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ISOLATION AND SCREENING OF ACETAMIDE DERIVATIVE AS A POTENT ANTIOXIDANT AGENT FROM *SATYRIUM NEPALENSE*

Monika Kawra[1, 2*] and Sarla Saklani[1]

[1]Department of Pharmaceutical Chemistry, HNBGU Srinagar, Garhwal (Uttarakhand), India;
[2]Uttarakhand Technical University (UTU), Dehradun (Uttarakhand), India.

Abstract

Plants served as the source of mankind prom past years. Different ancient Indian literatures and Ayurvedic literatures such as Charka Sanhita have elaborated the use and applications of medicinal plants. The Garhwal Himalayas are the repositories of such herbs and medicinal plants which are less explored till yet. These plants and herbs are the source of natural molecules known as phytochemicals having use in different pharmacological activities. The present investigation is about the isolation and characterization of novel molecule in the form of Acetamide derivative as determined via LC-MS/MS spectra from *Satyrium nepalense*. The studies revealed the isolated Acetamide derivative as a potent antioxidant agent as determined by different conventional procedures viz DPPH free radical scavenging activity, Total antioxidant activity determination, Percent inhibition of superoxide anion radicals and Percent Hydrogen peroxide free radicals inhibition activity. The study is the first ever report of the isolation, identification and antioxidant screening of Acetamide derivative from *Satyrium nepalense* from Garhwal Himalayas.

Key words : *Satyrium nepalense*, tubers extracts, antioxidant activity, Acetamide derivative, nutraceutical, antioxidant agent.

Introduction

The Indian Himalaya is home to more than 8000 species of vascular plants of which 1748 are known for their medicinal properties (Joshi *et al.,* 2017). These higher plants have played key roles in the lives of tribal peoples living in the Himalayan region by providing forest products for both food and medicine. From prehistorically time, the Himalayan flora has been in use for various purposes including some scientific therapeutic uses. The old Indian literature such as Rig-veda, Atherveda, Charka Sanhita, included various uses of plants of Himalaya region (Sharma *et al.,* 2011). Herbal derived anticancer constituents were described (Kawra *et al.,* 2019). Chemically, plants may have alkaloids, tannins, glycosides, steroids or other groups of compounds which may have a marked pharmaceutical role. These phyto-constituents give specific distinctiveness and properties to plants. Therefore, the analysis of these chemical constituents would help in determining various biological activities of plants. Functional groups and shape of molecules are responsible for the medicinal properties of bioactive compounds present in medicinal plants. LC-MS technique is an efficient technique used in pharmaceutical industry for isolation and identification of phytochemicals. Since the technique, LC–MS is able to ionize very large molecules, the upper scanning range of a mass analyser is more important here in comparison to GC–MS (Allouche *et al.,* 2016). LC-MS and LC-MS–MS techniques are very efficient for identification of phytochemicals such as alkaloids, coumarins, phenolic acids, flavonoids, isoflavonoids, terpenes, and steroids (Rahman, 2018). Natural antioxidants are commonly derived from plant sources and are known for their ability to diminish harm, resulted by some reactive species: oxygen, nitrogen, or even chlorine. Antioxidants are considered important nutraceuticals on account of many health benefits (Droge, 2002; Lee *et al.,* 2004). A number of scientific studies are addressing the varied health benefits of antioxidant supplementation in processes like stress, ageing, pathogen infestation, reduce cell damaging effects, apoptosis and neurological diseases of free

***Author for correspondence :** E-mail: kawra.monika8@gmail.com

radicals. Certain conventional and synthetic antioxidants like butylated hydroxytoluene (BHT) and butylated hydroxyl-anisole (BHA) reduces oxidative stress caused by the generation of free radicals. However, these synthetic antioxidants are reported dangerous to mankind as they may cause side effects, thus there is a need of alternate novel antioxidants which are non-toxic to human health (Ayoub *et al.,* 2017). Different natural molecules and phytochemicals were isolated and characterized as an antioxidant from plants of North West Himalayan Garhwal region of Uttarakhand (Mathur *et al.,* 2011a-d). *Satyrium nepalense* (Orchidaceae), also known as Salam mishri, is a medicinal herb found at higher altitudes (2400-3000 m) of the Indian Himalayan Region (IHR). Local inhabitants of Uttarakhand (India) commonly use this terrestrial herb as an energizing tonic. Decoction of tubers, roots and stems of this plant has been mainly used to treat various ailments such as diarrhoea, dysentery, fever, malaria and as a nutritional supplement since ancient times (Mishra, 2018). In the present study, active principle (antioxidant agent/compound) was isolated and identified from the methanolic extract of *Satyrium nepalense* via *conventional vitro* antioxidant procedures.

Materials and Methods

Sample Collection

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

Extraction procedure

The extraction procedure was utilized with some modifications (Bibi, 2016). The tubers were washed with running water followed by distilled water in order to remove dust and other contaminants. The material was dried under shade in indirect sunlight. 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), were 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.

