.3	18±0.2	10±0.3	10±0.2	12±0.4	14±0.4	10±0.3	9±0.2
2.	*S. typhi*	16±0.3	16±0.4	14±0.2	14±0.4	12±0.4	15±0.3	9±0.3	0	0	0
3.	*B. cereus*	12±0.5	18±0.5	20±0.4	14±0.2	14±0.3	14±0.2	18±0.4	10±0.2	11±0.4	11±0.4
4.	*S. aureus*	18±0.3	10±0.2	14±0.2	14±0.3	18±0.2	19±0.5	14±0.5	12±0.2	0	0
5.	*K. pneumoniae*	16±0.4	16±0.3	12±0.5	16±0.5	16±0.5	19±0.3	13±0.2	10±0.4	0	15±0.5

*(*ST-streptomycin, *CF-ciprofloxacin, *DO-doxycycline, *AM-ampicillin and *OF-ofloxacin)*

DISCUSSION

The use of various herbs and medicinal plants has a long history. The plant kingdom provides a useful source of new medicines, pharmaceutical entities and bioactive compounds that may be used not only for treating human diseases but also for enhancing animal production, health, food safety and quality, whilst conserving environment [21]. In regard to this the herb used in this study i.e. *N. sativa* is known widely and from ancient times for its miraculous therapeutic properties which are attributed because of its rich phytochemical composition belonging to nearly all the classes of secondary metabolites [22]. As plant tissue culture techniques have proved to be the factory of different or any specific metabolite so, in this present study, callus culture cultures of *N. sativa* were established from different parts of the *in vitro* grown seedlings so as to demonstrate their response towards callusing and towards different elicitors in respect to phytochemical accumulation. The results showed that the epicotyl explant had an excellent callusing potentials followed by leaf explant whereas, hypocotyl and root did not responded towards callusing [17]. This difference is due the response of different plant tissues differently to various growth promoting substances, nutrient medium, hormone balance etc [23]. Results of the study showed that elicitation of the epicotyl cultures by cellulose and $CoCl_2$ inhibited cell division and proliferation whereas control cultures along with pectin and $MnCl_2$ elicitated cultures showed early and fast cell division because of which the fresh weight of the elicited cells was always below or equal to the control values throughout the time of culture maintenance which is in favor of the results of previous studies on elicitation [24]. Whereas retarded growth was observed under $CoCl_2$ elicitation in both the cases of leaf and epicotyl which further ended up in lower fresh weight as stated in earlier study [25] where shoot growth increased with increasing concentrations of ethylene inhibitors, except for $CoCl_2$. Treatment with $CoCl_2$ did not result in good shoot organogenesis in *E. angustifolia*. In contrary to this elicitation both biotic and abiotic in leaf explant retarded cell division and proliferation as the callus initiation was seen earlier in the control cultures followed by pectin and $MnCl_2$ after the end of a month which is supported by the earlier work of Ajungla A *et al.,* where elicitation retarded the growth of root culture [26]. This response towards elicitation may again be tissue depended. The *in vitro* plant cultures are capable of producing and accumulating a lot of secondary metabolites[27] such as polyphenols (for example cumarins, flavonoids, tannins, stilbens, hydroxylderivates of cinnamic acid, etc.), alkaloids, terpenoids, and steroids. Studies have even revealed that the secondary metabolites produced by the plant organ cultures are very similar to the secondary metabolites of mother plants and so the results of the study showed the presence of phytochemicals in the calli which are actively reported in the crude extracts of the *N. sativa*. Several quantitative estimations and studies showed that the production of bioactive compounds can vary between differentiated and undifferentiated plant cells. For example, Tanwer *et al.*, [28] reported that calli of *S. acemella* produced a higher amount of sugars when compared with stem, leaves and roots of the intact plant. Study of Jana and Shekhawat [29] also showed that the callus cells of *A. graveolens* produced saponins, while the *in vitro* leaf cells failed to produce the same compound. The phytochemical results of earlier researchers are in the favor of TLC results of this study for screening of secondary metabolites which showed that alkaloids, flavonoids, phenols, cardiac glycosides and terpenoids were present in methanolic extracts [30]. Results have also demonstrated the variations in the presence of phytochemicals amongst the leaf and epicotyl extracts which are due to the differential allocation of the metabolites in different explants taken from the *in vitro* seedlings which have been reported as massive source of these compounds even more than the seeds [6, 31] and also due to the different elicitation. Alkaloid, flavonoid and terpenoid production was greatly induced by the $MnCl_2$ resulting in prominent spots on TLC more than the control as reported in the study of Nay Min Min Thaw Saw [32] which showed higher metabolite production under

