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Pharmacognostic and Phytochemical Standardization of Valuable Ethnomedicinal Himalayan Orchid *Satyrium nepalense* D. Don. (*Salam mishri*)

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ABSTRACT

Background: *Satyrium nepalense* D. Don (Orchidaceae) is an ethano-medicinal plant of the Himalayan region. Its tubers known as *Salam-mishri*, are used as an energizing tonic & aphrodisiac by local inhabitants of the Himalaya regions of India and other countries. It is also used in general debility, dysentery, diarrhea, nephritis, fever, backache, cuts, & wounds. **Objective:** Macroscopic, microscopic (T. S & Powder microscopy), physicochemical, phytochemical, and chromatographic studies of the tubers of *S. nepalense* D. Don. **Materials & Methods:** Genuine samples were collected from Surkunda hill region, Tehri Garhwal (Uttarakhand), authenticated, and organoleptic, microscopic, physicochemical, phytochemical, and chromatographic studies were done. **Results:** Genuine samples were taken. Physico-chemical parameters were within normal range. Glycosides, phenols, flavonoids, saponins, etc. were present in the sample. **Conclusion:** *Satyrium nepalense* D. Don (*salam-mishri*) is a valuable plant highly rich in phenolic content & flavonoids and should be further evaluated for traditional claims.

KEYWORDS: *Salam-mishri*, *Satyrium nepalense*, *Munjataka*, Himalayan orchids, ethano-medicinal, Surkunda hills.

1. INTRODUCTION

Satyrium nepalense D. Don (Family: Orchidaceae) is a valuable medicinal orchid species distributed over South Asia, includes the Himalayan region and the Western Ghats and mainly covering hilly regions of Nepal, India, Bhutan, Sri Lanka, and Myanmar.[1] In India, the species is mainly grow in grassy slopes at an altitude of 1000 to 4000m in the Himalayan region of Jammu and Kashmir, Himachal, Uttarakhand, Sikkim, Arunachal Pradesh, Meghalaya, and also in the hilly regions of Western Ghats including Kerala, Karnataka, Tamil Nadu, and Maharashtra.[2] *Satyrium nepalense* D. Don is an erect and glabrous terrestrial herb, about 30-60 cm high, rootstock with 2 oblong tubers, leaves usually 2-3, ovate-lanceolate with sheathing stem base, flowers are fragrant, pinkish 10-30cm long terminal spikes, capsules ovoid and ribbed.[3]

Its tubers are known as *Salam-mishri* by local inhabitants of Himalayan regions including Uttarakhand.[4] It is reported to be ethno-medicinally useful for treating various illnesses including malaria, fever, dysentery, diarrhea, weakness, etc. by consuming decoction of its tubers, roots and stems.[5] Its tubers are also used as a nutritional supplement.[6] This plant is rich in various phytoconstituents including alkaloids, flavonoids, saponins, and steroids.[7]

As per WHO, the three major attributes for standardization and quality control of herbal medicines include authenticity, purity and assay.[8] Hence, the present work is done to establish botanical and chemical standards like macroscopic, microscopic, physicochemical, phytochemical and chromatographic analysis of tubers of *Satyrium nepalense* D. Don, that would be helpful in preparing a monograph for the identification of genuine plant. The pictures of habit, habitat, inflorescence and tubers of *Satyrium nepalense* D. Don are shown in figure 1, 2, 3, and 4 respectively.

Figure 1: Habit.

Figure 2: Habitat.

Figure 3: Inflorescence.

Figure 4: Tubers.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

The solvents and chemicals used in this study were of analytical grade. Petroleum ether, chloroform, ethyl acetate, and ethanol were purchased from Merck (Mumbai, India). Gallic acid and quercetin were purchased from Sigma-Aldrich (St. Louis, MO, USA). A UV-visible spectrophotometer determined all spectrophotometric measures (Thermo- Fischer model Evolution 201).

