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Original Research

Satyrium nepalense, a high altitude medicinal orchid of Indian Himalayan region: chemical profile and biological activities of tuber extracts

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Abstract: The present study investigated antioxidant and antibacterial activities of 5 different extracts and derived fractions from the *S. nepalense* tubers. Identification of the most active fractions, their phytochemical characterization, total phenolic and flavonoid contents, and biological activities were also evaluated. Petroleum ether, chloroform, ethyl acetate, methanol, water extracts and methanol fractions were screened for their antibacterial activity at 10, 50 and 100 mg/mL doses against ten Gram-negative and Gram-positive bacterial strains by disc diffusion method. Their total antioxidant activity was measured by DPPH and ABTS assays. Identification of the main compounds was performed by LC-MS/MS. Methanol extract exhibited the highest antioxidant (IC_{50}= 30.79 µg/mL and 24.53 µg/mL for DPPH and ABTS, respectively) and antibacterial (MIC 71.5 to >100 µg/mL) activities in comparison with the other extracts. Levels of phenolics and flavonoids were also the highest in the same extract, i.e. 19.2 mg GAE/g and 11.20 mg QE/g, respectively. Phytochemical investigation of the active fractions of the methanol extract led to the isolation of gallic acid (19.04 mg/g) and quercetin (23.4 mg/g). Therefore, methanol extract showed an interesting potential for both antioxidant and antibacterial activities, thus deserving attention for future applications in the fields of medicinal plants and food supplements.

Key words: Himalayan orchids; Orchidaceae; Salam mishri; Antibacterial activity; Antioxidant activity; Phenylpropanoids.

Introduction

The healing herbs were the primary medicinal agents used by human kind. Plants play an important role in providing food for humans (1). Aromatic and medicinal plants have played a vital role as therapeutic agents for a long time and thus hold excessive economic value (2-4). Today, medicinal plant therapy is greatly used for treatment of many human and animal diseases (5-25).

Orchids comprise highly evolved and economically important plants bearing the most beautiful and attractive flowers in the plant kingdom. In the past 120 years, a number of botanists have reported the presence of orchids from the north Himalayan region, including *Cephalanthera longifolia* L., *Cypripedium cordigerum* D. Don., *Dactylorhiza hatagirea* D. Don, *Epipactis helleborine* L., *Epipactis royleana* L., *Goodyrea repens* (L.) R. Brown, *Listera ovata* (L.) R. Brown, *Spiranthes sinensis* (Persoon) Ames. *Satyrium nepalense* D. Don. and *Oreorchis micrantha* L. Most of these species are terrestrial and grow within 1595-4150 m of altitude (26).

A wide range of bioactive phytochemical and medicinal properties have been identified studying orchids. In fact, especially in India, orchids are prescribed for a variety of therapeutic uses in traditional medicines such as Ayurveda, Sidha and Unani. *Acampe praemorsa* (Roxb.) Hook.f, an epiphytic orchid, is used for treating rheumatism. Tubers of *Habenaria* spp. are being used to treat unconsciousness, and as vermicide and blood purifier. *Bauhinia variegata* L. is widely used in traditional medicine to treat a wide range of complains due to its many secondary metabolites. The phytochemical screening revealed that *Bauhinia variegata* contains terpenoids, flavonoids, tannins, saponins, reducing sugars, steroids and cardiac glycosides (27, 28).

Satyrium nepalense (Orchidaceae), also known as Salam mishri, is an endangered medicinal herb usually found at higher altitudes (2400-3000 m) of the Indian Himalayan Region (IHR). It is a terrestrial herb, commonly used by local inhabitants of Uttarakhand (India) as an energizing tonic and as an important medicine to cure different types of fever in traditional health care system of Uttarakhand. 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 (29). In the present study, phytochemical analysis and *in vitro* biological activities of five different extracts and resulting fractions from the *S. nepalense* tubers were carried out.

Materials and Methods

Chemicals and reagents

Solvents and chemicals used in this study were of analytical grade. Petroleum ether, chloroform, ethyl acetate, methanol and tween-80 were purchased from Merck (Mumbai, India). Ascorbic acid (AA), quercetin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), Trolox, potassium persulphate and kanamycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nutrient broth and nutrient agar were obtained from Himedia (Mumbai, India). All spectrophotometric measures were determined by a UV-visible spectrophotometer (Thermo Fischer model Evolution 201); MIC calculation was performed on a NanoStar Spectrophotometer (BMG LabTech).

Plant material

Satyrium nepalense D. Don tubers were collected from the Chamoli district, Uttarakhand (India), in August 2011. Samples were authenticated by Prof. J. K. Tiwari, Department of Botany, H.N.B. Garhwal (A Central) University, Srinagar, Uttarakhand. A voucher specimen (Pharma. Chem. 28/2011) was deposited in the Department of Pharmaceutical Chemistry, H.N.B. Garhwal (A Central) University, Srinagar, Uttarakhand for future reference. Plant tubers were cut into small pieces, dried in shade at room temperature and ground separately.

Extraction procedure

Dry powder (1 kg) of plant tubers was extracted consecutively with petroleum ether (PEESN), chloroform (CESN), ethyl acetate (EAESN), methanol (MESN) and water (WESN). The extraction procedure was performed 3 times for each solvent, for 72 h. Then, the mixture was filtered and the solvents were evaporated under reduced pressure yielding a solid residue.

Phytochemical screening

Phytochemical screening of different extracts was performed by using the standard methods for the qualitative analysis of phytochemicals such as alkaloids, glycosides, carbohydrates, proteins, phenolics, flavonoids, saponins, amino acids, steroids and triterpenoids (30-34).

