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# Estimation of Total Phenolic Content, Total Flavonoid Content, Antioxidant Activity and Molecular Docking Studies of *Stereocaulon piluliferum* Th. Fr

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**Abstract**

Secondary metabolites, along with most compounds found in lichens, possess unique qualities that contribute significantly to combating various diseases. The present research study aimed to assess the estimation of total phenolic content, total flavonoid content and antioxidant activity of a methanolic extract and molecular docking of chemical compound present in *Stereocaulon piluliferum*. The total phenolic content of the lichen extract was determined using the Folin-Ciocalteu reagent method, while the total flavonoid content was assessed using the aluminum chloride method. Similarly, antioxidant activity was evaluated through DPPH free radical scavenging. *S. piluliferum* displayed notable levels of total phenolic and flavonoid content at 50.92593 ± 0.12 mg GAE/g and 9.888 ± 0.21 mg QE/g, respectively. The methanolic crude extract of *S. piluliferum* demonstrated potent antioxidant properties with an $IC_{50}$ value of 111.95 ± 0.98 µg/mL. This study suggests that lichens, specifically *S. piluliferum*, could serve as a valuable natural source of antioxidants and showed the significant inhibition activity by exhibited a binding affinity of -8.5 kcal/mol of atranorin against alpha-glucosidase through computational analysis.

**Keywords:** Alpha-glucosidase, Atranorin, DPPH free radical scavenging, Inhibition activity, Lichen, Medicinal

## Introduction

A total of 45 lichen families, comprising 130 genera and 550 species, have been reported from various regions in Nepal, are classified according to altitude (Baniya, 2024). However, certain species can also adapt to lower elevations influenced by various environmental factors like moisture, temperature and substrate nutrients (Huang, 2010). *Stereocaulon puliferum* Th. Fr, a species of *Stereocaulon*, primarily grows on rocks or soil, features branched pseudopodetia with cylindrical, tapering, grey to ashy-green phylloclades; sacculate cephalodia; and globular apothecia that are orange to pale-orange in early development, often densely clustered. In mature *S. piluliferum*, typically only a few or a single apothecium is found in a terminal position, suggesting that many of the apothecial initials observed in youth fail to mature and degenerate instead (Awasthi, 1961). Transitional stages toward the palisade type, with elongated learning, can be observed within this species. Furthermore, *S. piluliferum* exhibits long multiseptate spores (Lamb, 1951).

Medicinal lichens have been utilized in therapies globally for centuries and continue to hold significance in medicine, possibly due to their chemical makeup. Among these compounds are also phenolic substances, encompassing phenolic acids (such as hydroxybenzoic and hydroxycinnamic acids), polyphenols (including hydrolyzable and condensed tannins) and flavonoids (Do et al., 2014). Antioxidants are substances that can hinder oxidation by interacting with free radicals, binding catalytic metals and neutralizing oxygen in biological environments (Halliwell & Gutteridge, 1984). The antioxidant capabilities of lichens and their secondary compounds remain largely unexplored. Recently, however, researchers have begun investigating the potential of lichens as sources of natural antioxidants (Aoussar et al., 2020; Behera et al., 2006; El-Garawani et al., 2020; Ersoz et al., 2017; Gulluce et al., 2006; Kosanić et al., 2016; Saha et al., 2021; Soundararajan et al., 2019).

The majority of phytochemicals found in lichens, such as phenolic compounds, dibenzofurans,

depsides, depsidones, pulvinic acid derivatives, lactones, and quinines, possess a high concentration of secondary metabolites (Yusuf, 2020), exhibit high potential of medicinal purpose for different biological activities like anti-diabetic (Mükemre et al., 2021), anticancer (Ingelfinger et al., 2020) and others. Previous studies have demonstrated the antiviral (Karagoz & Aslan, 2005), antioxidant (Ghate et al., 2013; Grujičić et al., 2014; Paudel et al., 2012;), antibacterial (Kosanić & Ranković, 2011; Kosanić et al., 2014) and antimutagenic properties (Agar et al., 2010; Kotan et al., 2011) of lichen metabolites, along with their anticancer effects against human melanoma, colon carcinoma (Ari et al., 2015), breast, and lung carcinoma (O'neill et al., 2010; Singh et al., 2013). *Stereocaulon* sp. also exhibited various biological activities, including antioxidant and antimycobacterial properties (Gupta et al., 2007).