2.2 Collection and Identification of plant material

The whole plant of *Satyrium nepalense* D. Don. with its inflorescence was collected from *Surkunda* Hill region, Uttarakhand, India in September 2019 for preparation of herbarium for its botanical identification. This plant was taxonomically specified and authenticated by BSI, Dehradun with reference number: BSI/NRC/Tech./Herb. (Ident.)/ 2019-20/776 and account no. 264 on 07/02/2020. The study's tubers were collected in July and August 2020. Plant tubers were thoroughly washed, dried under the sun for 2 days thereafter under shade at room temperature, and then ground into coarse powder.

2.3 Macroscopic and Microscopic study

The fresh and dried tubers were studied macroscopically for various organoleptic characteristics. For the microscopic study, T.S. was prepared, stained, and observed under a microscope.[9] Powder microscopy was done according to the method described by Kokate (2010).[10]

2.4 Physicochemical analysis

Physicochemical studies were done according to guidelines on quality control methods for medicinal plants by WHO.[11]

2.5 Phytochemical study

Chemical tests were carried out by using standard methods for various plant metabolites such as alkaloids, Flavonoids, Tannins, Saponins, etc.[12]

2.6 Thin layer chromatography (TLC)

Ethanol Extract of tubers of *S. nepalense* was taken as a sample. The TLC Profile of the drug sample was done on TLC plates coated with Silica Gel 60F254. The mobile solution used for TLC was Toluene: Ethyl Acetate: Formic acid (6:3.5:0.5). Visualization was done under Iodine vapors and Rf values were recorded.[13]

3. OBSERVATIONS AND RESULTS

3.1 Macroscopic study

The following table shows the organoleptic or macroscopic features of tubers of *S. nepalense* D. Don. (Figure 5) - **[Table No. 3.1]**

Table 3.1: Macroscopic features of tubers of *S. nepalense* D. Don.

S. No.	Character	Fresh tubers	Dried tubers
1.	Shape	Conical and tuberous	Conical.
2.	Size	Length- 4 - 6 cm. Width- 1.5 - 2.5 cm.	Length- 2-3 cm.Width- 0.5-1cm.
3.	Surface	Smooth but irregular	The external surface was found rough due to the presence of longitudinal wrinkles on drying
4.	Oduor	Aromatic	Aromatic
5.	Color	Creamish-white	Creamish – yellow
6.	Taste	Slightly mucilaginous	Sweet
7.	Fracture	Smooth and irregular.	Hard, irregular, and granulated, cut surface creamish white.

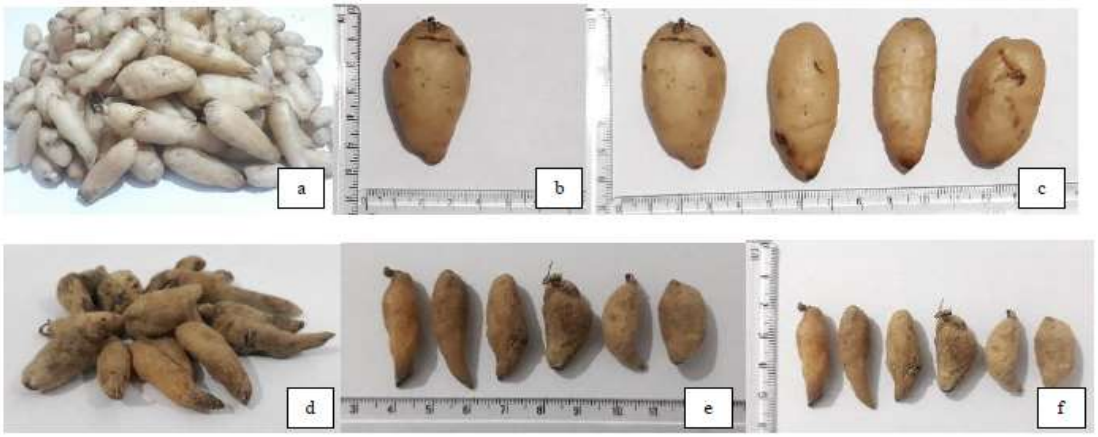

Figure 5: a) Fresh tubers, d) Dried tubers, b) & c) Length & Width of fresh tubers, e) & f) Length & Width of dried tubers.

