RESEARCH ARTICLE

Callus Induction and its Comparative Biochemical Studies from Seeds of *Nigella Sativa Linn.*

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ABSTRACT

Nigella sativa L. is a medicinal spice plant belonging to the family Ranunculaceae. It is an annual flowering herb and widely used medicinal plant throughout the world. *Nigella sativa* has several therapeutic effects which are attributed to its constituents like nigellicine, nigellidine, thymoquinone, dithymoquinone, thymol and carvacrol. Several beneficial pharmacological properties of this plant such as anti-oxidant, anti-bacterial, anti-histaminic, antihypertensive, hypoglycemic, anti-fungal, anti-inflammatory, anti-cancer and immunomodulatory effects were reported. The seeds of this plant have various health benefits in treating Diabetes, epilepsy, cancer, heart attack/damage, leukemia, etc. Thus*, in vitro* callus induction with different concentrations of growth regulators viz NAA, IAA, 2,4-D has been optimized and callus production was maximum at 2,4-D (3.0 mg/l). The highest total level of primary metabolites was found in callus culture of *Nigella sativa* as compared to the seeds of plant. Thus, the present study is an effort to raise *in vitro* callus and its comparative biochemical investigation with seeds for further pharmacological advantages.

Key words: Callus, Quantitative estimation, Pharmacological, Growth regulator, Metabolites.

INTRODUCTION

Medicinal plants have been extensively used to cure various health problems since ancient times. India is considered as the hub of spice cultivation, processing and export. Apart from adding color, flavour and taste, consumption of spices provide infinite health benefits. One such plant is *Nigella sativa*, which is small elegant annual herb distributed and cultivated all over India especially in Punjab, Himachal Pradesh, Gengetic plains, Bihar, Bengal, Assam and Maharashtra. Apart from India, the species is also grown in Syria, Lebanon, Israel and South Europe as well as in Bangladesh, Turkey, Middle-East and the Mediterranean basin[1]. *Nigella sativa* (Family Ranunculaceae) commonly known as black cumin, kunchika, kalika, kalaunji, kalvanijka have been used for thousands of years as a spice and food preservative, as well as a protective and curative remedy for several disorders. Traditionally, there is a common Islamic belief that black seed is a universal remedy for all ailments, but cannot prevent aging or death. Black seed is also known as the curative black cumin in the Holy Bible and is described as Melanthion by Hippocrates and Discords and as Gith by Pliny [2]. During the last two decades, many studies have been conducted, on the effect of *N. sativa* seed extracts on various body systems *in vitro* or *in vivo*. Seed extracts reveal a broad spectrum of pharmacological activities including immune-potentiating, antihistaminic, anti-diabetic, anti-hypertensive, anti-inflammatory and antimicrobial activities. Many of these activities have been due to the quinine constituents of the seed [3]. *N. sativa* has several therapeutic effects which are attributed to its constituents like nigellicine, nigellidine, thymoquinone, dithymoquinone, thymol and carvacrol [4]. Several beneficial pharmacological properties of this plant such as anti-oxidant, anti-bacterial, anti-histaminic, antihypertensive, hypoglycemic, anti-fungal, anti-inflammatory, anticancer and immunomodulatory effects were reported and different therapeutic properties such as reliving bronchial asthma, jaundice, hydrophobia, paralysis, conjunctivitis, piles, skin diseases, anorexia, headache, dysentery, infections, obesity, back pain, hypertension and gastrointestinal problems, have been described for the seeds of *N. sativa* and its oil [5] [6] . Due to

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tremendous medicinal properties, the demands of plant based drugs have been increased in pharmaceutical industries. Therefore *in vitro* callus from seeds have been raised. The aim of present study is to develop a protocol for *in vitro* callus induction and comparative biochemical studies on callus and seeds of *Nigella sativa* Linn.