Screening of the plant extracts for biological activities

Antioxidant activity

The antioxidant activity of different extracts of *S. nepalense* tubers was evaluated by 2,2-diphenyl-1-picrylhydrazyl-hydrate (DPPH) radical scavenging method as described by Tatsimo et al. (35). The samples were tested at concentrations of 10, 50, 100, 500 and 1000 µg/mL and results converted into percentage of antioxidant activity as follows:

% Inhibition = (Control absorbance - Test sample absorbance) / Control absorbance × 100

Ascorbic acid (AA) was used as positive control. A linear regression curve was established in order to determine the IC_{50} (µg/mL) values (36). In addition, samples were also assessed by using the 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical assay (37). Results were first expressed as percentage of radical inhibition, and then the IC_{50} was calculated as described above; in this case, Trolox was used as reference standard.

Antibacterial activity

Two different methods were employed for the determination of antimicrobial activities: the disc diffusion assay and the minimum inhibitory concentration (MIC, the lowest concentration of the sample that completely inhibites the microbial growth) of the samples against tested microorganisms. The disc diffusion method was carried out using nutrient agar (38-42). The extracts were tested *in-vitro* for their antibacterial activity against ten microorganisms: *Bacillus cereus* (1272), *Escherichia coli* (729), *Enterobacter gergoviae* (621), *Klebsiella pneumoniae* (432), *Salmonella entericatyphim* (98), *Shigella flexneri* (1457), *Staphylococcus aureus* (902), *Staphyloccus epidermidis* (435), *Streptococcus pyogenes* (1925) and *Escherichia coli* (443), purchased from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH) Sector 39-A, Chandigarh-160036 (India).

The cell suspension was standardized at the density of 530 nm and inoculated over the surface of agar medium using sterile cotton swab. A single disc was dipped into 10, 50 and 100 mg/mL stock solutions of plant extracts and placed in each plate. Then, all plates were incubated at 37 °C for 48 h. The zone of inhibition (ZOI) was measured in mm for each microorganism, three replications were performed.

Moreover, a broth dilution susceptibility assay was used for the determination of the MIC of the most active extract and fractions. All tests were carried out in Mueller Hinton broth (MHB). Bacterial strains were cultured overnight at 37 °C in Mueller Hinton agar (MHA). Test strains were suspended in MHB to give a standardized density measured as described above. Sample dilutions, ranging from 0.04 to 100.0 mg/mL, were prepared in a 96-well microtiter plate, including one growth control (MHB + solvent) and one sterility control (MHB + test sample). Plates were incubated under normal atmospheric conditions, at 37 °C for 24 h. The bacterial growth was indicated by the presence of a white "pellet" on the well bottom. Kanamycin (K, 0.04-5.0 mg/L) was used as control. The MIC determination was carried out by the assessment of turbidity by optical density readings at 600 nm, and expressed as mean of three replications ± standard deviation.

Total polyphenol content

Total phenolic content (F-C) of the *S. nepalense* methanol extract was determined with Folin-Ciocalteu method by monitoring the absorbance at 765 nm. Gallic acid was used as reference standard and values are

expressed as mg of gallic acid equivalents per gram of extract (GAE/g) (43-47).

Total flavonoid content of the *S. nepalense* methanol extract was assessed according to a modified colorimetric method (48) and the absorbance was measured at 510 nm. In this case, the total flavonoid content is expressed as mg of quercetin equivalents per gram of extract (QE/g).

Fractionation procedure for the active methanol extract of *S. nepalense* (MESN)

The methanol soluble fraction (50 g) of *S. nepalense* tuber was mixed with 10 g silica gel (Qualigen, 100-200 mesh) prepared in petroleum ether (60-80 °C). The column was subjected to diverse solvent systems: petroleum ether (100%), petroleum ether-chloroform (from 95:5 to 50:50), chloroform (100%), chloroform-ethyl acetate (from 95:5 to 50:50), ethyl acetate (100%) and ethyl acetate-methanol (from 99:1 to 95:5). Elutes were collected and, on the basis of their thin layer chromatography profiles, were combined into 8 groups (from SN-1 to SN-8). All groups were screened for their antioxidant and antibacterial activities. According to the bioassay-guided fractionation, SN-2 and SN-7 were found as the most active fraction and further analysed.

Identification of active constituents from the *S. nepalense* tuber active fractions

Identification of the main compounds was carried out by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Reverse phase chromatographic analyses were carried out under gradient conditions using an Accucore C18 column (100 × 3.0, 2.6 µm); the mobile phases were water containing 2% acetic acid (A) and methanol (B). The composition gradient started with 5% B for the first 2 min followed by a gradient to obtain 25% B at 10 min, 40% B at 20 min, 50% B at 30 min, 60% B at 40 min, 70% B at 50 min and 80% B at 60 min, according to the method described by Laghari et al. (48), with slight modifications. Mass spectra were acquired in positive ion mode, over the 200-800 *m/z* range. Source temperature was set at 120°C, capillary voltage 3300 eV and cone voltage 50 eV. LC-MS/MS spectra were acquired for the four most abundant ions observed in each MS spectrum. The mobile phases were filtered through 0.45 µm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use (49). Stock solutions of standard references were prepared in methanol at a concentration range of 0.031-0.250 mg/mL for quercetin and 0.006-0.250 mg/mL for gallic acid. Quantification was carried out by integration of the peaks using the external standard method, at 254 nm for gallic acid and 365 nm for quercetin. The flow rate was 0.8 mL/min and the injection volume was 40 µL. Chromatographic peaks were confirmed by comparing their retention times with those of reference standards and their fragmentation pattern.

Results and Discussion

Qualitative phytochemical analysis of various extracts from *S. nepalense* tuber

Primary phytochemical screening of five different extracts (PEESN, CESN, EAESN, MESN and WESN) of *S. nepalense* tuber was carried out by standard qualitative chemical tests (Table 1). The results showed the presence of carbohydrates, glycosides and flavonoids in MESN and WESN; phenolic compounds were present in EAESN and MESN, whereas alkaloids were absent in all tested samples. Saponins, proteins and amino acids were detected in WESN, whereas tannins and resins were found in CESN, EAESN and MESN. Unsaturated sterols/triterpenes were present in PEESN, CESN and MESN (Table 1). Therefore, the qualitative screening confirmed that, among the *S. nepalense* extracts, MESN was the richest in phytochemicals.