3.2 Microscopic study

3.2.1 Transverse section

Features that were seen in the Transverse section of tubers of *Satyrium nepalense* D. Don are mentioned below (Figure 6).

- Underground storage tubers are swollen roots enveloped in a velamen of 1-3 layers of elliptical or periclinal compressed cells.
- Tubers hairy or smooth.
- Exodermis 1-layered, constituting outer cortical margin.
- Cells containing starch granules.
- Vascularization polystelic comprising a ring of alternating xylem and phloem elements surrounding parenchymatous pith. They are surrounded by an endodermis and pericycle.

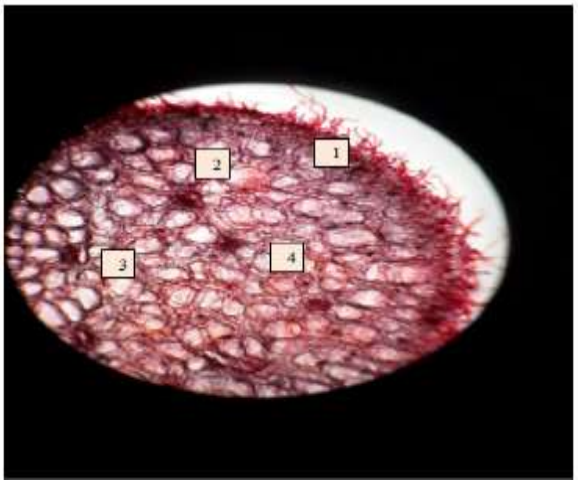

Figure 6: 1) Multiseriate epidermis with trichomes and 2-3 velamen layer, 2) Cortex, 3) & 4) Stele surrounded bypericycle containing vascular bundles.

3.2.2 Powder microscopy

The following table shows the features seen in the Powder microscopy (Figure 7): **[Table No. 3.2.2]**

Table 3.2.2 Features seen in the Powder microscopy.

Features	Powder of Tubers of *S. nepalense* D. Don.
Xylem vessels	+
Starch	+
Palisade cells	-
Oil globules	+
Parenchyma cells	+
Fibers	+

