# IN VITRO ANTIOXIDANT ACTIVITY OF AQUEOUS LEAF EXTRACT OF *SOLANUM NIGRUM* LINN.

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**Summary**

The objective of the present study was to establish *in-vitro* antioxidant activity of aqueous leaf extract of *Solanum nigrum* (Family: Solanaceae) in different *in-vitro* experimental methods like Diphenylpicrylhydrazyl (DPPH) radical, peroxide radical, superoxide radical and nitric oxide radical scavenging activity with reference to standard antioxidant ascorbic acid. The total antioxidant potential and reducing power were also determined. The plant extract shown the total antioxidant activity of 54.16 mg ascorbic acid equivalent/g as compared to 117.83 mg of the reference standard ascorbic acid. The reducing power of the extract was found to significant and in a concentration dependent manner. The test extract shown marked antioxidant activity with an $IC_{50}$ value of 165 µg/mL for DPPH radical, 472 µg/mL for superoxide radical, 417 µg/mL for $H_2O_2$ radical and 483 µg/mL for nitric oxide radical. Hence basing on the above results it was concluded that the aqueous extract of leaves of *S.nigrum* showed significant antioxidant activity.

**Keywords:** *Solanum nigrum,* free-radical, antioxidant, DPPH.

## Introduction

The ROS readily attack and induce oxidative damage to various biomolecules including proteins, lipids, lipoproteins and DNA (1). This oxidative damage is a crucial etiological factor implicated in several chronic human diseases such as diabetes mellitus, cancer, atherosclerosis, arthritis, neurodegenerative diseases and also in the ageing process (2-5). Under normal circumstances, the deleterious reactions triggered by these ROS are detoxified and controlled by a system of enzymic (superoxide dismutase, catalase and glutathione peroxidases) and non- enzymic antioxidants which eliminate pro-oxidants and scavenge free radicals (6) and there is equilibrium between the ROS generated and the antioxidants present. However, owing to ROS overproduction and/or inadequate antioxidant defense, this equilibrium is hampered favouring the ROS upsurge that culminates in oxidative stress. Epidemiological studies have found that the intake of antioxidants such as Vitamin C reduces the risk of coronary heart disease and cancer (7).Natural antioxidants, especially phenolics and flavonoids, are safe and also bioactive. Therefore, in recent years, considerable attention has been directed towards identification of plants with antioxidant ability that may be used for human consumption.

*Solanum nigrum* Linn. (Solanaceae) commonly known as Black Berried Nightshade is a common herb found in disturbed habitats, distributed throughout India, Ceylon and all temperate and tropical regions of the world. The plant has a great medicinal value; the leaves are known to be used to treat headache & diseases of nose (8), ringworm (9), heart & liver ailments, wounds & burns (10), toothache (11). The ethnomedical information cited that hot aqueous extract of dried leaves is used for its antidiabetic (12), antiviral (13), antipyretic, anticonvulsant, sedative, antimalarial, antispasmodic & diaphoretic (14), molluscicidal (15), anti-bronchitis & anti-gastralgia (16) activities.The *Kondh* tribes of Orissa, India use the hot aqueous extract of the fruits and leaves as a folk medicine for the treatment of diabetes mellitus. The seed diet has been reported to possess significant hypoglycemic and hypocholesterolemic activity in rats (17). The leaves are reported to contain several constituents e.g. flavonols like Quercetin, Hyperoside (18), Steroids and alkaloids like Sitosterol, Solamargine, Stigmastrol, Campesterol, Cholesterol (19), Solasodine (20) and Sapogenin like Tigogenin (21).In our previous work, the plant extract is reported to possess phenolic and flavonoid

contents (22). The present work aims to confirm the antioxidant potential of the extract in in-vitro using different extensive experimental methods.

## Methods

### Chemicals

All the solvents used in the study were of highest commercially available analytical grade purity and were procured from S.D. Fine Chemicals Limited, Mumbai, India. 1, 1-diphenyl-2-picrylhydrazyl (DPPH), sodium nitropruside (SNP), nitro blue tetrazolium (NBT), nicotinamide adenine dinucleotide (NADH), ascorbic acid, phenazine methosulphate, Griess reagent, trichloroacetic acid, ammonium molybdate, potassium ferricyanide, naphthyl ethylene diamine dihydrochloride and other chemicals were obtained from Sigma Chemical Company, Mumbai, India.