Antioxidant activity of *S. nepalense* extracts

The concentration of antioxidant(s) needed to decrease the initial free radical concentration by 50% (IC_{50}) is a parameter widely used to measure the antioxidant activity. A lower IC_{50} value corresponds to a higher antioxidant power (50). The IC_{50} values of extracts from *S. nepalense* tuber measured by DPPH method ranged from 90.66 to 30.79 µg/mL, in WESN and MESN, respectively (Table 2). In particular, the decreasing order of extract antioxidant power was as follows: AA > MESN > EAESN > CESN > PEESN > WESN. It was previously highlighted that at least two *in vitro* assays should be carried out for the evaluation of extract antioxidant capacity; for this reason, in order to confirm the results obtained by the DPPH test, the ABTS method was used. Noteworthy, ABTS decolourisation assay is highly efficient for both hydrophilic and lipophilic antioxidants (51). The extracts were able to reduce the ABTS radical in a concentration-dependent manner. IC_{50} values of *S. nepalense* extracts ranged from 24.53 ± 2.01 to 157.18 ± 10.81 µg/mL, in MESN and PEESN, respectively (Table 2). MESN confirmed to be the most active extract.

Antibacterial activity of *S. nepalense* extracts

The extract antibacterial activity was assessed against a panel of ten foodborne pathogens (including Gram-positive and Gram-negative species) at the dose of 10, 50 and 100 mg/mL, and the results expressed as zone of inhibition (ZOI, in mm) were compared with the activity of the positive control, kanamycin (5 mg/mL) (Table 3). MESN at 100 mg/mL exhibited a significant antibacterial activity against both Gram-positive and Gram-negative bacteria as *B. cereus* (12 mm, MIC = 75.2 µg/mL), *E. coli* 729 (8 mm, MIC = 91.5 µg/mL), *E. gergoviae* (7 mm, MIC = >100 µg/mL), *K. pneumonia* (6 mm, MIC = >100 µg/mL), *S. entericatyphim* (6 mm, MIC = >100 µg/mL), *S. flexneri* (7 mm, MIC = >100 µg/mL), *S. aureus* (12 mm, MIC = 71.5 µg/mL), *S. epidermidis* (7 mm, MIC = 95.0 µg/mL), *S. pyogenes* (12 mm, MIC = 80.6 µg/mL) and *E. coli* 443 (8 mm, MIC = 99.5 µg/mL). MESN at 50 mg/mL did not show any antibacterial activity against three microorganisms (*E. gergoviae, S. entericatyphim* and *S. flexneri*), whereas, at 10 mg/mL, it was only effective against *S. aureus* (6 mm). PEESN exhibited antibacterial effects in the range of 6-11 mm; at 100 and 50 mg/mL, this extract showed antibacterial activity only against four (*E. coli*, *S. flexneri*, *S. aureus* and *E. coli*) and two (both *E. coli*) microorganisms, respectively, whereas, at 10 mg/mL, PEESN did not show any significant activity. WESN, CESN and EAESN exhibited ZOI in the range

Table 1. Phytochemical screening of *Satyrium nepalense* tuber extracts.

Secondary metabolites	PEESN[a]	CESN	EAESN	MESN	WESN
Carbohydrates					
a. Molish test	(-)	(-)	(-)	(+)	(+)
b. Fehling test	(-)	(-)	(-)	(+)	(+)
c. Benedict test	(-)	(-)	(-)	(+)	(+)
Glycosides					
a. Keller-Killani test	(-)	(-)	(-)	(+)	(+)
b. Legal test	(-)	(-)	(-)	(+)	(+)
Alkaloids					
a. Mayer's test	(-)	(-)	(-)	(-)	(-)
b. Dragondroff test	(-)	(-)	(-)	(-)	(-)
Flavonoids					
a. Shinoda test	(-)	(+)	(+)	(+)	(+)
b. Ammonia	(-)	(-)	(+)	(+)	(+)
Phenolic compounds					
a. Ferric chloride test	(-)	(-)	(+)	(+)	(-)
Protein and Amino acids					
a. Millon's test	(-)	(-)	(-)	(-)	(+)
b. test	(-)	(-)	(-)	(-)	(+)
c. Biuret Test	(-)	(-)	(-)	(-)	(+)
Saponins					
a. Foam test	(-)	(-)	(-)	(-)	(+)
Tannins					
a. Pyrogallol and catechol	(-)	(+)	(+)	(+)	(-)
b. Gallic acid	(-)	(+)	(+)	(+)	(-)
Unsaturated sterol/Triterpenes					
a. Liebermann Burchard test	(+)	(+)	(-)	(+)	(-)
b. Salkowiskis test	(+)	(+)	(-)	(+)	(-)
Resins	(-)	(+)	(+)	(+)	(-)

[a]PEESN, petroleum ether extract; CESN, chloroform extract; EAESN, ethyl acetate extract; MESN, methanol extract; WESN, water extract.

Table 2. Antioxidant activity of *Satyrium nepalense* tuber extracts.

