# Isolation and identification of an antioxidant constituent from *Satyrium nepalense* (Himalayan Orchid)

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***Abstract***
*Plants are the bio-factories of natural molecules and compounds which are the important source of well-known herbal drugs and formulations. In spite of several known molecules, still there are lot which are yet unexplored. These molecules may have different pharmacological properties.*

*The present study was performed in order to isolate and identify the novel compound SNP2 from Satyrium nepalense (Himalayan Orchid) for determination of in vitro antioxidant activity via conventional procedures. The results determined SNP2 as Gluconic acid molecule via LC-MS/MS technique. The molecule possessed significant antioxidant activity as determined in the study and thus can be utilized as a promising candidate in category of an antioxidant and nutraceutical agent.*

**Keywords:** Antioxidant activity, *Satyrium nepalense* (Himalayan Orchid), Gluconic acid, Nutraceutical agent.

## Introduction

Himalayas are well known for their richest hot spot of biodiversity in the world. The Indian Himalaya Region harbors approximately 8644 plant species belonging to 1748 families. These plant species are known as medicinal plants and maximum species have been reported around up to 1800 m altitudinal range[1]. Natural products, including plants, animals and minerals have been the basis of treatment of diseases from time immemorial. Medicine is next to the three basic needs required by human in order to survive or make their life comfortable. Use of herbal medicine is as old as human race itself. Medicinal plants are one of the most important components of the forests of Himalaya and are well known for their efficacy in coping with various diseases[2].

Uttarakhand, also known as "Dev Bhomi" lies in the western Himalayan region and is famous for its rich variety of herbs, medicinal and aromatic plant species. Garhwal Himalaya is one of the richest floristic zones of India and contains more than 300 species of medicinally important plants[3].

Different medicinal plants from Garhwal region of North West Himalaya were investigated for antimicrobial, antioxidant and anti-inflammatory activities[4-7]. The rural communities in Chamoli, the remote district of Uttarakhand, have their own way of living with in social and cultural moorings. Irrespective of their simplicity and complexity, these communities hold rich traditional knowledge on medicinal plants. The Orchidaceae is a diverse and widespread family of flowering plants, with blooms that are often colourful and fragrant. Beautiful flower orchids are becoming a rarity, losing out to human greed. Orchids are not only important for their aesthetic value but also because they work as ecological indicators. Orchids are mysterious in many ways. These are increasingly being cultivated throughout the world. The incredible shapes and colours of their flowers and their long vase-life have attracted many generations of mankind. 1141 species of orchids in 166 genera are recorded from India[8].

In Uttarakhand, total of 72 genera with 236 species of orchids are recorded[9]. Taking all the monocotyledonous families into account, Orchidaceae is the 2nd largest family after Poaceae in Uttarakhand. Orchids are popular for their healing properties too. *Satyrium nepalense* is a medicinal orchid also known as Salam mishri, usually found at higher altitudes (2400-3000 m) of the Indian Himalayan Region (IHR). It is a terrestrial herb, commonly used by native people of Uttarakhand as folk remedy against various complaints. Decoction of tubers, roots and stems of the plant has been used in various infectious diseases and also as a nutritional supplement since ancient time. It is also used as a food, tonic, in diarrhoea, malaria and dysentery[10].

In the present study antioxidant constituent from the methanolic extract of *Satyrium nepalense* has been isolated and identified. Methanolic extracts of *Satyrium nepalense* exhibit remarkable antioxidant and antibacterial activities among the tested extracts possibly due to the presence of phenolic acids and flavonoids, in particular gallic acid and quercetin as confirmed by LC-MS/MS analysis[11].

## Material and Methods

**Sample Collection:** Tubers of *Satayrium nepalense* were collected from Chamoli district of Uttarakhand at an altitude of 2000-2800 meters and identified by the Botanical Survey of India, Dehradun, Uttarakhand, India.

**Extraction procedure:** The extraction procedure was utilized with some modifications[12]. The tubers were washed with running water and then with distilled water to remove dust and other contaminants. They were then shade dried at an average temperature. The plant material was coarsely powdered with the help of an electric blender and passed via

sieve no. 40 and stored in a closed container for further use. Different organic solvents (petroleum ether, chloroform, methanol, and water) were used for the extraction of polar and non-polar organic compound. The powdered material (100 g) of *Satyrium nepalense* (Tubers) was first extracted with petroleum ether using Soxhlet apparatus for 72 h at room temperature and then successively extracted with chloroform, methanol, and water. All extracts were concentrated and dried by using vacuum rotary evaporator to evaporate solvents, while the concentrated extracts were kept in desiccators until further used.

**Fractionation procedure for the extraction of active principle methanol extract of *S. nepalense*:** The methanol soluble fraction (50 g) of *S. nepalense* tuber was mixed with 10 g silica gel (Qualigen, 100- 200 mesh) prepared in chloroform. The column was subjected to diverse solvent systems: chloroform (100%), chloroform-methanol (from 95:5 to 50:50). Elutes were collected on the basis of their thin layer chromatography profiles. These were combined into 10 groups (from SNP1 to SNP10). Fraction SNP2 was crystallized at room temperature and further identified by LC-MS/MS.