Sclereids	+
Calcium oxalate	+
Stone cells	-

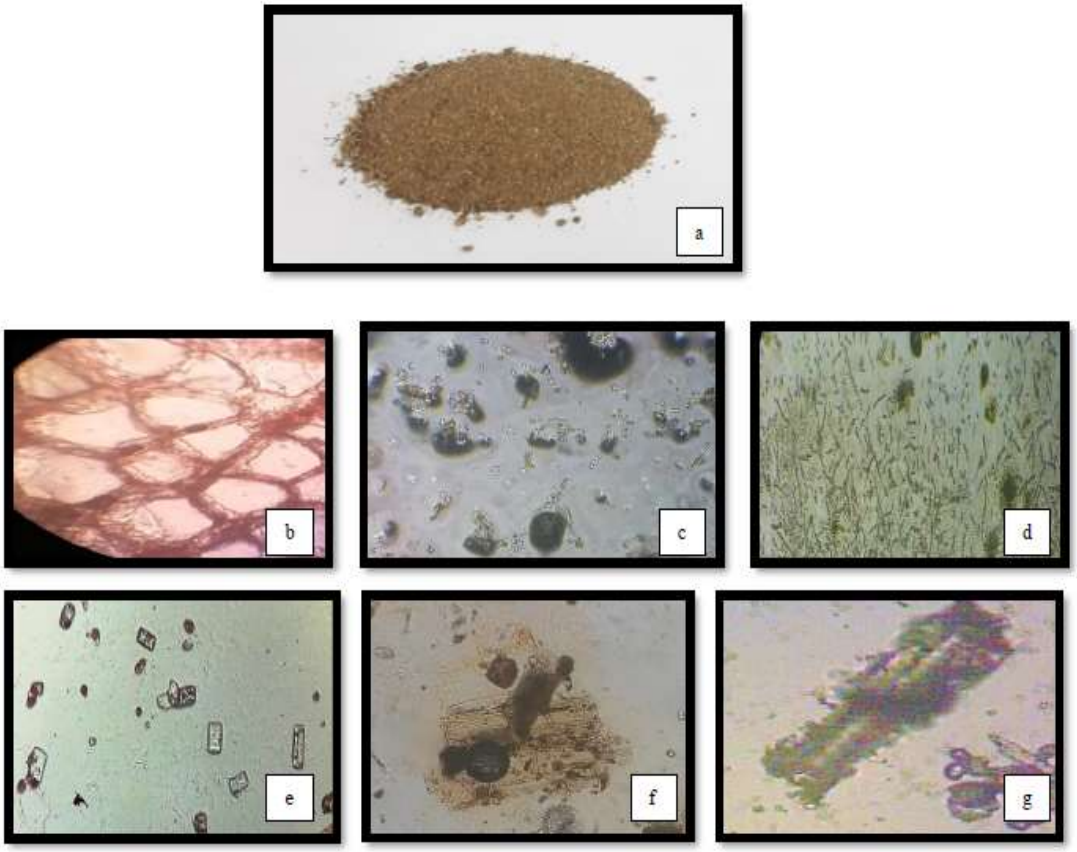

Figure 7: a) Powder of sample, b) Parenchymatous cells, c) Oil globules, d) Calcium oxalate, e) Starch grains, f) Sclerieds, g) Trachieds.

3.3 Physicochemical study

The following table shows the different physicochemical parameters of the sample drug. **[Table no. 3.3]**

Table 3.3: Observed values of physicochemical parameters.

S. No.	Observations	Units	Observed value
1.	Foreign matter	%w/w	0
2.	Moisture content (LOD)	%w/w	6.9
3.	Total ash	%w/w	4.03
4.	Acid-insoluble ash	%w/w	1.1
5.	Water soluble ash	%w/w	2.23
6.	pH value	%w/v	5.9
7.	Water soluble extractive	%w/w	20.18
8.	Alcohol soluble extractive	%w/w	4.31
9.	Petroleum ether soluble extractive	%w/w	4.4

3.4 Phytochemical study

The phytochemical study of this plant was performed for the detection of active constituents in six extracts i.e., PEESN (Petroleum ether extract of *S. nepalense*), CESN (Chloroform extract of *S. nepalense*), EAESN (Ethyl acetate extract of *S. nepalense*), HAESN (Hydroalcoholic extract of *S. nepalense*), EESN (Ethanolic extract of *S. nepalense*), AESN (Aqueous extract of *S. nepalense*).

Test	PEESN	CESN	EAESN	HAESN	EESN	AESN
1. For carbohydrates						
Fehling test	-	-	-	+	+	+
Benedict test	-	-	-	+	-	+
Molish test	-	-	-	+	+	+
2. For alkaloids						
Dragondroff test	-	-	-	-	-	-
Mayer's test	-	-	-	-	-	-
Hager's test	-	-	-	+	-	+
Wagner test	-	-	-	+	+	-
3. For amino acids						
Ninhydrin test	-	-	-	+	+	+
4. For proteins						
Biuret test	-	-	-	+	-	+
Millon's test	-	-	-	-	+	+
Xanthoprotic test	-	-	-	+	+	-
5. For saponin						
Foam test	-	-	-	-	-	+
6. For glycosides						
Borntragor's Test	-	-	-	-	-	-
Keller Killani Test	-	-	-	+	+	+
7. For phenolic compounds						
Phenolic test	-	-	-	+	+	+
8. For flavonoids						
Shinoda Test	-	+	+	+	+	+
Ammonia Test	-	-	+	+	+	+
9. For steroids						
Salkowaski test	+	+	-	+	+	-
Liebermann Burchard test	+	+	-	+	+	-
10. For tannins						
Ferric chloride test	-	-	-	+	-	-
Lead acetate test	-	-	+	+	+	+
Pot. dichromate test	-	-	-	+	+	+