S. nepalense tuber extracts	DPPH (IC_{50} values in µg/mL)[a]	ABTS (IC_{50} values in µg/mL)
PEESN[b]	79.56 ± 9.31	157.18 ± 10.81
CESN	59.33 ± 4.43	68.85 ± 3.40
EAESN	45.39 ± 7.55	37.14 ± 2.98
MESN	30.79 ± 2.51	24.53 ± 2.01
WESN	90.66 ± 6.95	135.24 ± 8.24
AA	21.30 ± 1.66	--
Trolox	--	13.85 ± 1.26

[a]DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid). [b]PEESN, petroleum ether extract; CESN, chloroform extract; EAESN, ethyl acetate extract; MESN, methanol extract; WESN, water extract; AA, ascorbic acid. Results are expressed as mean ± standard deviation (n=3).

of 6-10 mm and possessed higher antimicrobial activity than PEESN. In comparison to standard drug (kanamycin 5 mg/mL), only MESN showed potent antibacterial effects against all microorganisms at the concentration of 100 mg/mL. In general, the decreasing order of the extract antibacterial activity was as follows: K > MESN > CESN > EAESN > WESN > PEESN (Table 3). The MIC of K against tested bacteria ranged between 0.4 and 3.8 mg/mL.

Qualitative phytochemical screening and biological activity assessment suggested that MESN had the highest number of secondary metabolites as well as the highest antioxidant and antibacterial activities in comparison to the other extracts. Hence, it was selected for further analyses.

Total polyphenolic contents

The result of the total phenolic content of MESN, using Folin-Ciocalteu method, is presented in Figure 1. The concentration of phenols in the examined plant extracts was expressed as mg of gallic acid equivalent per gram of extract (GAE/g), (the standard curve equation: $Y = 0.108X + 0.191$, $R^2 = 0.977$). The concentration of phenols was 19.2 ± 0.12 mg GAE/g of extract.

The content of total flavonoids was measured by aluminium chloride colorimetric technique and the results were expressed as mg of quercetin equivalent per gram of extract (QE/g) (standard curve equation: $Y = 0.144X + 0.094$, $R^2 = 0.996$). The concentrations of flavonoids were 11.20 mg QE/g of extract (Figure 1).

Fractionations of methanol extract (MESN)

MESN was fractionated in different combinations of solvents by column chromatography, leading to the isolation of eight fractions named SN-1, SN-2, SN-3, SN-4, SN-5, SN-6, SN-7 and SN-8. All isolated fractions

Table 3. Antibacterial activity of *Satyrium nepalense* tuber extracts.

Bacteria	MTCC (Code)	Zone of inhibition (mm)															K	MIC[d] MESN
		PEESN[b]			CESN			EAESN			MESN			WESN				
		10[c]	50	100	10	50	100	10	50	100	10	50	100	10	50	100		
BC[a]	1272	-	-	-	-	-	07	-	-	10	-	06	12	-	-	-	18	75.2 ± 1.6
EC	729	-	06	11	06	06	08	06	07	09	-	06	08	-	07	10	22	91.5 ± 1.3
EG	621	-	-	-	-	-	07	-	-	-	-	-	07	-	-	-	13	>100
KP	432	-	-	-	06	08	06	-	-	10	-	06	06	-	-	-	17	>100
SENT	98	-	-	-	-	-	-	-	-	-	-	-	06	-	-	08	13	>100
SF	1457	-	-	07	-	-	-	-	06	07	-	-	07	-	-	09	10	>100
SA	902	-	-	06	-	09	10	-	06	06	06	07	12	-	06	08	24	71.5 ± 1.8
SEPID	435	-	-	-	-	-	10	-	-	07	-	05	07	-	06	09	13	95.0 ± 1.2
SP	1925	-	-	-	-	-	08	-	-	07	-	06	12	-	-	-	12	80.6 ± 1.0
EC	443	-	06	11	06	07	07	06	07	09	-	06	08	-	07	07	24	99.5 ± 1.08

[a]BC= *B. cereus;* EC= *E. coli;* EG= *E. gergoviae;* KP= *K. pneumonia;* SENT= *S. entericatyphim;* SF= *S. flexneri;* SA= *S. aureus;* SEPID= *S. epidermidis;* SP= *S. pyogenes.* [b]PEESN, petroleum ether extract; CESN, chloroform extract; EAESN, ethyl acetate extract; MESN, methanol extract; WESN, water extract. [c] =mg/mL; K= kanamycin 5 mg/mL. [d]MIC, minimum inhibitory concentration, results are expressed as µg/mL ± standard deviation.

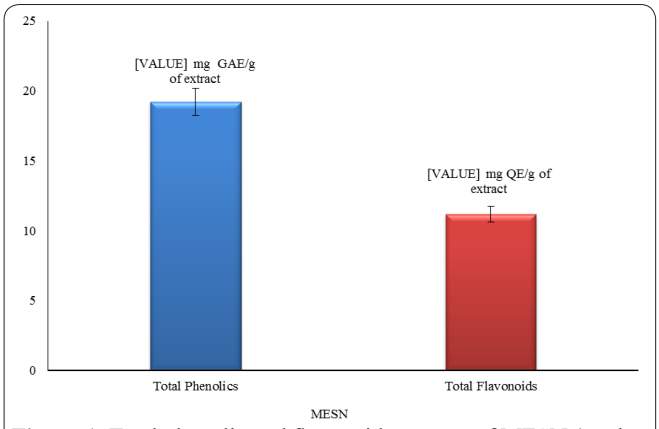

Figure 1. Total phenolic and flavonoid contents of MESN (methanol extract of *Satyrium nepalense* tuber); results (mean ± standard deviation) are expressed as mg of gallic acid equivalents per gram of extract (GAE/g) and mg of quercetin equivalents per gram of extract (QE/g).

were further screened for biological activities.