Recent research has focused on exploring natural antioxidants sourced from a variety of origins, including plants, micro and macro-algae, macromycetes and lichens. This study specifically investigates the estimation of total phenolic and flavonoid content, antioxidant activities and *in silico* study of *S. piluliferum*.

## Materials and Methods

### *Specimen collection and identification*

The lichen was collected from Champadevi hill which lies on the southwest part of Kathmandu Valley at the height of 2278 meter above sea level, which was the study area as shown in Figure 1. The species initially wasn't recognized in the field but was later identified at the National Herbarium and Plant Laboratories (KATH), Godawari, Lalitpur, Nepal with voucher code no: LIC011.

### *Materials*

Methanol ($CH_3OH$), dimethylsulfoxide (DMSO), sodium carbonate ($Na_2CO_3$), aluminium trichloride ($AlCl_3$) and triple de-ionized water from a local vendor. Folin-Ciocalteu reagent (FCR), quercetin, gallic acid and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were sourced from Sigma-Aldrich in Germany.

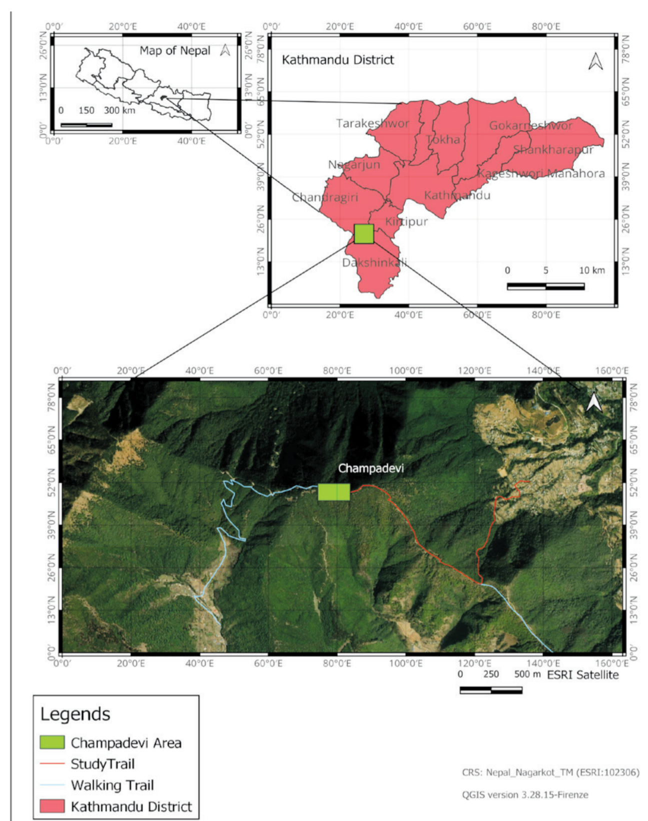



**Figure 1:** Location map of collection area, Champadevi Hill, Kathmandu, Nepal

### *Methods*

**Methanol extract preparation:** The collected lichen materials were shade dried before being grind to a fine powder and stored in airtight papers. The sample powder was combined with 90% methanol in a clean and dry conical flask via the cold-percolation method. Subsequently, filtrates were collected in a flask using filter paper. The residue was subjected to extraction by evaporating the solvent and it was concentrated at 45°C using a rotary evaporator. The remaining filtrate was remained in a water bath at 37°C to obtain the pure dried sample. The extract's weight was measured to calculate the yield percentage and it was stored in the refrigerator at 4°C for further investigation.

**Phytochemical screening:** The crude lichen extracts were examined for the presence of phytochemicals such as alkaloids, flavonoids, hormones, glycosides, terpenoids, saponins, proteins, polyphenols, and carbohydrates. The crude extracts underwent a phytochemical screening process (Harborne, 1998).