3.4.1 Total phenolic content

Total phenolic content was estimated by using the Folin-Ciocalteu method by monitoring the absorbance at 765 nm. Gallic acid was used as reference standard and values were expressed

as mg of gallic acid equivalents per gram of extract (GAE/g).[14] The concentration of phenols was 16.46 mg GAE/g of extract. The following table shows the absorbance value at different concentrations of Gallic acid and the total phenolic content of the sample. [Table no.3.4.1] Calibration cure of gallic acid is shown in figure 8.

Table 3.4.1: Determination of total phenolic content.

S. No.	Sample	Concentration	Absorbance
1.	Gallic Acid	10 µg/ml	0.246
2.	Gallic Acid	20 µg/ml	0.468
3.	Gallic Acid	40 µg/ml	0.647
4.	Gallic Acid	60 µg/ml	0.967
5.	Test Sample	25% solution	0.354
Total phenolic content = 16.46± 0.12 (mg of gallic acid equivalent /gm sample)			

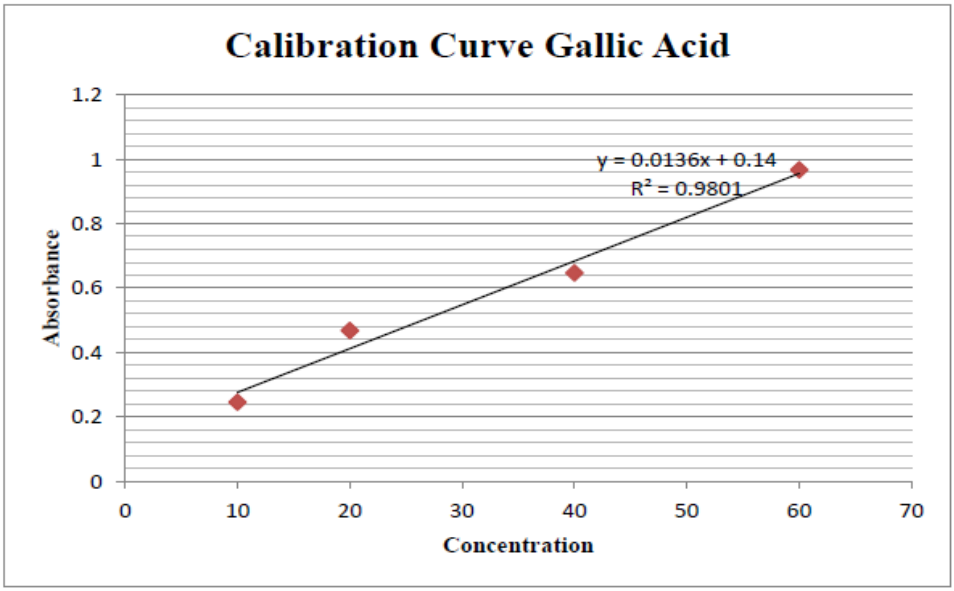

Figure 8: Calibration curve of gallic acid.

3.4.2 Total flavonoid content

The total flavonoid content was measured by aluminium chloride colorimetric technique and the absorbance was measured at 510 nm.[15] The total flavonoid content is expressed as mg of quercetin equivalents per gram of extract (QE/g). The concentration of flavonoids was 34mg QE/g of extract. The following table shows the absorbance value at different concentrations of Quercetin and the total flavonoid content of the sample. [Table no.3.4.2] Calibration curve of quercetin is shown in figure 9.

Table 3.4.2: Determination of total flavonoid content.