treatment. Pectin treatment has also helped in the production of the estimated metabolites in the epicotyl calli more than the leaf callus which is justified by studies which have demonstrated that pectin was found responsible for enhancing oleanolic acid accumulation in *Calendula officinalis* cell suspension cultures [33]. It has also been reported that the addition of pectin to chitosan-treated cell cultures had positive effects on cell growth and amaranthin accumulation in *Chenopodium rubrum* cells [34]. Amongst the abiotic elicitors $MnCl_2$ treated epicotyl callus proved to be the best source of metabolite production followed by the $CoCl_2$ treatment which showed varying induction and inhibition of metabolites. The results of the study are justified by the previous results where $CoCl_2$ (0.3 mg/L), employed to the media has helped the production of taxane (39.75 mg/L) in cell suspension cultures of *Taxus baccata* [35]. Similarly $CoCl_2$ elicitation in epicotyl gave distinct spots of phenols more than any other metabolites which was supported by the results of Ahmed Madi Waheed AL-Mayahi [36]. The inhibitory activity caused by plant or plant callus extracts towards the microbes generally depends upon the concentration of the extracts, type of plant part used and microbes tested [37]. The distribution and concentration of secondary metabolites responsible for inhibitory activity varies according the plant and its parts [38, 39]. This may be a reason for the variation in the inhibitory activity of different extracts of *N. sativa* against the bacterial strains where maximum inhibition was marked by $MnCl_2$ epicotyl followed by pectin epicotyl and pectin leaf callus extracts. The anti-bacterial results of the present study are in agreement with the previous findings that methanolic plant extracts give higher antimicrobial activities [40-42]. The higher antimicrobial activity of the callus extract could be either related to the production of a compound/group of compounds produced only in undifferentiated callus cells or may be produced in higher amounts in these cells when compared to differentiated cells as reported in the previous studies [43-45] suggesting that in vitro cell cultures contain potential active antimicrobial components. Further elicitation also played a remarkable role which showed higher or similar amount of metabolite production along with antibacterial activity when control calli were compared to the pectin and $MnCl_2$ elicitation. Results of this study against the pathogenic bacteria showed higher zone of inhibitions when compared to the study of Kamal *et al.,* [46] on the inhibitory activity caused during germination of the same plant.

CONCLUSION

This study is the first report showing the stimulatory effects of elicitors on the growth and production of important metabolites like terpenoids (thymoquinone and thymol), flavonoids, phenols, alkaloids and glycoside in epicotyl and leaf calli of *N. sativa*. Our results showed that production of phytochemicals importantly terpenoids in the calli of *N. sativa* can be modified by elicitors such as pectin and $MnCl_2$. Usefulness of these callus extracts were further demonstrated on different pathogenic bacterial strains where they have given significant amount of inhibition proving their strong antibacterial property. Increased phytochemical accumulation in response to pectin and $MnCl_2$ demonstrated that these elicitors can be evaluated in experimental botany and the pharmaceutical industry intended for biosynthesis of important terpenoids like Thymoquinone and Thymol in *N. sativa*. Therefore it could be concluded that the results of this study have revealed clear potentiality of *N. sativa* callus extracts as an alternative source for antimicrobial drug.

CONFLICT OF INTEREST

Conflict of interest declared none.

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