Antioxidant activity of MESN fractions

DPPH radical-scavenging method was used to evaluate the antioxidant activities of diverse fractions due to its simplicity, sensitivity and reproducibility (Table 4). Among all tested fractions, SN-2 had the highest antioxidant activity (34.18 µg/mL IC_{50}), comparable to that of ascorbic acid used as standard (25.83 µg/mL IC_{50}), while SN-3 (233.68 µg/mL IC_{50}) had the lowest activity. Furthermore, SN-7 was the second most active fraction (44.7 µg/mL IC_{50}). These results were also confirmed by ABTS assay (Table 4). In fact, in this case, fractions SN 2 and 7 exhibited the lowest IC_{50} values (19.21 ± 0.84 and 29.92 ± 1.87 µg/mL, respectively), comparable with Trolox used as standard (13.85 ± 1.26 µg/mL). Slight differences were observed among the IC_{50}values of the less active fractions, when the results of the two antioxidant tests were compared, thus showing a high grade of correlation between these assays (51).

Antibacterial activity of MESN fractions

Results obtained for the antimicrobial activity of MESN fractions, using disc diffusion method, showed that all the bacterial strains were sensitive to the SN-2 and SN-7 fractions (Table 5). At the dose of 10, 50 and 100 mg/mL, it was found that SN-2 fraction (range: 6-18 mm) together with SN-7 fraction (range: 6-16 mm) showed the highest ZOI against all microorganisms. At the same concentrations, SN-1 fraction showed

Table 4. Antioxidant activity of MESN[a] fractions.

MESN fractions	DPPH[b] (IC_{50} values in µg/mL)	ABTS (IC_{50} values in µg/mL)
SN-1	92.40 ± 1.04	115.48 ± 9.35
SN-2	34.18 ± 0.24	19.21 ± 0.84
SN-3	233.68 ± 0.18	198.25 ± 10.14
SN-4	119.24 ± 0.80	99.87 ± 6.59
SN-5	47.80 ± 0.44	39.14 ± 3.74
SN-6	147.77 ± 0.77	197.25 ± 9.98
SN-7	44.7 ± 0.29	29.92 ± 1.87
SN-8	64.34 ± 1.25	76.25 ± 2.58
AA[c]	25.83 ± 1.06	--
Trolox	--	13.85 ± 1.26

[a]MESN, methanol extract of *Satyrium nepalense* tuber. [b]DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid). [c]AA, ascorbic acid. Results are expressed as mean ± standard deviation (n=3).

Table 5. Antibacterial activity of MESN[a] fractions.

Bacteria	MTCC (Code)	Zone of inhibition (mm)																								K	MIC[d] SN-2	MIC SN-7
		SN-1			SN-2			SN-3			SN-4			SN-5			SN-6			SN-7			SN-8					
		10[c]	50	100	10	50	100	10	50	100	10	50	100	10	50	100	10	50	100	10	50	100	10	50	100			
BC[b]	1272	07	08	10	10	11	13	-	-	14	-	06	12	-	-	-	06	08	09	10	13	13	07	09	10	20	80.0 ± 1.6	87.1± 1.8
EC	729	10	12	14	13	14	18	14	14	16	-	06	08	-	06	07	06	07	07	12	13	14	10	11	13	24	90.0 ± 1.3	82.6 ± 1.1
EG	621	-	-	-	07	08	08	-	-	08	-	-	-	-	-	-	-	-	06	-	09	09	-	-	-	12	>100	>100
KP	432	09	10	11	07	13	14	-	08	12	-	06	06	-	-	-	06	09	09	10	10	10	09	07	07	16	>100	>100
SENT	98	-	-	-	-	06	08	-	-	07	-	-	-	-	-	06	-	-	-	-	06	09	-	-	06	11	>100	>100
SF	1457	-	-	-	06	06	07	-	-	09	-	-	-	-	-	06	-	-	-	-	07	07	-	07	07	10	84.2 ± 0.8	72.3 ± 1.9
SA	902	09	09	10	10	14	17	-	-	14	-	10	12	-	11	14	-	-	-	09	13	16	09	09	09	22	90.2 ± 1.7	69.6 ± 2.0
SEPID	435	-	-	-	06	08	08	-	-	07	-	06	07	-	-	-	08	09	09	12	12	13	10	12	13	13	75.0 ± 2.1	81.4 ± 1.6
SP	1925	-	-	-	-	06	07	-	-	-	-	-	07	-	-	-	09	09	09	-	-	09	-	-	06	10	>100	>100
EC	443	13	13	14	14	16	18	06	08	15	-	06	08	-	06	06	8	10	10	13	14	14	13	14	14	21	88.0 ± 1.8	96.3 ± 1.9

[a]MESN, methanol extract of *Satyrium nepalense* tuber. [b]BC= *B. cereus;* EC= *E. coli;* EG= *E. gergoviae;* KP= *K. pneumonia;* SENT= *S. entericatyphim;* SF= *S. flexneri;* SA= *S. aureus;* SEPID= *S. epidermidis; SP= S. pyogenes.* [c]mg/mL; K= kanamycin 5 mg/mL. [d]MIC, minimum inhibitory concentration, results are expressed as µg/mL ± standard deviation (n=3).

ZOI between 7 and 14 mm against *B. cereus*, *E. coli* (both 729 and 443 strains), *K. pneumonia* and *S. aureus*. Only SN-3 fraction at 100 mg/mL exhibited ZOI between 7 and 16 mm against all bacterial strains except *S. pyogenes*. SN-4 and SN-5 fractions were less active against all microorganisms with similar ZOI. SN-6 fraction was effective against *B. cereus*, *E. coli*-729, *E. coli*-443, *K. pneumonia*, *S. epidermidis* and *S. pyogenes*. This fraction was very little effective against *E. gergoviae* (6 mm) and it had no activity when tested against *S. entericatyphim*, *S. flexneri* and *S. aureus*. At 100 mg/mL concentration, SN-8 fraction produced a high ZOI when tested against all microorganisms except *E. gergoviae*. In summary, the decreasing order of antibacterial activity of fractions at 100 mg/mL was as follows: K > SN-2 > SN-7 > SN-8 > SN-3 > SN-6 > SN-4 > SN-1 > SN-5 (Table 5).