**Identification of crystallized constituents from the *S. nepalense* tuber fractions by LC-MS/MS:** Identification of the main compounds was carried out by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The LC-MS instrument used was of Agilent Technologies India Pvt. Ltd., Bangalore. The method utilizes single quadrupole (SQ) LC/MSD. The machine used was an auto sampler, column heater and a photo-diode array (PDA) detector. The column used for the study was a reversed phase RP C18 (150 X 3.0 mm, 2.5 µm). The column temperature was kept constant at 40˚C. The two types of mobile phases were used as 2mM ammonium acetate mixed in water as mobile phase A and acetonitrile as mobile phase B.

Chromatographic separation was achieved with following gradient program: 0 minute – 5%B; 1 minute – 5%B; 15 minutes - 97%B; 20 minute – 97% B; 21 minute – 5%B; 25 minute – 5%B. The flow rate of 0.4 mL/minute was maintained. The control and treated samples were dissolved in a mixture of water and methanol (60:40 *v/v*) to prepare 1 mg/mL stock solution.

An aliquot of 2 µL of the stock solution was used for analysis by LC-ESI-MS and the total run time was maintained for 25 minutes. Mass spectrometric analysis was accompanied on a Triple Quad (Waters Quattro Premier XE, USA) mass spectrometer equipped with an electro spray ionization (ESI) source with the following parameters: electrospray capillary voltage 3.5 kV; source temperature 100˚C; desolvation temperature 350˚C; cone voltage 30 V; desolvation gas flow 1000 L/h and cone gas flow 60 L/h. Nitrogen was used in the electro-spray ionization source. The multiplier voltage was set at 650 V. LC-MS was taken in positive and negative ionization mode and with the full scan (*m/z* 50-1400). The total ion chromatogram was recorded.

**Methods for determination of antioxidant activity**

**DPPH free radical scavenging activity:** Different solutions of the active principle SNP2 for the DPPH test[13] were prepared by re-dissolving 0.2 g of sample in 10 ml of the specific solvent. The working solution of DPPH solution was prepared after mixing 0.025 g of DPPH in 1000 ml of methanol. From the above working solution of DPPH, 2 ml of the DPPH solution was mixed with 40 µl of each of the sample solution and transferred to a cuvette. The reaction solution was monitored at 515 nm after an incubation period of 30 minutes at room temperature using a UV-Visible Systronics spectrophotometer. The inhibition percentage of the absorbance of DPPH solution was calculated using the following equation:

Inhibition %= (AbsT=0 min -AbsT=30 min)/ AbsT=0 min ×100

where AbsT=0 min was recorded as absorbance of DPPH at zero time and AbsT=30 minutes was recorded as the absorbance of DPPH after 30 minutes of incubation.

Ascorbic acid (0.5 mM) was dissolved in methanol and used as a standard to convert the inhibition capability of active constituent solution to the ascorbic acid equivalent. IC50, concentration of the sample required to scavenge 50% of DPPH free radicals was also determined.

**Superoxide Anion Radical Scavenging Activity:** Superoxide anion radical scavenging Activity was measured with some modifications[14]. The active constituent was mixed with 3 ml of reaction buffer solution (pH 7.4) containing 1.3 µM riboflavin, 0.02 M methionine and 5.1 µmolar NBT. The reaction solution was illuminated by exposure to 30W fluorescent lamps for 20 minutes and the absorbance was measured at 560 nm using a spectrophotometer. Ascorbic acid was used as positive control and the reaction mixture without any sample was used as negative control. The superoxide anion radical scavenging activity (%) will be calculated as:

$$\frac{\text{Ao} - \text{As}}{\text{Ao}} \times 100$$

where Ao = absorbance of positive control and As = absorbance of sample.

**Scavenging of Hydrogen peroxide ($H_2O_2$):** Percent scavenging of $H_2O_2$ was determined[15]. A solution of $H_2O_2$ 40 mM was prepared in phosphate buffer (pH, 7.4). $H_2O_2$ concentration was determined spectrophotometrically from absorbance at 230 nm by using UV-VIS spectrophotometer. Active constituent was added to $H_2O_2$ solution. The absorbance of $H_2O_2$ at 230 nm was observed after 10 minutes against a blank solution containing phosphate buffer without

$H_2O_2$. Ascorbic acid was used as a positive control. The % scavenging $H_2O_2$ was determined as:

$$\frac{Ao - As}{Ao} \times 100$$

where A0 = the absorbance of positive control and As = the absorbance of sample.

## Results and Discussion

The SNME extract was fractionated in different combinations of solvents by column chromatography leading to the isolation of ten fractions named SNP1, SNP2, SNP3, SNP 4, SNP5, SNP6, SNP7, SNP8, SNP9 and SNP10. The crystallized fraction SNP2 was identified as gluconic acid molecule having *m/z* value 195. The results are shown in figure 1.