S. No.	Sample	Concentration	Absorbance
1.	Quercetin	50 µg/ml	0.093
2.	Quercetin	100 µg/ml	0.175
3.	Quercetin	150 µg/ml	0.229
4.	Quercetin	200 µg/ml	0.747
5.	Test Sample	25% solution	0.041
Total phenolic content= 34 (mg of quercetin equivalent /gm sample)			

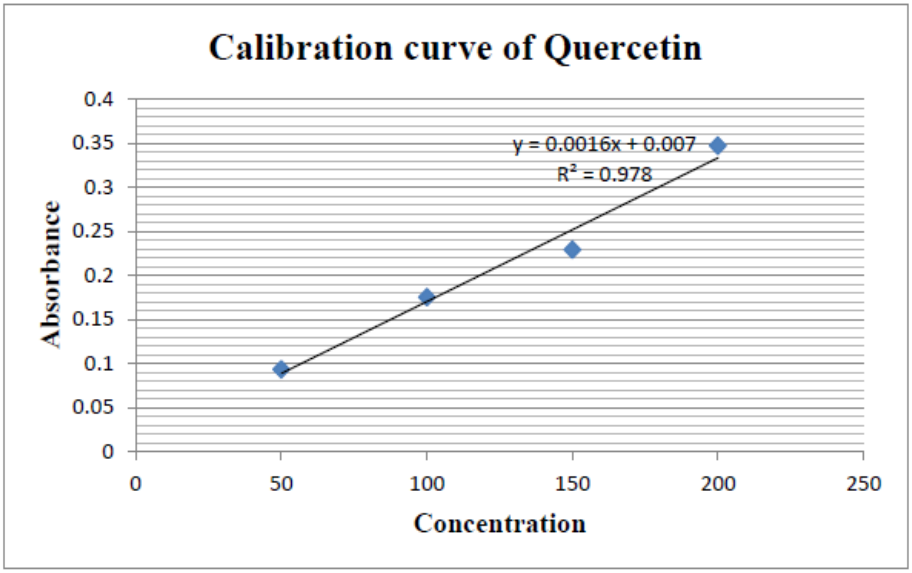

Figure 9: Calibration curve of Quercetin.

3.5 Thin Layer Chromatography (TLC)

The total no. of spots seen were 06 with Rf values 0.45, 0.47, 0.56, 0.61, 0.71, and 0.97.

Figure 10: TLC plate with six spots.

4. DISCUSSION

Based on a detailed organoleptic study of both fresh and dried tubers, it was found that the shape of some fresh tubers was conical and some were tuberous with rough surfaces due to longitudinal ridges on them. After drying shape was conical with longitudinal margins. Fresh tubers were 4-6cm in length and 1.5 to 2.5 cm in diameter. Dried tubers were 2-3 cm in length and 0.5 – 1 cm in width. Tubers were less aromatic when fresh and found to have a strong aroma after grounding them in powder form when dried. The taste of fresh tuber was acrid at the beginning which felt sweet later on. It was slightly mucilaginous too. Dried tuber powder was sweet in taste. The fracture of the fresh tuber was smooth but irregular, cut surface was bright white. After drying, it was hard to break, the cut surface was irregular and granulated and was creamish-white in color.

Microscopic study (T. S) of tubers of *Satyrium nepalense* D. Don. had shown multiple layered epidermis consisting of 1-3 layers of velamen which is the typical feature of an orchid tuber. Tubers were hairy. Exodermis 1-layered, constituting outer cortical margin. Cells contained starch granules. Vascularization polystelic. Each meristele was found to comprise a ring of alternating xylem and phloem elements surrounding the parenchymatous pith. They were surrounded by an endodermis and pericycle. In powder microscopy**,** the powder of the tubers was creamish brown in colour. Parenchymatous cells, oil globules, calcium oxalate crystals, starch grains, sclereids, and tracheids were found in the powdered sample examined under the microscope.