### Plant Materials

Fresh and mature plant of *Solanum nigrum linn*. was collected from Konark, Orissa, India and the plant was and authenticated by taxonomist, Dr. A. K. Pradhan, Professor, Department of Botany, PPD Mahavidyalaya, Tigiria, Cuttack, Orissa, India. A voucher specimen (Regdn. No. SPS/SOAU/2009/008) has been preserved in the institution herbarium of School of Pharmaceutical Sciences, Siksha 'O' Anusandhan University for future reference. After due authentication, fresh matured leaves were collected in bulk, cleaned thoroughly with distilled water, followed by shade drying. The shade dried leaves were powdered in an electrical grinder. The powdered part was kept in a nylon bag in a deep freezer until the time of use.

### Preparation of the extract

Powdered plant material (550 g) was refluxed with 1500 ml of distilled water for 48 h, after defatting with petroleum ether (60-80 $^0$C). Following filtration and concentration in a rotary evaporator, a dark brown viscous residue was obtained, with yield value of 21.52% w/w.

### Antioxidant Studies

### Evaluation of Total antioxidant activity

The assay was done according to Prieto *et al,* 1999 (23). The antioxidant activity of the extract was evaluated through the principle of the formation phosphomolybdenum complex. In this method, an

aliquot of 0.4 ml of sample solution (100 ppm in methanol) was mixed in a vial with 4 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The blank was prepared by replacing the sample with 0.4 ml of methanol. The vials were capped and incubated in a water bath at 95 $^0$C for 90 mins. After cooling the samples at room temperature, the absorbances were measured at 695 nm against the blank. The antioxidant activity was expressed relative to that of ascorbic acid.

**Assay of Reducing Power**

This Ferric Reducing Antioxidant Power (FRAP Assay) of the extract was performed based on the method Yildrim et al 2000; Lu and Foo (24). The assay mixture i.e. 1 ml of plant extract solution (final concentration 100- 500 mg/l) was mixed with 2.5 ml phosphate buffer( 0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide [ K3Fe(CN6)] (10g/l), then mixture was incubated at 50 degree C for 20 minutes. Two and one-half, 2.5 ml of trichloroacetic acid (100g/l) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 ml of the supernatant solution was mixed with 2.5 ml of distilled water and 0.5 ml Fecl3 (1g/l) and absorbance measured at 700nm in V-600 UV-Visible Spectrophotometer (JASCO Ltd, JAPAN). Ascorbic acid was used as standard and phosphate buffer used as blank solution. The absorbances of the final reaction mixture were expressed as mean ± standard error mean. Increased absorbance of the reaction mixture indicates stronger reducing power.

**Free Radical Scavenging Activity**

**DPPH Scavenging Activity**

The free radical scavenging capacity of the aqueous extracts of *Solanum nigrum* was determined using DPPH (1, 1-diphenyl-2-picryl-hydrazyl) (25-27). DPPH solution (0.004% w/v) was prepared in 95% methanol. The extract was mixed with 95% methanol to prepare the stock solution (100 mg/100mL). The concentration of this extract solution was 100 mg /100 ml or 1000µg/ml. From stock solution 1ml, 2ml, 3ml, 4ml & 5ml of this solution were taken in five test tubes & by serial dilution with same solvent were made the final volume of each test tube up to 10 ml whose concentration was then 100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml & 500µg/ml respectively. Freshly prepared DPPH solution (0.004% w/v) was added in each of

these test tubes containing extract (100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml & 500µg/ml) and after 10 min, the absorbance was taken at 517 nm using V-600 UV-Visible Spectrophotometer (JASCO Ltd, JAPAN). Ascorbic acid was used as a reference standard and dissolved in distilled water to make the solution with the concentration 100µg/ml. Control sample was prepared containing the same volume without any extract and reference ascorbic acid. 95% methanol was used as blank. % scavenging of the DPPH free radical was measured using the following equation:

% DPPH radical-scavenging = [(Absorbance of control - Absorbance of test Sample) / (Absorbance of control)] x 100

**Superoxide free-radical scavenging activity**

Measurement of superoxide anion ($O_2^-$) scavenging activity of extracts was based on the slight modified method described elsewhere (28, 29). $O_2^-$ radicals are generated non-enzymatically in Phenazine methosulphate–Nicotinamide adenine dinucleotide phosphate (PMS–NADH) systems by the oxidation of NADH and assayed by the reduction of NBT. In this experiment, the superoxide radicals were generated in 1 mL of Tris-HCl buffer (16 mM, pH 8.0) containing NBT (50 µM) solution and NADH (78 µM) solution. The reaction was started by adding PMS solution (10 µM) to the mixture. The reaction mixture was incubated at 25°C for 5 min, and the absorbance at 560 nm in a spectrophotometer was measured against blank samples. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following formula:

(%) I = (A0 – A1) / (A0) x 100

where A0 was the absorbance of the control and A1 was the absorbance of extract and the standard compound.