Column fractionation for the extraction of active principle

The methanolic 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 varying in different concentrations *i.e.* variable ratios, 95:5 v/v to 50:50 v/v. Elutes were collected on the basis of their thin layer chroma-tography profiles. These were combined into 10 groups (from SNP1 to SNP10). Fraction, SNP1 was crystallized at room temperature and further identified by LC-MS/MS.

Identification of active principle in column fraction via LC-MS/MS

Identification of the compound (SNP1) was car-ried 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 quadruple (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 40ÚC. The two types of mobile phases were used as 2 mM 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 a 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 100^0C; desolvation temperature 350^0C; 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 SNP1 for the DPPH test (Fargare, 1995) 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

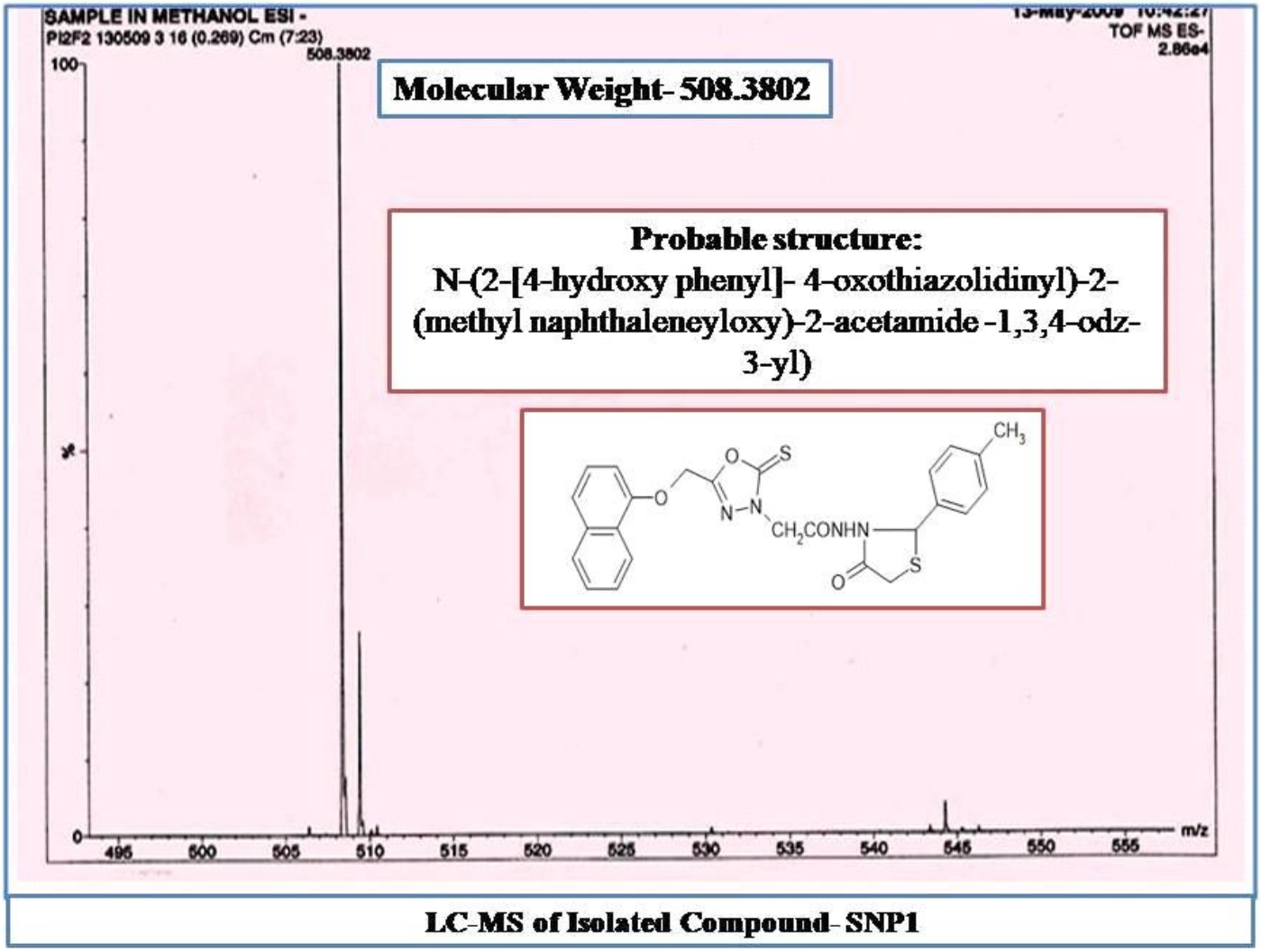

Fig. 1: LC-MS/MS spectra of SNP1 (Acetamide derivative).

Table 1: Antioxidant activity assays of isolated Acetamide derivative (SNP1).

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)
SNP1	8.23±0.028	2.24±0.022	86.56±0.035	67.12±0.027
Standard (Ascorbic acid)	11.08±0.034	1.85±0.047	86.56±0.038	85.23±0.03

*SNP1, Acetamide derivative; *±SD; Level of significance, $p<0.05$.