The most active fractions obtained, i.e. SN-2 and SN-7, were subjected to MIC measurement against bacterial strains (Table 5). In particular, the SN-2 fraction showed the lowest MIC value when tested against of *S. epidermidis* (75.0 ± 2.1 µg/mL), while SN-7 was more effective against *S. aureus* (69.6 ± 2.0 µg/mL). The two fractions were more active than the extract MESN, indicating an enrichment of the active compounds.

Identification and quantification of compounds from active isolated fractions by LC-MS/MS

On the basis of *m/z* determination and retention times in comparison with the standards, and taking into account the main fragments observed in the MS/MS spectra, the structures of the most abundant compounds present in the two active fractions (SN-2 and SN-7) were determined as quercetin (23.4 ± 0.03 mg/g) and gallic acid (19.04 ± 0.01 mg/g) (Table 6). Quercetin was identified by its *m/z* at 303.0 (Figure S1), whereas gallic acid showed a characteristic peak at 168.7 and another at 124.7 that corresponds to the loss of the carboxylic function (Figure S2).

In the current study, the preliminary phytochemical screening supported the presence of various bioactive constituents' asglycosides, tannins, sterols/triterpenes, phenolics and flavonoids *in S. nepalense*. Plants are rich in a variety of secondary metabolites and, therefore, it is pivotal to identify the phytochemical components of local medicinal plants usually prescribed by herbalists in the treatment of diseases. The presence or the absence of certain phytochemicals could be useful to explain some of the biological activities of some plant extracts. For example, the biological properties of flavonoids, apart from their antioxidant effects, include protection against allergies, inflammation, platelet aggregation, microbes, ulcers, toxins, viruses and tumors (52, 53). These compounds can serve as natural antibiotics, which help the body to fight microbial infections (54).

MESN was found to be the most active extract due to its strong antioxidant and antibacterial effects, and, after MESN fractionation, SN-2 and SN-7 fractions were found to be the most active. The LC-MS/MS analysis confirmed the presence of gallic acid (a phenolic acid) and quercetin (a flavonoid) in SN-2 and SN-7 fractions of MESN. Antioxidant and antibacterial activities of gallic acid and quercetin have been previously reported (55-58). Therefore, it can be hypothesized that MESN and its fractions (SN-2 and SN-7) possess significant biological activities possibly due to the presence of gallic acid and quercetin. To the best of our knowledge, there is no previous report showing the antioxidant and antibacterial activities of *S. nepalense* extracts.

This study has demonstrated that the methanol extract of *S. nepalense* exhibits 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. However, further studies will be carried out in order to identify minor bioactive phytochemicals and possible applications in the fields of pharmaceutics and food supplements.

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Conflict of Interest

There is no conflict of interest.

References

1. Raeisi S, Ojagh SM, Sharifi-Rad M, Sharifi-Rad J, Quek SY. Evaluation of *Allium paradoxum* (MB) G. Don. and *Eryngium caucasicum* trauve. Extracts on the shelf-life and quality of silver carp (*Hypophthalmichthys molitrix*) fillets during refrigerated storage. Journal of Food Safety 2017; 37(3): e12321.

2. Sharifi-Rad J, Hoseini-Alfatemi SM, Sharifi-Rad M, Teixeira da Silva JA. Antibacterial, antioxidant, antifungal and anti-inflammatory activities of crude extract from *Nitraria schoberi* fruits. 3 Biotech 2015; 5(5): 677-684.

Table 6. Quantification of gallic acid and quercetin in SN-2 and SN-7 fractions of active MESN[a]

Compounds	SN-2 fraction Quantity (mg/g)	SN-7 fraction	Parent ion	Collision energy (V)
Gallic acid	19.04 ± 0.01	-	168.7	10
Quercetin	-	23.4 ± 0.03	303.0	10

[a]MESN, methanol extract of *Satyrium nepalense* tuber. Results are expressed as mean ± standard deviation (n=3).