**Total phenolic content:** The lichen extract underwent total phenolic content (TPC) estimation and was determined using the Folin-Ciocalteu reagent method, with slight modification (Khan et al., 2018). For the reaction mixture, 20 µL of extract (1 mg/mL), 100 µL of FC reagent (10% in distilled water) and 80 µL of 1 M $Na_2CO_3$ were combined. The mixture was then incubated in the dark at room temperature for 20 minutes. The maximum absorbance was measured at 765 nm. A standard solution of gallic acid underwent the same treatment. To prepare the stock solution of 1000 µg/mL concentration, 1 mg gallic acid was dissolved in 1 mL of water. The stock solution was diluted further to a final concentration of 10, 20, 30, 40, 50, 60, 70, and 80 µg/mL. For the total phenolic content test, different concentrations of gallic acid were used as a positive control. The calibration curve was established based on the measured absorbance, and the total phenolic amount was reported as gallic acid equivalent (mg of GAE/g of lichen extract).

**Total flavonoid content:** Total flavonoid content (TFC) was assessed utilizing the aluminum chloride method with minor adjustments (Chang et al., 2002). Specifically, 20 µL of lichen extract (1 mg/mL), 110 µL of distilled water, and 60 µL of ethanol were combined, and an initial reading was obtained at 415 nm. Subsequently, 5 µL each of $AlCl_3$ and $CH_3CO_2K$ were simultaneously introduced, followed by a 30-minute incubation at room temperature. The peak absorbance was recorded at 415 nm. This procedure was also applied to the Quercetin standard solution for which a stock solution with a concentration of 154 µg/mL was made by dissolving 1.54 mg of quercetin in 10 mL of water. The stock solution of quercetin was diluted to provide various concentrations of 10, 20, 40, 60, 80, and 100 µg/mL. The flavonoid concentration in the plant extract was quantified as milligrams of quercetin equivalents per gram (mg QE/g).

### *Antioxidant activity*

The assessment of antioxidant activity utilized the DPPH free radical scavenging method, with minor adjustments (Polu et al., 2017). In detail, 100 µL of differing concentrations of crude extract (100 µg/mL) were mixed with 100 µL of DPPH solution (0.1 mM) in triplicate within a 96-well plate. Following this, the mixture underwent a 30-minute incubation period in darkness at room temperature. Absorbance measurements were subsequently obtained at 517 nm using a microplate reader. The percentage of inhibition was calculated using the following formula:

$$\text{Inhibition} = \left(\frac{A_c - A_s}{A_c}\right) \times 100\%$$

Where Ac and As are the absorbance of the control and sample respectively

### *Computational experiment*

Atranorin, a compound present in all *Stereocaulon* species (Oset, 2014) showed significant inhibition activity against alpha-glucosidase (Devi, 2019; Karunaratne et al., 2014) was obtained in SDF format from the PubChem portal (Kim & Bolton, 2024). The ligand's structure was optimized using Avogadro software (Hanwell et al., 2012) and its resulting structure was saved in PDB format. The protein structure of alpha-glucosidase (PDB ID 5ZCC, resolution 1.70 Å) (Auiewiriyanukul et al., 2018) was retrieved from the Protein Data Bank (www.rscb.org), also in PDB format. The ligand and protein structures were converted from PDB to PDBQT format by using AutoDock Tools (Morris et al., 2009). To validate the docking protocol, the native ligand was docked into the active site of the protein structure, as shown in Figure 2. The RMSD was found to be less than 2 Å.

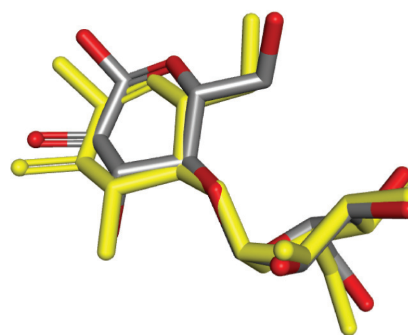


**Figure 2:** Superimposition of native ligand in crystalline structure (brown-red) with native ligand in docked structure (yellow); RMSD <2 Å

For the molecular docking study, Protein rigid docking was performed using AutoDock Vina (Trott & Olson, 2010). The dimensions of the box for the receptor (PDB ID 5ZCC) were set to 30 Å x 30 Å x 30 Å, and the center coordinates were set to 4.395696, 50.355826, and 73.584043, respectively. The exhaustiveness was set to 48, and the energy range was set to 4.