Further the antioxidant activities of the molecule gluconic acid were determined by conventional methods. The results showed that the molecule gluconic acid possessed significant antioxidant activity as determined by the conventional methods shown in table 1 and figure 2. The standard antioxidant used was Ascorbic acid (1 mg/ml) solution prepared in sterilized DMSO. The results revealed that SNP2 molecule (Gluconic acid) in 1 mg/ml concentration showed IC50 – 15.67 µg/ml in comparison to ascorbic acid (1 mg/ml) which showed IC50 value as 11.08 µg/ml as determined by DPPH assay. It is meant that lower is the IC50 value, more is the antioxidant activity. Further, antioxidant activity was determined by total antioxidant activity assay (by taking the absorbance at 695 nm).

The results showed absorbance value of SNP2 (A695=0.88) in comparison to ascorbic acid (A695=1.85). This assay states that higher is the absorbance value, higher is the antioxidant activity. The antioxidant activities were also determined by percent inhibition of superoxide anion radical and percent inhibition of peroxides via hydrogen per oxide assay. The results revealed that SNP2 molecule possessed 75.12% inhibition of superoxides in comparison to ascorbic acid which showed 85.56% inhibition. Simultaneously, SNP2 possessed 53.78% inhibition of peroxide in comparison to ascorbic acid which possessed 85.23% inhibition. The results of all the conventional procedures were found to be correlated with each other. The previous findings also established the antioxidant properties of gluconic acid[16-22].

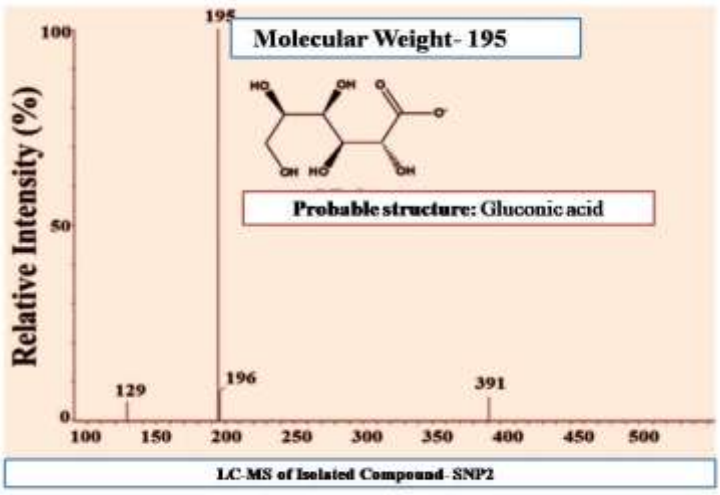


**Figure 1: LC-MS/MS of SNP2 (Gluconic acid)**

**Table 1**
**Antioxidant activity assays of isolated Gluconic acid molecule (SNP2)**

| Extracts and Standard (1 mg/ml) | Assays of determination of antioxidant activity | | | |
|---|---|---|---|---|
| | DPPH free radical scavenging activity (IC50) | Total antioxidant activity (A695) | Superoxide anion radical scavenging activity (Percent inhibition) | Scavenging of hydrogen peroxide (Percent inhibition) |
| SNP2 | 15.67±0.056 | 0.88±0.035 | 75.12±0.047 | 53.78±0.04 |
| Standard (Ascorbic acid) | 11.08±0.034 | 1.85±0.047 | 86.56±0.038 | 85.23±0.03 |

*SNP2, Gluconic acid; *±SD; Level of significance, $p<0.05$

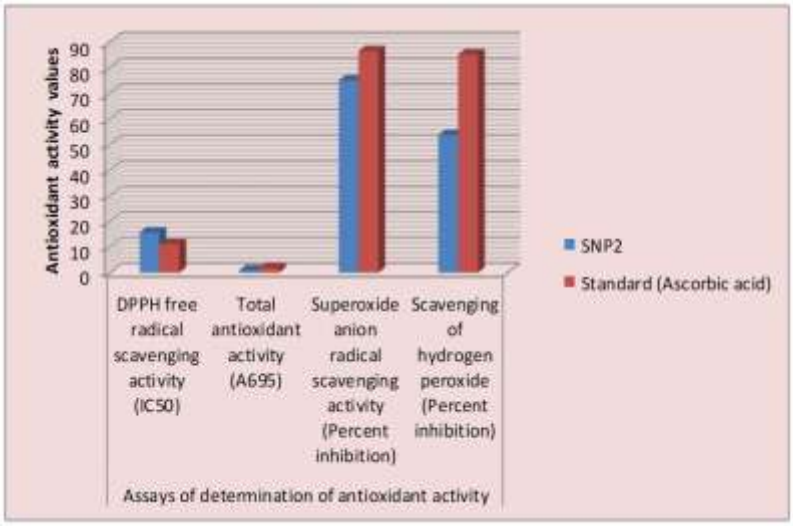


**Figure 2: Graphical representation of antioxidant activities as determined by conventional procedures**

## Conclusion

The results of the present study concluded that SNP2 molecule (Gluconic acid) possessed significant antioxidant activities as determined by conventional methods. The molecule gluconic acid thus can be utilized as a significant antioxidant and as a natural cure in the form of nutritional supplement and effective nutraceutical in maintaining healthy body and prevention against different diseases.

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