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Identification of Gene and evaluation of biological activities in berries of *Solanum nigrum* (Linn.)–a medicinal plant of North India

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A B S T R A C T

Solanum nigrum is a small-sized shrub which is distributed throughout India, Ceylon, and all temperate and tropical zones of the world. The fruit is described as highly medicinal being used as laxative, alternative, aphrodisiac, tonic, diuretic; improves appetite and taste; useful in disease of heart and eye, dysentrery, hiccough, vomiting, asthama, bronchitis, fever, urinary discharges and improving the voice and also useful in erysipelas and rat bite, hydrophobia. The aim of this study was to identify the gene which is responsible for the high antioxidant and antimicrobial activity in berries of *Solanum nigrum* (*S. nigrum*) by employing fast and accurate molecular approach to facilitate early detection of two RAPD groups from leaves of red and dark violet berries of *S. nigrum*. A unique band was found present in dark violet berries of *S. nigrum* responsible for higher biological efficacy as compared red berries. The water extract of *S. nigrum* dark violet berries showed highest free radical scavenging activity in DPPH assay. The ethanol extract of *S. nigrum* berries exhibited higher antioxidant potential than water extract in FRAP assay whereas the water extract was found to be most effective in βCL assay**.** The water extract showed very wide zone of inhibition against *streptococcus salvarius* (19.16 ± 0.27 mm in red berries and 37.44 ± 0.29 mm in Dark violet berries) and *Fusarium oxyporum* f.sp-lini (17.71 ± 0.36 mm in red berries and 33.11 ± 0.30 mm in Dark violet berries) at 500 ppm concentration as compared to other strains. This study showed that water and ethanol extracts have powerful antioxidant activity in the berries of *S. nigrum* to that of various antioxidant systems *in vitro*. It also showed antimicrobial activity in water extract of fruits against microbes under test. Moreover, these extracts can be used as easily acceptable source of natural antioxidants, as a possible food supplement or may be useful in pharmaceutical applications.
Keywords: Gene, RAPD, antioxidants, antibacterial activity, *Solanum nigrum*, *Fusarium.*

A R T I C L E I N F O

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1. Introduction

Solanum nigrum (Linn.) family: *Solanaceae*, commonly known as 'Black nightshade' , makoy, deadly nightshade, is a popular traditional medicinal plant, extensively used in India and other parts of the world to cure liver disorders, chronic skin ailments (Psoriasis and ringworm) fevers, diarrhoea, eye diseases, hydrophobia etc. It is native to Eurasia (http://en.wikipedia.org/wiki/Solanum_nigrum).It possesses medicinal properties like antimicrobial, antioxidant, cytotoxic properties, antiulcerogenic, and hepatoprotective activity [20, 32, 9]. *Solanum nigrum* is a potential herbal alternative as anti-cancer agent and one of the active principles reported to be responsible for this action is Diosgenin [36,35, 32]. The berries and leaves are mainly used for medicinal purposes. They are also useful in otopathy, ophthalmopathy, rhinopathy and hepatitis. Decoction of the plant depresses the CNS (central nervous system) and reflexes of the spinal cord [22,27]. The Kondh tribes of Orissa, India use hot aqueous extract of the fruits and leaves as a folk medicine for the treatment of diabetes mellitus [24]. In spite of the economic and medicinal value of *S. nigrum*, no serious attention has been paid for the gene identification and taxonomical identification at the molecular level. This is a prerequisite for the exploitation of the vast genetic variability helding in improvement of the quality and quantity of its drug contents. Studies on genetic diversity within species is undertaken through introduction of molecular analysis techniques, such as randomly amplified polymorphic DNA (RAPD) analysis [37,38]. RAPD analysis is a multilocus arbitrary fingerprinting technique that can be used for determining genetic relationships of various species [31,33,12]. The present study was intended to evaluate the biological activity and identification of gene responsible for the higher biological activity between dark violet and red berries of *Solanum nigrum* (Linn.) using RAPD-PCR method.