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

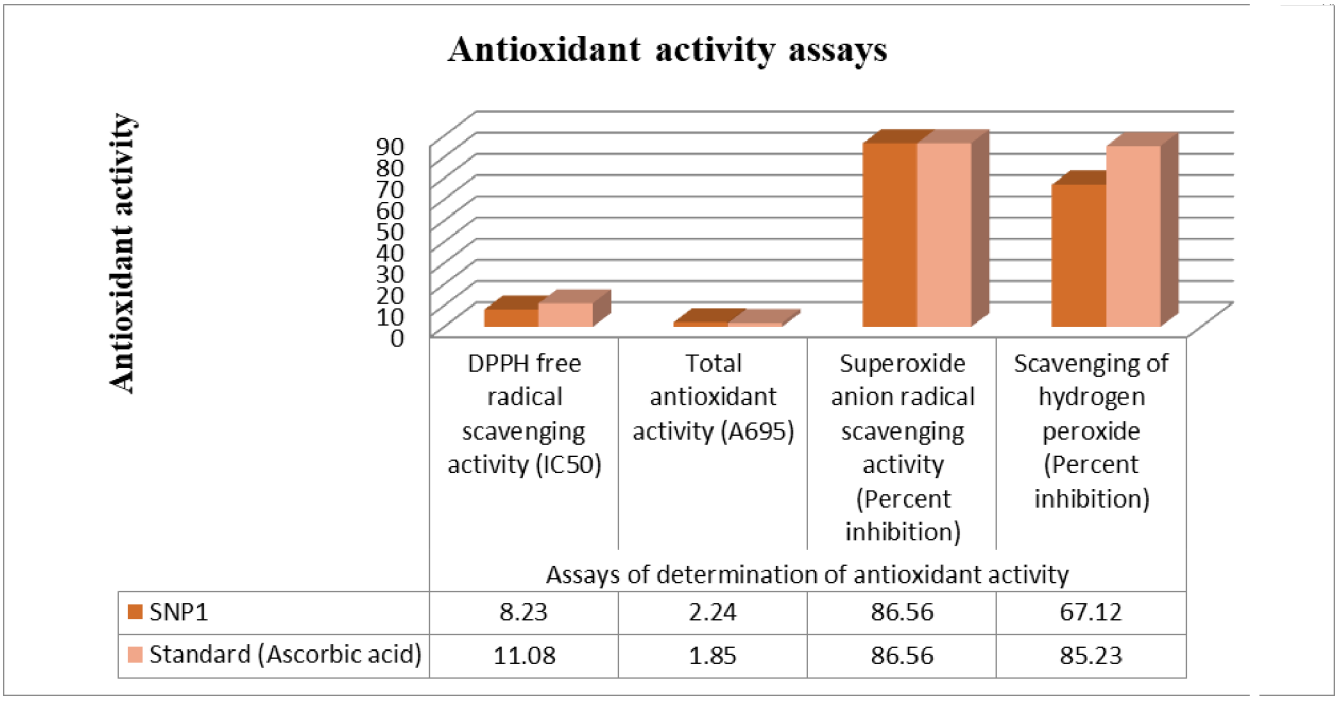

Fig. 2: Graphical representation of antioxidant activity assays of isolated Acetamide derivative (SNP1).

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 (Duan *et al.,* 2006). 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.

$\frac{A_O - A_S}{A_O} \times 100$ The superoxide anion radical scavenging activity (%) will be calculated as:

Where, Ao = absorbance of positive control

As = absorbance of sample

Scavenging of Hydrogen peroxide (H_2O_2)

Percent scavenging of H_2O_2 was determined (Prieto *et al.,* 1999). A solution of H_2O_2 40 mM was prepared in phosphate buffer (pH, 7.4). H_2O_2 concentration was determined spectro-photometrically 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:

$$\frac{A_O - A_S}{A_O} \times 100$$

Where, A0 = the absorbance of positive control

As = the absorbance of sample

Results

The SNME extract was fractionated in different combinations of solvents by column chromatography, leading to the iso-lation of ten fractions named SNP1, SNP2, SNP3, SNP 4, SNP5, SNP6, SNP7, SNP8, SNP9 and SNP10. The crystallized fraction SNP1 was identified as Acetamide derivative molecule broadly identified as N-(2-[4-hydroxy phenyl]-4-oxothiazolidinyl)-2-(methyl naphthaleneyloxy)-2-acetamide-1,3,4-odz-3-yl) having *m/z* value 508.3802. The results are shown in Figure 1. The antioxidant activities of SNP1 (Acetamide derivative) were determined by conventional procedures as mentioned in reference with the standard antioxidant, Ascorbic acid. The results were found to be significant with $p<0.05$ (Table 1 and Fig. 2).

Discussion

The present investigation revealed, SNP1 molecule

as an Acetamide derivative as determined by LC-MS/MS spectra. The results of antioxidant activity revealed the acetamide derivative as a potent antioxidant agent. The results of the present study correlate with the previous findings (Saturnino *et al.,* 2010; Olgen *et al.,* 2013; Jamkhandi *et al.,* 2013).

Conclusion

The results of the study concluded that, Acetamide derivative isolated and purified from *Satyrium nepalense* can be utilized as a potent natural antioxidant agent and in formulation of effective nutraceuticals.

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