**$H_2O_2$ scavenging activity**

Scavenging of $H_2O_2$ by the extract was determined by the method of Ruch *et al*, 1989 (30). One millilitre of the test extract solution [prepared in phosphate buffered saline (PBS)] was incubated with 0.6 ml of 4mM $H_2O_2$ solution (prepared in PBS) for 10 min. The absorbance of the solution was measured at 230 nm against a blank solution containing the extract without $H_2O_2$. The concentration of $H_2O_2$ was spectrophotometrically determined from absorption at 230 nm using the molar absorptivity of 81 $M^{-1}$ $cm^{-1}$.

**NO scavenging activity**

Nitric oxide (NO) was generated from sodium nitropruside (SNP) and measured by Greiss reaction (31). SNP in aqueous solution at physiological pH instinctively generates NO, which intermingles with oxygen to generate nitrite ions that can be anticipated by Greiss reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride). Scavengers of NO with oxygen leading to reduced production of NO. SNP (5 mM) in phosphate buffer saline (PBS) was mixed with different concentration of (100-500 µg/mL) drug dissolved in suitable solvent and incubated at 25$^{o}$C for 150 min. The above samples were reacted with Greiss reagent. The absorbance of chromophore created during diazotization of nitrite with sulphanilamide and following coupling with naphthyl ethylene diamine was read at 546 nm and referred to the absorbance of standard solution of potassium nitrite treated in the same way with Greiss reagent.

**Statistical analysis**

All the experimental results were expressed as mean ± SEM of three parallel measurements. The significancy is measured by using t' test, $p < 0.05$, considered as significant.

## Results and Discussion

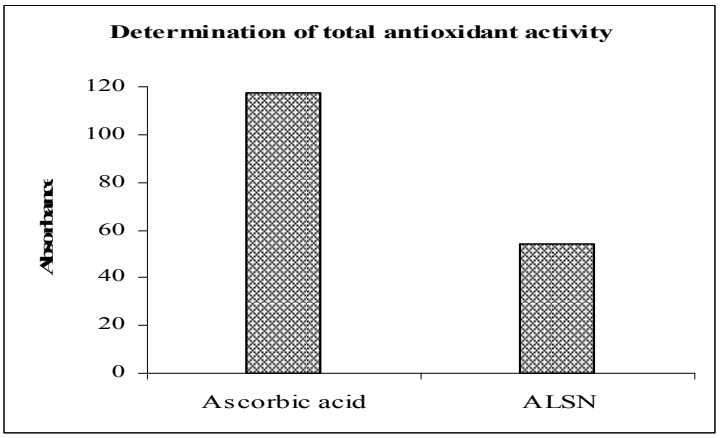
**Total antioxidant activity and assay of Reducing Power**

The leaf extract of *S. nigrum* was carried out using distilled water as solvent and found that the yield value of the extract is 21.52% (w/w). The said plant extract is reported to possess the phenolic content (33.83 µg of pyrocatechol equivalent /500mg) and flavonoid content (5.86 mg equivalent of quercetin /gm) (22). It is well known that the presence of polyphenols and flavonoids in plants, mainly responsible for their dynamic antioxidant activity, the obtained amount of total phenolics & flavonoids in the extract indicated the extract to possess a high antioxidant activity.