2. Materials and Methods

Plant Material and Extraction Procedures:

Solanum nigrum (Linn.) berries (Red and Dark Voilet) were collected from cantonment area of Kanpur, Uttar Pradesh, India. An initial quality evaluation of plant material was carried out as per the guidelines on herbal quality control [39] and a voucher specimen (C2/Chem/DAV/12) has been deposited in Department of Chemistry, Dayanand Anglo Vaidic (DAV) College, Kanpur, Uttar Pradesh, India for further reference. The water and ethanol extracts of berries (Red and Dark Voilet) were prepared according the method of Oke & Mhamburger (2002). DNA extraction from leaves of equal age belonging to Dark violet and red berry of plants was done using hexadecyltrimethyl ammonium bromide (CTAB) extraction method with certain modifications. The procedure with modification was used for detecting RAPD products.

Chemicals:

The chemicals used were 2,2-diphenyl- 1-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-s-triazine (TPTZ), β-carotene (Sigma- Aldrich, USA), ascorbic acid (Merck Darmstadt, Germany)**,** tris HCl, sodium acetate trihydrated, glacial acetic acid, ferric chloride hexahydrated ($FeCl_3.6H_2O$), ferrous sulphate heptahydrated ($FeSO_4.7H_2O$), ethanol and linoleic acid, tween 20, butylated hydroxyl toluene (BHT), and other chemicals used were of analytical grade and were obtained from either Sigma-Aldrich or Merck.

Test Microorganisms and Growth Media:

The microbiological samples *Staphylococcus aureus* and *Streptococcus salivarius* were collected from Microbiology division of Shivam Dental Hospital, Kanpur, Uttar Pradesh, India. The isolates were identified according to published guidelines of Burneti et al. 1994. The fungal isolates *Fusarium oxysporum* f sp. Lini splini and *Aspergillus flavus*, were taken from plant pathology laboratory, Dayanand Anglo Vaidic College, Kanpur,Uttar Pradesh, India. The bacterial and fungal strains were maintained on Mueller–Hinton agar (MHA) and Potato dextrose agar (PDA) plates respectively at 4 °C.

DPPH Free Radical Scavenging Assay:

The free radical scavenging capacity of water and ethanol extract of *S. nigrum* berries were evaluated as per the methodology of Blois [7] as elaborated by Elmastas [14, 15]. DPPH solution (0.1 mM) was prepared and its 3 ml DPPH solution was added to 0.1 ml of water or ethanol at different concentrations (viz. 0.25, 0.5, and 1.0 mg/ml). The absorption was measured at 515 nm up to 30 min or until it

remained constant. The scavenging capacity of DPPH radical was calculated using the following formula [18]: Percent inhibition of DPPH = $A_{control} - A_{sample} / A_{control} X 100$. Where $A_{control}$ is the absorbance of the control reaction and A_{sample} is the absorbance in presence of water or ethanol extract. Ascorbic acid was used as standard.

Ferric Reducing Antioxidant Power (FRAP) assay:

The FRAP value was calculated using the formula described by Benzie and Strain [5,6] based on reduction of Fe^{+3} TPTZ to a blue coloured Fe^{+2} TPTZ. The FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ and 20 mM $FeCl_3.6H_2O$ in a ratio 10:1:1 at 37°C. The absorbance readings were taken after 0.5s and every 15s until 4min and the absorbance was measured at 593nm. The change of absorbance $\Delta A = A_{4min} - A_{0min}$ was calculated and compared to ΔA of Fe^{+2} standard solution. The antioxidant potential of samples was determined from a standard curve and plotted using $FeSO_4.7H_2O$ at a concentration range between 200 and 1000 µM.

β - Carotene Linoleate Bleaching (βCL) Assay:

Total antioxidant activity of *S. nigrum* berries (Dark violet and red) extracts and standards (BHT) were measured by standard methods [21]. One mililitre of β-carotene solution (0.2 mg/ml chloroform) was pipetted into a round bottom flask (100 ml) containing 0.02 ml of linoleic acid and 0.2 ml of 100 % Tween-20. The mixture was then evaporated at 40^0C for 10 min by rotary evaporator to remove chloroform. Then the mixture was immediately diluted with 100 ml of distilled water. The distilled water was added slowly to the mixture with vigorous agitation to form an emulsion. Five ml of emulsion was transferred into test tube containing 0.2 ml of samples in 80 % methanol at different tested concentrations.