3. Sharifi-Rad J, Hoseini-Alfatemi S, Sharifi-Rad M, Miri A. Phytochemical screening and antibacterial activity of different parts of the *Prosopis farcta* extracts against methicillin-resistant *Staphylococcus aureus* (MRSA). Minerva Biotecnologica 2014; 26(4): 287-293.
4. Sharifi-Rad M, Mnayer D, Flaviana Bezerra Morais-Braga M et al. *Echinacea* plants as antioxidant and antibacterial agents: From traditional medicine to biotechnological applications. Phytotherapy Research 2018; doi: 10.1002/ptr.6101.
5. Sharifi-Rad J, Hoseini-Alfatemi SM, Sharifi-Rad M, Setzer WN. Chemical composition, antifungal and antibacterial activities of essential oil from *Lallemantia royleana* (Benth. In Wall.) Benth. Journal of Food Safety 2015; 35(1): 19-25.
6. Sharifi-Rad M, Varoni EM, Salehi B et al. Plants of the Genus *Zingiber* as a Source of Bioactive Phytochemicals: From Tradition to Pharmacy. Molecules 2017; 22(12): 2145.
7. Sharifi-Rad J, Salehi B, Varoni EM et al. Plants of the *Melaleuca* genus as antimicrobial agents: from farm to pharmacy. Phytotherapy Research 2017; 31(10): 1475-1494.
8. Sharifi-Rad J, Salehi B, Stojanović-Radić ZZ et al. Medicinal plants used in the treatment of tuberculosis-Ethnobotanical and ethnopharmacological approaches. Biotechnology Advances 2017; doi: 10.1016/j.biotechadv.2017.07.001.
9. Sharifi-Rad J, Salehi B, Schnitzler P et al. Susceptibility of herpes simplex virus type 1 to monoterpenes thymol, carvacrol, p-cymene and essential oils of S*inapis arvensis* L., *Lallemantia royleana* Benth. and *Pulicaria vulgaris* Gaertn. Cellular and Molecular Biology (Noisy le Grand) 2017; 63(8): 42-47.
10. Salehi B, Zucca P, Sharifi-Rad M et al. Phytotherapeutics in cancer invasion and metastasis. Phytotherapy Research 2018; 1-25: doi:10.1002/ptr.6087.
11. Salehi B, Ayatollahi S, Segura-Carretero A et al. Bioactive chemical compounds in *Eremurus persicus* (Joub. & Spach) Boiss. essential oil and their health implications. Cellular and Molecular Biology (Noisy le Grand) 2017; 63(9): 1-7.
12. Sharifi-Rad J, Ayatollahi SA, Varoni EM et al. Chemical composition and functional properties of essential oils from *Nepeta schirazziana* Boiss. Farmacia 2017; 65(5): 802-812.
13. Sharifi-Rad J, Sureda A, Tenore GC et al. Biological activities of essential oils: From plant chemoecology to traditional healing systems. Molecules 2017; 22(1): 70.
14. Sharifi-Rad J, Soufi L, Ayatollahi S et al. Anti-bacterial effect of essential oil from *Xanthium strumarium* against shiga toxin-producing *Escherichia coli*. Cellular and Molecular Biology (Noisy-le-Grand, France) 2016; 62(9): 69-74.
15. Stojanović-Radić Z, Pejčić M, Stojanović N, Sharifi-Rad J, Stanković N. Potential of *Ocimum basilicum* L. and *Salvia officinalis* L. essential oils against biofilms of *P. aeruginosa* clinical isolates. Cellular and Molecular Biology (Noisy-le-Grand, France) 2016; 62(9): 27-32.
16. Sharifi-Rad M, Tayeboon G, Sharifi-Rad J, Iriti M, Varoni E, Razazi S. Inhibitory activity on type 2 diabetes and hypertension key-enzymes, and antioxidant capacity of *Veronica persica* phenolic-rich extracts. Cellular and Molecular Biology (Noisy-le-Grand, France) 2016; 62(6): 80-85.
17. Sharifi-Rad M, Tayeboon G, Miri A et al. Mutagenic, antimutagenic, antioxidant, anti-lipoxygenase and antimicrobial activities of *Scandix pecten-veneris* L. Cellular and Molecular Biology (Noisy-le-Grand, France) 2016; 62(6): 8-16.
18. Sharifi-Rad M, Iriti M, Gibbons S, Sharifi-Rad J. Anti-methicillin-resistant *Staphylococcus aureus* (MRSA) activity of Rubiaceae, Fabaceae and Poaceae plants: A search for new sources of useful alternative antibacterials against MRSA infections. Cellular and Molecular Biology (Noisy-le-Grand, France) 2016; 62(9): 39-45.
19. Sharifi-Rad J, Mnayer D, Tabanelli G et al. Plants of the genus *Allium* as antibacterial agents: From tradition to pharmacy. Cellular and Molecular Biology (Noisy-le-Grand, France) 2016; 62(9): 57-68.
20. Sharifi-Rad J, Mnayer D, Roointan A et al. Antibacterial activities of essential oils from Iranian medicinal plants on extended-spectrum β-lactamase-producing *Escherichia coli*. Cellular and Molecular Biology (Noisy-le-Grand, France) 2016; 62(9): 75-82.
21. Sharifi-Rad J, Fallah F, Setzer W, Entezari RH, Sharifi-Rad M. *Tordylium persicum* Boiss. & Hausskn extract: A possible alternative for treatment of pediatric infectious diseases. Cellular and Molecular Biology (Noisy-le-Grand, France) 2016; 62(9): 20-26.
22. Bagheri G, Mirzaei M, Mehrabi R, Sharifi-Rad J. Cytotoxic and Antioxidant Activities of *Alstonia scholaris*, *Alstonia venenata* and *Moringa oleifera* Plants From India. Jundishapur Journal of Natural Pharmaceutical Products 2016; 11(3): e31129.
23. Sharifi-Rad M, Varoni EM, Iriti M et al. Carvacrol and Human Health: A Comprehensive Review. Phytotherapy Research 2018; doi: 10.1002/ptr.6103.
24. Salehi B, Mishra AP, Shukla I et al. Thymol, thyme and other plant sources: health and potential uses. Phytotherapy Research 2018 ; doi: 10.1002/ptr.6109.
25. Salehi B, Kumar NVA, Şener B, Sharifi-Rad M, Kılıç M, Mahady GB, Vlaisavljevic S et al. Medicinal Plants Used in the Treatment of Human Immunodeficiency Virus. International Journal of Molecular Sciences 2018; 19(5): 1459.
26. Shapoo GA, Kalo Z, Singh S, Ganie AH, Pader B. Evaluation of diversity and habitat types of some orchid species growing in Kashmir Himalaya. Citeseer 2014;10: 8-13.
27. Joshi G, Tewari LM, Lohani N, Upreti K, Jalal JS, Tewari G. Diversity of orchids in Uttarakhand and their conservation strategy with special reference to their medicinal importance. Report and Opinion 2009; 1(3): 47-52.
28. Sharma P, Mahajan N, Garg P, Singh G, Dadhwal S, Sharma S. *Malaxis acuminata*: a review. International Journal of Research in Ayurveda and Pharmacy 2011; 2(422): 425.
29. Babbar SB, Singh DK. Protocols for *In Vitro* Mass Multiplication and Analysis of Medicinally Important Phenolics of a Salep orchid, *Satyrium nepalense* D. Don ("Salam Mishri"). Protocols for *In Vitro* Cultures and Secondary Metabolite Analysis of Aromatic and Medicinal Plants, Second Edition 2016: 1-11.
30. Harborne A. Phytochemical methods a guide to modern techniques of plant analysis: Springer Science & Business Media; 1998.
31. Wagner H, Bladt S. Plant drug analysis: a thin layer chromatography atlas: Springer Science & Business Media; 1996.
32. Khandelwal KR. Practical pharmacognosy: Pragati Books Pvt. Ltd.; 2008.
33. Bateman RM, Hollingsworth PM, Preston J, Yi-Bo L, Pridgeon AM, Chase MW. Molecular phylogenetics and evolution of Orchidinae and selected Habenariinae (Orchidaceae). Botanical Journal of the Linnean Society 2003; 142(1): 1-40.
34. Kumari P, Singh BK, Joshi GC, Tewari LM. Veterinary Ethnomedicinal Plants in Uttarakhand Himalayan Region, India. Ethnobotanical Leaflets 2009; 10: 1312-27.
35. Tatsimo SJN, de Dieu Tamokou J, Havyarimana L et al. Antimicrobial and antioxidant activity of kaempferol rhamnoside derivatives from *Bryophyllum pinnatum*. BMC Research Notes 2012; 5(1): 158.
36. Omri Hichri A, Mosbah H, Majouli K et al. Chemical composition and biological activities of *Eruca vesicaria* subsp. longirostris essential oils. Pharmaceutical Biology 2016; 54(10): 2236-2243.
37. Armentano MF, Bisaccia F, Miglionico R et al. Antioxidant and proapoptotic activities of *Sclerocarya birrea* [(A. Rich.) Hochst.] methanolic root extract on the hepatocellular carcinoma cell line