The total antioxidant activity and the ferric reducing power of test extract and standard drug ascorbic acid were investigated by using, different in vitro methods and are presented in Fig. 1 and 2 respectively. Fig.1 shows that the plant extract was found to have total antioxidant activity of 54.16 mg ascorbic acid equivalent/gm in comparison to that of the reference standard ascorbic acid which

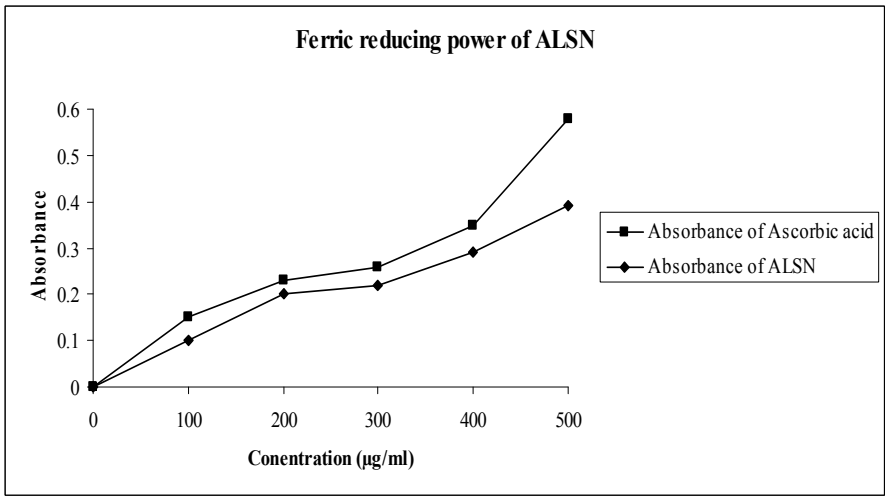
registered 117.83 mg ascorbic acid equivalent/gm. Similarly, Fig.2 represented the reductive capabilities of the aqueous extract compared to that of ascorbic acid and found that the extract potentiate in reducing the $Fe^{3+}$ / ferricyanide complex to the ferrous form ($Fe^{2+}$) which was monitored by measuring the formation of Perl's Prussian blue at 700nm. The reducing power of the extract was found to be significant and in a concentration dependent manner.

**Fig. 1. Determination of Total Antioxidant activity of *Aqueous Leave extracts of S.nigrum***




Values are expressed in MEAN ± S.E.M (n =3).

**Fig. 2. Determination of Ferric reducing power of *Aqueous Leave extracts of S.N (ALSN)***




Values are expressed in MEAN ± S.E.M (n =3).

**Free Radical Scavenging Activity**

The capacity of *S. nigrum* leaf extract to scavenge DPPH, O2•−, •OH and NO were measured and the results are shown in Table-1.

The aqueous extract of *S. nigrum* scavenges DPPH radical in a concentration dependent manner. The antioxidants react with DPPH, a purple colored stable free radical and convert it into a colorless α-α-diphenyl-β-picryl hydrazine. The amount of DPPH reduced could be quantified by measuring a decrease in absorbance at 517 nm. The leaf extract of *S. nigrum* significantly and concentration dependently reduced DPPH radicals. However at a concentration of 500µg/ml, the extract significantly ($p< 0.001$) scavenged 94.0 % of DPPH radicals and had an $IC_{50}$ value of 165µg/ml. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. It has characteristic absorbance maxima at 517 nm, which is widely used to evaluate the free radical scavenging effect of natural antioxidants (32).

Many researchers have reported positive correlation between free radical scavenging activity and total phenolic compound. DPPH radical scavenging activity increased with the increase of phenolic compound content (33-35). The DPPH scavenging ability of the extract may be attributed to its hydrogen donating ability.

The primary free radical in most biological systems is Superoxide ($O_2$•−). Although $O_2$•− itself is quite uncreative compared to the other radicals, but it can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals (36). From the investigations, it was found that the *S. nigrum* leaf extract scavenged $O_2$•− significantly and in a concentration dependent manner. The $O_2$•− scavenging activity was determined by Phenazine methosulphate/NADH-NBT system wherein $O_2$•− derived from dissolved oxygen by Phenazine methosulphate/NADH coupling reaction reduces NBT. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anions in the reaction mixture. The test extract exhibited a maximum of 56.4% superoxide scavenging activity with a significant extent ($p<0.01$) at a concentration of 500 µg/ml and the $IC_{50}$ value is 417µg/ml. The spontaneous or catalytic dismutation of $O_2$•− leads to the formation of $H_2O_2$, which in the presence of a transition metal ion like $Fe^{3+}$,

decomposes into •OH radicals, a highly damaging species in free radical pathology (37). The extract is found to scavenge 57.6 % of $H_2O_2$ and in a significant extent ($p<0.001$) at 500 µg/ml with a calculated $IC_{50}$ value of 472µg/ml. However, as compared to DPPH; $O_2$•−, $H_2O_2$ and NO were weakly scavenged by the extract.