The tubes were then gently agitated and incubated at 45ºC for 2 hrs. The absorbance of samples was measured at 470 nm using a spectrophotometer at initial time (t =0) against a blank, consisting of an emulsion without β-carotene. The standards at the same concentration were used for comparison. 0.2 ml of 80% methanol in 5 ml of the above emulsion was used as control. Observations were carried out at 15 min interval. Antioxidant activity was measured in terms of successful bleaching of β-carotene in percentage using the formula: $\% AA = [1-A_0 - A_t / A^0_0 - A^0_t] X 100$, Where A_0 and A^0_0 are the absorbance values measured at initial time of the incubation for samples and control respectively, while A_t and A^0_t were absorbance values measured in the samples or standard and control at t = 120 min.

Antimicrobial Assay:

The antimicrobial activity was evaluated by food poison technique described by Nene and Thapliyal [28]. Water and ethanol extracts were used to prepare 100, 200 and 500 ppm concentrations of nutrient agar for antibacterial and PDA for antifungal assay. Control plates were poured with the respective medium without plant material. 16 hrs culture of *Staphylococcus aureus and Streptococcus salivarius were* diluted with sterile physiological saline solution (PS: 0.85% w/v sodium chloride) so that a concentration of inoculum approximately 108 cfu mL^{-1} could be achieved. Whatman filter paper disc of 5 mm diameter was dipped in this bacterial culture and was placed in the centre of the above prepared nutrient agar plate. PDA petriplates containing different concentrations of plant material were inoculated with 5 mm mycelial disc of fungal species from 7 day old culture grown on PDA. The inoculated plates were incubated at 25 ± 10ºC till fungus covered the control plates. Colony diameter was recorded and percent inhibition in each treatment was calculated. The zones < 8 mm were not considered significant.

Random Amplified Polymorphic DNA (RAPD):

DNA extraction from leaves of equal age belonging to Dark violet and red berry plants was done at Innovation life sciences, Vivek Khand, Gomti Nagar Lucknow, Uttar Pradesh, India using hexadecyltrimethyl ammonium bromide (CTAB) extraction method with certain modification performed by the procedure with modification was used for detecting RAPD products. The PCR reaction mixture was prepared in disposable microamp tubes of 0.2 ml capacity. Amplification reaction was performed in a Bio RAD Gene cycler TM (Bio-RAD, USA). All transfers of PCR ingredients were done with sterile disposable micropipette tips. Each 25 µl reaction mixture contained 4 µl of 10 X reaction buffer (10mM Tris-Hcl, pH8.3 and 50mM Kcl), 3mM $MgCl_2$, 01 unit of tag DNA polymerase, 0.1 mM d NTP mix (all reagents from Genei, India), 0.8 mM of 10 mer primer and approximately 50 mg of sample DNA.

PCR amplification conditions were used as follows: first cycle of 3 steps, denaturation at 90ºC for 1 min the annealing step at 36ºC for 30 sec and 1 min extension at 72 ºC followed by second cycle of 3 steps, denaturation at 94ºC for 5 sec, annealing step at 36 ºC for 15 sec and at 72^0C for 1 min for template extension followed 45 cycles and last cycle was extension step of 7min at 72^0C followed hold at 4^0C. Amplified products (18µl) were mixed with 2 µl of 6x loading dye (0.25% bromophenol blue, 0.25% Xylene cyanol and 40% sucrose, W/V). Amplification products were analysed by electrophoresis on 1.2 % agarose gel containing 0.2 µg/ml of ethidium bromide in 1 X TBE buffer pH 8.0 along with standard molecular weight 1 Kb Gene ruler. Electrophoresis was carried out at a constant voltage 60V for three hrs, visualized under UV trans-illuminator and photographed.