MATERIALS AND METHODS

Mature seeds of *N. sativa* were collected from Jaipur and adjoining areas and were used for the present study. After removal of the seed coat, the de- coated seeds were surface sterilized by washing in 3-5 drops of Teepol (commercial bleach solution) 15 min, then rinsed with distilled water for 3-4 times. The rinsed seeds were treated with 0.1% H_2SO_4 (1 min.) to break the dormancy of seed and then rinse with distilled water to remove H_2SO_4 and then surface sterilized in 0.1% mercuric chloride for 3 min followed by washing in sterile water 3-4 times. Seeds were then placed on MS (Murashige and Skoog) basal medium [7] for callus induction. Cultured flasks were incubated in culture camber. The temperature of chamber was maintained at 25 ± 1^0 C using air conditioner and light intensity (1200 lux) was provided from fluorescent tubes (40 watt) and incandescent bulbs (40 watts). A photoperiod of 16h light was provided. The cultures were observed and examined every week and final morphogenetic data were recorded. For isolation and quantification of primary metabolites seeds of *N. sativa* along with callus tissue were dried and powdered for evaluation of various primary metabolites.

PRIMARY METABOLITES
(i) Carbohydrates

(a)Total Soluble Sugars
Extraction
The dried experimental plant material (50 mg each) was homogenized in pestle and mortar with 20 mL of 80% ethanol separately and left overnight. Each sample was centrifuged at 1200 rpm for 15 minutes; the supernatants were collected separately and concentrated on a water bath using the method of Dubois *et al.,*[8]. Distilled water was added to make up the volume up to 50 mL and processed further for quantitative analysis.

(b) Starch
Extraction

The residual mass obtained after extraction of total soluble sugars of each of the test samples was suspended in 5 mL of 52% perchloric acid (Mc Cready *et al.,* [9]). Later, 6.5 mL of water was added to each sample and the mixture was shaken vigorously for 5 minutes.

Quantitative Estimation
1 mL of aliquot of each sample was used for the estimation of carbohydrates using the phenol sulphuric acid method of Dubois *et al.,* [8]. A standard regression curve of standard sugar (glucose) was prepared. A stock solution of glucose (100μg mL^{-1}) was prepared in distill water. From this solution, 0.1 to 0.8 mL was pipette out into eight separate test tubes and volume was made up to 1 mL with distilled water. These tubes were kept on ice; 1 mL of 5% phenol was added in each tube and shaken gently. 5 mL of conc. Sulphuric acid added was rapidly poured so that the steam hits the liquid and tubes were gently shaken during the addition of the acid. Finally the mixture was allowed to stand on water bath at $26-30^0$C for 20 minutes. The characteristics yellow orange colour was developed. The optical density was measured at 490 nm using spectrophotometer (Carl Zeiss, Jena DDR, VSU 2 P), after setting for 100% transmission against a blank (distilled water). Standard regression curve was computed between the known concentration of glucose and their respective optical density, which followed Lambert Beer's Law. All samples were analyzed in the same way as described above and contents of total soluble sugars and starch were calculated by computing optical density of each of the samples with standard curve.

(ii) Proteins
Extraction
The test sample (50 mg each) were separately homogenized in 10 mL of cold 10% trichloro acetic acid (TCA) for 30 min and kept at 4^0C for 24 hours. These mixtures were centrifuged separately and supernatants were discarded. Each of the residues was again suspended in 10 mL of 5% TCA and heated at 80^0 C on a water bath for 30 minutes. The samples were cooled centrifuged and supernatants of each were discarded. The residue was then washed with distilled water, dissolved in 10 ml of 1N NaOH and left overnight at room temperature.