### *ADMET prediction*

The ADMET properties of the ligand were analyzed using the ADMETlab 2.0 (Xiong et al., 2021) webserver while their toxicity was assessed through the ProTox II (Banerjee et al., 2018) webserver.

## Results and Discussion

### *Phytochemical analysis*

Phytochemical screening of *S. piluliferum* was revealed by the presence of alkaloids, flavonoids, tannins/phenols, glycosides, and saponins, absence of volatile oil and terpenoids as represent in Table 1.

**Table 1:** Phytochemical screening of crude extract of *S. piluliferum*

| S. N. | Phytochemical | *S. piluliferum* |
|---|---|---|
| 1. | Alkaloids | + |
| 2. | Flavonoids | + |
| 3. | Volatile oil | - |
| 4. | Glycosides | + |
| 5. | Terpenoids | - |
| 6. | Saponin | + |
| 7. | Phenolic/Tannin | + |

Note: (+) denote presence; (–) denote absence

### *Determination of total phenolic content (TPC)*

Phenols are the primary components in secondary metabolites and play a crucial role in the biological functions of plants. In this investigation, the phenolic concentration in *S. piluliferum* was determined to be 50.925 ± 0.12 mg GAE/g. This outcome was derived utilizing calibration curves constructed from standard Gallic acid, exhibiting an $R^2$ value of 0.986. The equation of the straight line was y = 0.0054x + 0.021, as illustrated in Figure 3 below. Our analysis revealed that the total phenolic content (TPC) in the methanol extract was notably higher compared to a prior study on the methanol extract of *Lobaria pulmonaria*, which reported a total phenolic content was of 87.9 mg/g, Similarly, for *Usnea longissimi*, the phenolic content was found to be 38.6 mg/g (Odabasoglu et al., 2004).

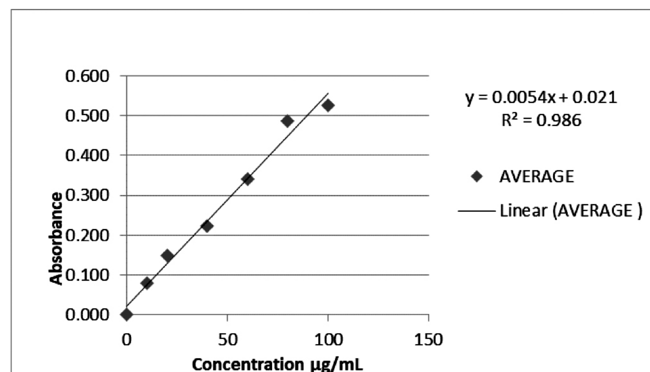



**Figure 3:** Calibration curve for total phenolic content of *S. piluliferum*

### *Determination of total flavonoid content (TFC)*

In this study, the flavonoid content in *S. piluliferum* was 11.51±0.02 mg QE/g, which showed a correlation with the linear equation obtained: y = 0.0179x + 0.0132, with an $R^2$ value of 0.9894 in Figure 4.

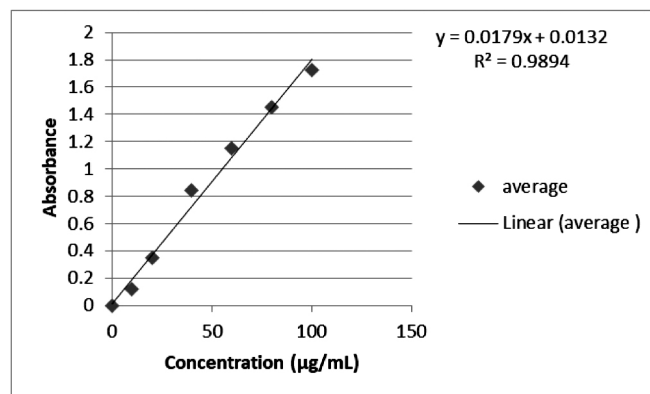



**Figure 4:** Total flavonoid content calibration curve of *S. piluliferum*

The TFC in the methanol extract yielded noteworthy findings when compared to those reported in previous research articles where *Evernia prunastri* methanolic extract TFC value was 5.84 ± 0.03 mg RE/g of DW and 0.05 ± 0.03 mg RE/ g of DW was found in the water extract. Similarly, the methanolic extract of *Ramalina lacera* was 3.97 ± 0.3 mg RE/g of DW, and in the aqueous extract, TFC was 0.01 ± 0.03 mg RE/ g of DW (Naama et al., 2023).