Statistical Analysis

The assays were carried out in triplicate and the results were expressed as means ± standard errors. The differences between the antioxidant and antimicrobial activity of the extracts were analysed using analysis of variance (ANOVA). The statistical analyses were carried out using Origin 8 (Northampton, MA01060, USA).

Table 1: *In-vitro* Antioxidant activity of berries of *Solanum nigrum* (linn.) in terms of percent inhibition of DPPH free radical using DPPH free radicals scavenging assay. (Mean ± SE).

Entry	Concentration (mg/ml)	Black Fruit		Red fruit		Ascorbic acid
		Water extract	Ethanol extract	Water extract	Ethanol extract	
01	0.10	9.15 ± 0.33[a]	8.71 ± 0.33[a]	9.21 ± 0.65[a]	11.12 ± 1.07[a]	10.39 ± 0.93[a]
02	0.25	26.39 ± 0.90[b]	19.15 ± 0.57	28.84 ± 1.17[b]	17.66 ± 0.84	23.74 ± 0.50[b]
03	0.50	53.86 ± 1.01	41.12 ± 0.68[c]	47.96 ± 0.59[c]	45.55 ± 1.42[c]	43.18 ± 0.22[c]
04	1.00	96.98 ± 0.51[ab]	85.90 ± 0.66[d]	85.39 ± 0.92[d]	74.22 ± 1.11	94.67 ± 0.28[ab]

Table 2: *In-vitro* Antioxidant activity of berries of *Solanum nigrum* (linn.) in terms of Fe(II) micromole per litre using Ferric reducing antioxidant potential (FRAP) assay. (Mean ± SE).

Entry	Concentration (mg/ml)	Black Fruit		Red fruit		Ascorbic acid
		Water extract	Ethanol extract	Water extract	Ethanol extract	
01	0.10	73.36 ± 1.91	57.60 ± 0.83	44.50 ± 3.70[a]	42.08 ± 2.55[a]	398 ± 2.85
02	0.25	170.07 ± 5.07	137.47 ± 1.76[b]	114.86 ± 11.44[b]	105.91 ± 6.23	761.32 ± 1.46
03	0.50	357.84 ± 13.30	291.03 ± 3.55[c]	246.915 ± 5.94[c]	222.91 ± 4.72[c]	876.7 ± 1.78
04	1.00	723.05 ± 6.06	501.35 ± 4.45[ab]	500.97 ± 11.44[ab]	458.50 ± 11.62[ab]	1859.25 ± 4.03

Table 3: *In-vitro* Antioxidant activity of berries of *Solanum nigrum* (linn.) using β - carotene linoleate bleaching (βCL) assay. (Mean ± SE).

Entry	Concentration (mg/ml)	Black Fruit		Red fruit		BHT
		Water extract	Ethanol extract	Water extract	Ethanol extract	
01	0.10	8.62 ± 0.42[a]	6.19 ± 0.59[b]	8.03± 0.54[a]	6.63 ± 0.43[b]	9.48 ± 1.06[a]
02	0.25	26.49 ± 0.94	20.33 ± 0.62[c]	19.02 ± 0.52[c]	14.90 ± 0.89	20.54 ± 2.67[c]
03	0.50	45.03± 0.77	40.20 ± 0.67[d]	37.95 ± 0.73[d]	34.99± 0.95	42.57 ± 1.62[d]
04	1.00	89.91± 0.85	80.06± 0.59[ab]	80.94± 0.60[ab]	74.84± 1.34	88.46 ± 3.11

Table 4: *In- vitro* Antimicrobial activity of water extracts of *Solanum nigrum* (linn.) Berries in terms of zone of inhibition in millimeter (mm). (Mean ± SE