*S. nigrum* leaf extract at a concentration of 500 µg/ml also quenched 51.7% NO released by a NO donor, SNP in a significant manner ($p<0.01$) showing the $IC_{50}$ value is 483µg/ml. Incubation of SNP solution in PBS at 25 $^0$C for 150 min resulted in the release of NO. The extract effectively and dose dependently decreased the release of NO (Table-1). Control experiments showed that, even at high concentrations, the extract did not interfere with the reaction between nitrite and Griess reagent. ROS like $O_2$•− may react with NO and give rise to various other reactive nitrogen species (RNS) such as $NO_2$, $N_2O_4$, peroxynitrite. Both ROS and RNS together attack and damage various cellular molecules. Virtually all cellular components including lipids, proteins, nucleic acids, carbohydrates are susceptible to oxidative damage (38). *S. nigrum* leaf extract, owing to its radical scavenging ability may provide protection against oxidative damage induced to the biomolecules: proteins and lipids.

## Conclusions

Overall, it could be concluded that *S. nigrum* leaves bear a potent antioxidant activity due to their phyto-constituents which in turn scavenge free radicals. The preliminary studies showed the presence of a number of polyphenols and flavonoids, which may be responsible for its antioxidant activities.

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**TABLE – Determination of 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide ($O_2$•−), hydrogen peroxide ($H_2O_2$) and nitric oxide (NO) scavenging activity of *ALSN***

| Treatment & Concentration | DPPH | $O_2$•− | $H_2O_2$ | NO |
|---|---|---|---|---|
| | % of control | % of control | % of control | % of control |
| **Control** | 100.0 ± 5.4 (00) | 100.0 ± 4.2 (00) | 100.0 ± 3.7 (00) | 100.0 ±2.7 (00) |
| **Ascorbic acid** | | | | |
| 100 µg/ml | 29.6 ± 2.1[c] (70.4) | 43.3 ± 3.7[b] (56.7) | 61.4 ± 4.8 (38.6) | 77.4 ± 3.6 (22.6) |
| 200 µg/ml | 13.2 ± 1.5[c] (86.8) | 37.1 ± 4.8[b] (62.9) | 45.7 ± 3.5 (54.3) | 68.3 ± 3.9[a] (31.7) |
| 300 µg/ml | 3.4 ± 0.41[c] (96.6) | 29.4 ± 2.6[c] (70.6) | 38.6 ± 2.4[b] (61.4) | 59.1 ± 2.4[b] (40.9) |
| 400 µg/ml | 0.00 (100) | 23.9 ± 3.1[c] (76.1) | 27.2 ± 1.7[c] (72.8) | 56.7 ± 3.8[b] (43.3) |
| 500 µg/ml | 0.00 (100) | 18.7 ± 2.3[c] (81.3) | 21.5 ± 1.8[c] (78.5) | 33.5 ± 4.9[c] (66.5) |
| **ALSN** | | | | |
| 100 µg/ml | 57.4 ± 4.8[a] (42.6) | 92.5 ± 7.4 (7.5) | 97.3 ± 3.1 (2.7) | 91.2 ± 5.7 (8.8) |
| 200 µg/ml | 46.8 ± 4.1[a] (53.2) | 83.7 ± 5.9 (16.3) | 91.5 ± 4.8 (8.5) | 88.3 ± 6.1 (11.7) |
| 300 µg/ml | 31.5 ± 4.7[a] (68.5) | 68.9 ± 4.2[a] (31.1) | 82.7 ± 5.0 (17.3) | 73.5 ± 5.3 (26.5) |
| 400 µg/ml | 17.3 ± 3.2[b] (82.7) | 51.3 ± 3.2[a] (48.7) | 67.9 ± 4.6[a] (32.1) | 67.9 ± 3.5[a] (32.1) |
| 500 µg/ml | 6.0 ± 1.1[c] (94.0) | 43.6 ± 2.7[b] (56.4) | 42.4 ± 4.7[c] (57.6) | 48.3 ± 3.9[b] (51.7) |
| $IC_{50}$ | 165 | 417 | 472 | 483 |

Values are expressed in MEAN ± S.E.M (n =3). Values expressed in the parenthesis indicate % scavenging activity.
(t-value denotes statistical significance at [a]$p<0.05$, [b]$p<0.01$ and [c]$p<0.001$ respectively, in comparison to control group).

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