HepG2. BioMed Research İnternational 2015; 2015.
38. Kerr J. Manual of clinical microbiology. BMJ Publishing Group; 2004.
39. Janssen A, Scheffer J, Svendsen AB. Antimicrobial activity of essential oils: a 1976-1986 literature review. Aspects of the test methods. Planta Medica 1987; 53(05): 395-398.
40. Singleton VL, Orthofer R, Lamuela-Raventós RM. [14] Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. Methods in enzymology 1999; 299: 152-178.
41. Benli M, Güney K, Bingöl Ü, Geven F. Antimicrobial activity of some endemic plant species from Turkey. African Journal of Biotechnology 2007; 6(15).
42. Russo D, Bonomo M, Salzano G, Martelli G, Milella L. Nutraceutical properties of *Citrus clementina* juices. Pharmacologyonline 2012; 1(1): 84-93.
43. Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. American Journal of Enology and Viticulture 1965; 16(3): 144-158.
44. Davies K. Annual plant reviews, plant pigments and their manipulation. Vol 14: John Wiley & Sons; 2009.
45. Zou Y, Lu Y, Wei D. Antioxidant activity of a flavonoid-rich extract of *Hypericum perforatum* L. *in vitro*. Journal of Agricultural and Food Chemistry 2004; 52(16): 5032-5039.
46. Russo D, Malafronte N, Frescura D et al. Antioxidant activities and quali-quantitative analysis of different *Smallanthus sonchifolius* [(Poepp. and Endl.) H. Robinson] landrace extracts. Natural Product Research 2015; 29(17): 1673-1677.
47. Ibrahim MM, Sahli AAAA, Alaraidh IA, Al-Homaidan AA, Mostafa E, El-Gaaly G. Assessment of antioxidant activities in roots of Miswak (*Salvadora persica*) plants grown at two different locations in Saudi Arabia. Saudi Journal of Biological Sciences 2015; 22(2): 168-175.
48. Laghari AH, Memon S, Nelofar A, Khan KM, Yasmin A. Determination of free phenolic acids and antioxidant activity of methanolic extracts obtained from fruits and leaves of *Chenopodium album*. Food Chemistry 2011; 126(4): 1850-1855.
49. Costantino V, Fattorusso E, Mangoni A et al. Tedanol: A potent anti-inflammatory ent-pimarane diterpene from the *Caribbean Sponge* Tedania ignis. Bioorganic & Medicinal Chemistry 2009; 17(21): 7542-7547.
50. Rice-Evans C, Miller N, Paganga G. Antioxidant properties of phenolic compounds. Trends in Plant Science 1997; 2(4): 152-159.
51. Dudonné S, Vitrac X, Coutiere P, Woillez M, Mérillon J-M. Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC assays. Journal of Agricultural and Food Chemistry 2009; 57(5): 1768-1774.
52. Georgewill O, Georgewill U. Evaluation of the anti-inflammatory activity of extract of *Vernonia amygdalina*. Asian Pacific Journal of Tropical Medicine 2010; 3(2): 150-151.
53. Simplice FH, Arm AB, Roger P, Emmanuel AA, Pierre K, Veronica N. Effects of *Hibiscus asper* leaves extracts on carrageenan induced oedema and complete Freunds adjuvant-induced arthritis in rats. Journal of Cell and Animal Biology 2011; 5(5): 66-68.
54. Morris CJ. Carrageenan-induced paw edema in the rat and mouse. Inflammation protocols: Springer; 2003: 115-121.
55. Sautebin L, Ialenti A, Ianaro A, Di Rosa M. Endogenous nitric oxide increases prostaglandin biosynthesis in carrageenin rat paw oedema. European Journal of Pharmacology 1995; 286(2): 219-222.
56. Ali SS, Kasoju N, Luthra A et al. Indian medicinal herbs as sources of antioxidants. Food Research International 2008; 41(1): 1-15.
57. Albayrak S, Aksoy A, Sagdic O, Hamzaoglu E. Compositions, antioxidant and antimicrobial activities of Helichrysum (Asteraceae) species collected from Turkey. Food Chemistry 2010; 119(1): 114-122.
58. Alves MJ, Ferreira IC, Froufe HJ, Abreu R, Martins A, Pintado M. Antimicrobial activity of phenolic compounds identified in wild mushrooms, SAR analysis and docking studies. Journal of Applied Microbiology 2013; 115(2): 346-357.