Entry	Pathogens	Concentrations of Water Extract					
		Red Berries			Black Berries		
		100 ppm	200 ppm	500ppm	100ppm	200ppm	500ppm
1	*Aspergillus flavus*	2.36 ± 0.23	7.13 ± 0.27[a]	11.29 ± 0.35[b]	7.13 ± 0.60[a]	14.25 ± 0.31[ab]	20.41 ± 0.28
2	*Fusarium Oxyporum* f.sp-*lini*	6.10 ± 0.38	12.34±0.37[b]	17.71 ± 0.36	13.27±0.57[ab]	27.50 ± 0.23	33.11 ± 0.30
3	*Staphylococcus aureus*	5.75 ± 0.19[c]	11.08 ± 0.34[b]	18.86 ± 0.42	8.07 ± 0.33[a]	13.96 ± 0.13[ab]	23.87 ± 0.15
4	*Streptococcus salivarius*	5.11± 0.13[c]	13.04 ± 0.19[ab]	19.16 ± 0.27	14.47 ± 0.35[ab]	26.05 ± 0.27	37.44 ± 0.29
5	Blank	-	-	-	-	-	-

3. Results and Discussion

DPPH Free Radical Scavenging Assay:

The extracts found in increasing order of inhibition of DPPH were Ethanol extract < Ascorbic acid <Water extract (Dark violet berries), (Table 1.) and Ethanol extract < Water extract < Ascorbic acid (red berries), (Table 1.) at all concentrations. The results showed that % inhibition of DPPH free radicals increased according to their concentrations. There was a significant difference ($P < 0.05$) between percent inhibition of DPPH of extracts and their different concentrations. The free radical scavenging activity of *S. nigrum* was significantly much higher than the reported values for *Ocimum basilicum* [19]. Water extracts of *S. nigrum* dark violet berries showed highest antioxidant activity in this assay. Hydrogen donor capacities of polyphenols for DPPH radicals were found proportional to the number of hydroxyl groups [23,30] and the amount of

inactivated DPPH radical was found proportional to the concentration of added flavonoids. The observed lowest inhibition values of extract may be due to the fact that DPPH radical is a long lived less reactive radical, which reacts only with very reactive phenolic and other antioxidants.

Ferric Reducing Antioxidant Power (FRAP) assay:

The ethanol extract of *S. nigrum* berries exhibited higher antioxidant potential than the water extract ones. Increasing order of reducing ability was found as Water extract < Ethanol extract < Ascorbic acid (dark violet berries) (Table 2.) and Water extract < Ethanol extract < Ascorbic acid (red berries) (Table 2.) at 1mg/ml. $FeSO_4.7H_20$ was used for calibration ($R^2 = 0.98$).

The reducing properties are associated with presence of compounds which exerted their action by breaking free radical chain through donating a hydrogen atom [17,13].This suggested presence of inhibiting compounds as a result of FRAP activity in different extracts. The FRAP assay is widely used in evaluation of antioxidant components in dietary polyphenols. The antioxidant activity increases proportionally to polyphenol contents and according to recent reports, a highly positive relationship between total phenols and antioxidant activity appears to be the trend in numerous plant species [1].

β - Carotene Linoleate Bleaching (βCL) Assay:

The antioxidant activities of solvent extracts in βCL assay were observed in increasing order as Ethanol extract < BHT <Water extract (Dark violet berries) and Ethanol extract < Water extract < BHT (red berries) in all concentrations (Table 3). Water extract was found to be the most effective. It is clear that the presence of antioxidants in berries of *S. nigrum* extracts reduced oxidation of β-carotene. The control sample oxidized most rapidly. There were significant differences (p<0.05) between different extracts and standards with same concentrations. Amin I et al. 2002 [4] also found similar results in water and alcoholic extract while working with seaweeds like species of *Laminaria*, *Undaria* and *Hijiki*.