Quantitative Estimation
Each of the above samples (1 mL) was taken and the total protein content was estimated using the

spectrophotometer through method of Lowry *et al.,*[10]. A regression curve was prepared. A stock solution of BSA (Sigma Chem. Co., St. Louis, USA) was prepared in 1N NaOH (1 mgL^{-1}). Eight concentrations (ranging from 0.1 to 0.8 mgL^{-1}) were separately measured in test tube and volume of each sample was made to1 mL by adding distilled water. To each, 5 mL of freshly prepared alkaline solution (Prepared by mixing 50 mL of 2% Na$_2$CO$_3$ in 0.1 N NaOH and 1 mL of 0.5 % CuSO$_4$ .5H$_2$O in 1% Sodium potassium tartarate) was added and kept at room temperature for 10 minutes. In each sample 0.5mL of Folin-Ciocalteau reagent (commercially available reagent was diluted with equal volume of distilled water just before use) was added rapidly with immediate mixing and optical density of each sample was measured after 30 minutes at 750 nm using spectrophotometer against blank. Five replicates of each concentration were taken and average value was plotted against their respective concentrations to compute regression curve.

All samples were processed in the same manner and the concentration of the total protein content in each sample was calculated by referring the optical density of each sample with standard curve. Five replicates of each concentration were taken and their mean value was calculated.

(iii) Lipids
Extraction and Quantification
The test sample were dried, powdered and 100mg was macerated with 10 mL distilled water, transferred to a conical flask containing 30 mL of chloroform and methanol (2/1:v/v); Jayaraman [11]. The mixture was thoroughly mixed and left overnight at room temperature in dark for complete extraction. Later, 20 mL of chloroform mixed with 2 mL of water were added and centrifuged. Two layers were separated, the lower layer of chloroform, which contained all the lipids, was carefully collected in the preweighed glass vials and the colored aqueous layer of methanol which contained all the water soluble substances and thick interface layer were discarded in each test sample. The chloroform layers dried *in vacuo* and weighed. Each treatment was repeated thrice and their mean values were calculated.

(iv) Phenols
Extraction
The deproteinized test materials (200 mg each) were macerated with 10 mL of 80% ethanol for 2 hours, and left overnight at room temperature. The mixtures were centrifuged and the supernatants were collected separately and maintained up to 40 mL by adding 80% ethanol .

Quantitative Estimation
Total phenol content in each sample was estimated by spectrophotometer method of Bray and Thorpe [12]. It includes the preparation of a regression curve of standard phenol (Tannic acid). A stock solution of tannic acid was prepared by mixing 40 mg of standard phenol in 1 mL of 80%ethanol. Eight concentrations ranging from 0.1 to 0.8 mL were prepared in the test tube and volume was raised to 1mL by addition of 80% ethanol. To each test tube , 1mL of Folin-Ciocalteau reagent (commercially available reagent was diluted by distilled water in 1:2 ratio just before use) and 2 mL of 20% sodium carbonate solution was added and then mixture was shaken thoroughly. The samples were placed in boiling water for 1 min and cooled under running water. These reaction mixtures were diluted to 25 mL by adding distilled water and optical density was read at 750 nm against a blank. The optical density of each sample was plotted against the respective concentration of total phenols to compute regression curve. The concentrations in the test samples were calculated by referring the respective optical density of test sample against standard curve of tannic acid.

RESULTS AND DISCUSSION
Callus induction
MS medium supplemented with different concentrations of various auxins and cytokinins showed different response for callus induction. Seed showed maximum callus formation on MS medium supplemented with 2,4-D (3.0 mg/L). The callus produced was green, compact and friable. Results are presented in Fig. A.
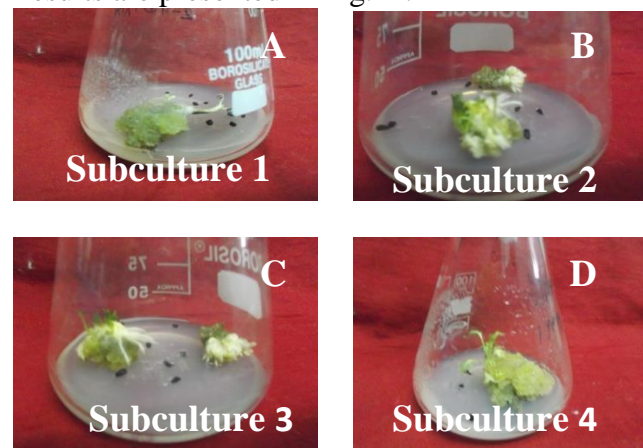