All physicochemical parameters including foreign matter, moisture content, ash values, extractive values, and pH were assessed and were within normal range. **Foreign matter** in the test sample was 0% w/w. It was free from mechanical impurities. **Moisture content** is a critical indicator of the quality of the material. The moisture content of the test sample was 6.9 % w/w. **pH** value plays a significant role in the compatibility of the drug with body fluid, site of action, and stability of the drug. pH value of the test sample was 5.9% w/v. **Extractive value** is directly relative to the strength or potency of the drug which is estimated in different solvents. The water-soluble extractive value found in the test sample was 20.18 % w/w, Alcoholic soluble extractive value was found to be 4.13 % w/w. Petroleum ether extractive value had been found 4.4 % w/w. **Ash value** is the indicator of the presence of inorganic & and earthy matter in the plant. The total ash in the test sample was 4.03 % w/w. The acid insoluble content indicates the presence of silicaceous matter and heavy metals test sample

and it had been found 1.1 %w/w. Water soluble ash estimated the inorganic water-soluble salt in the sample It had been found 2.23 %w/w.

Phytochemical screening of six different extracts (PEESN, CESN, EAESN, HAESN, EESN, and AESN) of *S. nepalense* tuber was carried out by standard qualitative chemical tests. The results showed the presence of carbohydrates, glycosides, and flavonoids in EESN, AESN, and HAESN; phenolic compounds were present in HAESN, EAESN, and EESN, whereas alkaloids were present in HAESN. Saponins, proteins, and amino acids were detected in AESN and HAESN, whereas tannins were found in HAESN, CESN, EAESN, and EESN. Unsaturated sterols/triterpenes were present in HAESN, PEESN, CESN, and EESN.

The result of **the total phenolic content**, using the Folin-Ciocalteu method, is presented in Figure 4.1. 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: $Y = 0.0136X + 0.14$, $R^2 = 0.9801$). The concentration of phenols was 16.46 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: $Y = 0.0016X + 0.007$, $R^2 = 0.978$). The concentration of flavonoids was 34 mg QE/g of extract.

Polyphenols possess antioxidant activity because of the structure of their functional groups. They were found abundant in several hydroxyl groups and serve as sources of hydrogen ion donors to free radicals. This influences several mechanisms of antioxidant activity such as scavenging radicals and metal ion chelation ability. This might be responsible for its energizing and rejuvenating potential for maintaining health.[16] They act by suppressing ROS formation by either inhibiting enzymes involved in their production, scavenging of ROS, or upregulation or protection of antioxidant defenses.[17] Flavonoids are a group of natural substances with variable phenolic structures. They are found in fruits, vegetables, grains, bark, roots, stems, flowers, tea and wine. Flavonoids are now considered an indispensable component in a variety of nutraceutical, pharmaceutical, medicinal, and cosmetic applications. This is attributed to their anti-oxidative, anti-inflammatory, anti-mutagenic, and anti-carcinogenic properties coupled with their capacity to modulate key cellular enzyme function. Flavonoids can be used as potential steroid-genesis modulators against three enzymes 3β-hydroxysteroid dehydrogenase (HSD), 17β-HSD, and aromatase of the steroid-genesis pathway.[18] Thin layer chromatography establishes the phytochemical fingerprint

profiling in drugs for identity. T.L.C. plate of the test sample showed 6 spots under iodine vapour visualization. Calculated Rf values were - 0.45, 0.47, 0.56, 0.61, 0.71, 0.97.

5. CONCLUSION

Satyrium nepalense D. Don (*salam-mishri*) is a valuable plant that is being used in the traditional system of medicine as an energizing tonic, aphrodisiac, and in diseases like diarrhea, dysentery, low backache, nephritis, cuts, wounds, etc. A Pharmacognostical study showed that a genuine plant sample was taken for the study. Typical characters including the velamen layer were seen in organoleptic and microscopic studies. Physico-chemical parameters were within normal limits indicating good collection and processing of the test drug. The extractive value of hydro-alcoholic extract was found maximum which can be used for further experiments after more evaluation. Phytochemical tests showed the presence of polysaccharides, amino acids, phenols, flavonoids, saponins, tannins, steroidal glycosides, etc. It was found rich in phenols and high content of flavonoids which might be responsible for its therapeutic action by reducing ROS formation and preventing cellular integrity. This study will be helpful for further pharmacological approaches.

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Conflict of interest statement

We declare that we have no conflict of interest.

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