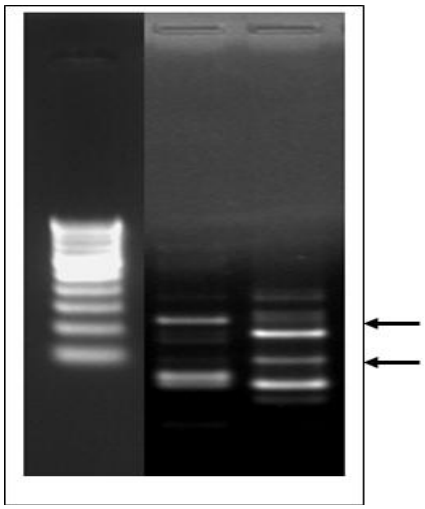

Figure 1: RAPD profile of leaves of *Solanum nigrum*

Antimicrobial Activity:

The preliminary antimicrobial screening of *S. nigrum* (Dark violet and red berries) in water extract gave relatively wide inhibition zones against test strains as compared to positive control (Table 4). The tested microbes belonging to indigenous or resident oral microorganisms can lead to infectious dental diseases. More than 25% of world cereals have been found contaminated with known mycotoxins (especially aflatoxins) and more than 300 fungal metabolites have been reported to be toxic to man and animals [25]. The two fungal species viz *Fusarium oxysporum* f sp. Lini splini was isolated causing wilt of *Linum ustitatissimun* (linseed) and the other *Aspergillus flavus* was a common aflatoxin producing contaminant of food materials. Aflatoxins are the most notorious of the mycotoxins causing acute and chronic toxicoses in foodstuff [11].

The water extract showed very wide zone of inhibition against *streptococcus salvarius* (19.16± 0.27mm in red berries and 37.44± 0.29mm in Dark violet berries) and *Fusarium oxyporum* f.sp-lini (17.71 ± 0.36 mm in red berries and 33.11± 0.30 mm in Dark violet berries) at 500 ppm concentration. Water extract was found very less effective against *Aspergillus flavus* (02.36 ± 0.23 mm) at 100 ppm concentration. The water extract of dark violet berries showing positive results were found to contain alkaloids [34,10], flavonoids [16,26], phenols [2] & terpenoids [3]. In this screening, water extract demonstrated antimicrobial activity which support claims related to the topical use of *S. nigrum* berries extract against dental diseases and also for food preservation.

Gene identification of *Solanum nigrum* (Linn.) varieties based on Random Amplified Polymorphic DNA (RAPD):

In the present study, RAPD marker viz., RAB-05 was used for distinguishing two varieties of *Solanum nigrum*, one producing black berries & another red berries, from Kanpur, Uttar Pradesh, India. Two distinct RAPD profiles were obtained using RAB-05 primer for the two varieties of *S. nigrum* (Fig 1.) Thus, the two varieties of *S. nigrum* can be differentiated at the initial molecular level using random primer RAB-05. Moreover, preliminary analysis of the RAPD profiles also revealed some prominent RAPD markers that can be explored for developing sequence characterized amplified region (SCAR) markers for differentiating the two varieties.

The results of antioxidant property (from all three assays) for both varieties of *S. nigrum* showed that black fruits have comparatively higher antioxidant activity than red fruits. Our data from random analysis by polymerase chain reaction suggested two points. First, both varieties are

different at genome level as it produces different PCR products with same primer. Second, the unique PCR product present in black fruited plants could have direct or indirect impact on the plant metabolites synthesis and accumulation responsible for better biological activity of the plant. The monomorphic band may be responsible for the presence of higher concentration of biological active compounds such as Flavonoids, Alkaloids and phenolic compounds.

4. Conclusion

The results obtained in this study clearly showed that water and ethanol extracts have powerful antioxidant activity against various antioxidant systems *in vitro.* Moreover, these extracts can be used as easily acceptable source of natural antioxidants and as a possible food supplement or may be useful in pharmaceutical applications. The efficacy of water extracts of *S. nigrum* (Linn.) for all the pathogens tested, as well as its higher antioxidant property portends the polarity based potential in biochemicals which are present in the berries. Monomorphic band, also isolated using RAPD, may be responsible for the higher biological activity of the berries. Further studies related to the identification and evaluation of natural antioxidant compounds from berries extracts would give further impetus to the antioxidant therapy by providing new drug candidates.

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