Fig (A): Callusing of *N. sativa* **seed explants during four subcultures 2,4-D (3.0 mg/L).**

Table 1: Total level of primary metabolites (mg/gdw) in various seeds and callus culture of *Nigella sativa*

Plant part	Total soluble sugars	Starch	Protein	Lipids	Phenol
Seeds	54.00 ±0.93	27.46 ±0.83	285.00 ±1.33	29.00 ±0.82	3.90 ±0.04
Callus	79.00 ±0.97	31.39 ±0.67	351.09 ±0.93	47.00 ±0.79	4.70 ±0.23

Primary metabolites

Preliminary phytochemical tests are helpful in analysing the chemical constituents in the plant material that may well lead to their quantitative estimation. Only preliminary work was carried out by some researchers like Kumar *et al.*,[13] and Sandhya and Grampurohit [14]. Preliminary phytochemical studies were carried out which included extractive value estimation, ash analysis, fluorescence analysis, and powder analysis by the treatment of various chemical reagents. Realising the significance of these preliminary phytochemical analysis in medicinal plant species phytochemical investigation on different plant species have been carried out such as *Commiphora wightii* [15], *Digera muricata* [16] and *Coleus aromaticus* [17]. Carbohydrates are major energy and carbon sources in plants. These play a vital role in the life of plants and animals both as structural elements and in maintenance of functional activity. Several workers have studied carbohydrate contents of various medicinal plant species viz. *Prunella vulgaris* [18]. In the present studies, total soluble sugar, starch, protein, lipid and phenol was maximum in callus as compared to seeds. Results are represented in Table-1. Khandelwal *et al.* also observed highest sugar contents in leaf as compared to other *in vivo* and *in vitro* tissues of *Mitragyna parvifolia* [19]. Similar results were observed by Sharma and Sarin in *Sesamum indicum* [20]. According to Sharma [21] the presence of higher levels of starch in intact plant part might be due to more storing capacity to escape the drought conditions. The accumulation of starch may be due to the presence of sucrose in the medium [22] or due to the presence of continuous light [23]. Total proteins were found to be higher in callus than seed. Since, during differentiation the cells are quantitatively changing their activities, new proteins have to be synthesized, thus the protein concentration was high before differentiation. Similar observation was also recorded by Singh *et al.*, [24]. Phenolic compounds are main constituents of many plant species and play an important role in the regulation of plant growth and development. These compounds have a variety of functions in plants like defence mechanism against microbial attack through phytoalexins, control of enzyme, lignification, auxin-activity and cell-wall synthesis. During the present studies, it was observed that among all samples callus had maximum total phenolic contents as compared to seed. Phenolic compounds have been studied in different plant species like *Tylophora indica* [25], *Hamelia patens* and *Mitragyna parvifolia* [19]. Singh *et al.,* reported higher amount of total phenols in non-differentiating callus than in differentiating callus of *Commiphora wightii* [15]. Lipids are found more in callus as compared to seeds.

CONCLUSION

The results of quantitative estimation of primary metabolites from seed and callus of *Nigella sativa* revealed that the callus is a potential source of various active phytoconstituents present in it and which contribute medicinal as well as physiological properties of the plants. These may be used as an alternative to sort out the above mentioned problems as the metabolites of *N. sativa* have shown tremendous medicinal and pharmaceutical properties. Hence, it could be concluded by these findings that we can enhance these pharmaceutically important compounds easily which can be used as natural drugs in future.

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