### Antioxidant activity

In this study, the methanol extract of *S. piluliferum* exhibited potent inhibitory activity against DPPH, with an $IC_{50}$ value of 111.95 ± 0.98 µg/mL which was nearer with standard value of quercetin 83.27 ± 1.26 µg/mL. These findings are depicted in percentage inhibition which is shown in the bar graph below (Figure 5). Furthermore, a previous study was observed that the $IC_{50}$ value of the methanol extract of *Cladia aggregata* was 124.24 ± 1.23 µg/mL (Rosli et al., 2020) indicating low inhibition as compared with our currently studied research work.

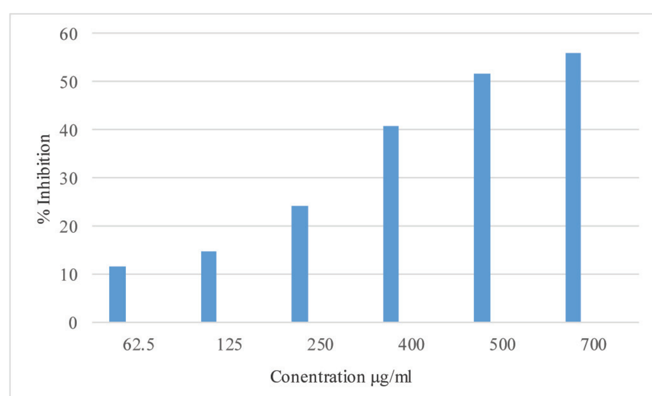



**Figure 5:** Antioxidant of *S. piluliferum* in percentage inhibition

### Molecular docking analysis

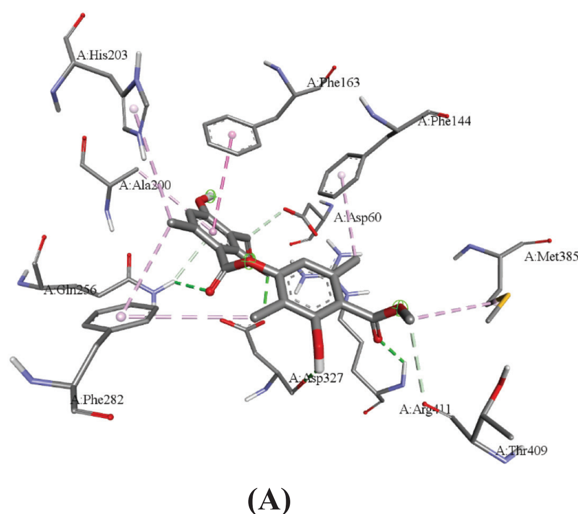
After validating the docking protocol, molecular docking was performed between the protein and atranorin. Atranorin exhibited a binding affinity of -8.5 kcal/mol, compared to the native ligand's -8.4 kcal/mol. The interaction of protein and ligand was analyzed from Pymol and Discovery Studio software (Dassault Systèmes, 2017; Schrodinger, 2015). It formed three hydrogen bonds with GLN256 (2.72 Å), ASP327 (2.45 Å, 2.54 Å), and ARG411 (2.53 Å), as depicted in Figure 6, significantly contributing to the stability of the complexes, as supported by previous reports (Bhaumik et al., 2024; Macabeo et al., 2020). Additionally, C-H bonds, pi-donor hydrogen bonds, alkyl, pi-alkyl, pi-pi stacking and Van der Waals interactions were also observed, as detailed in Table 2.

**Table 2:** Types of interaction with amino acid residue of protein

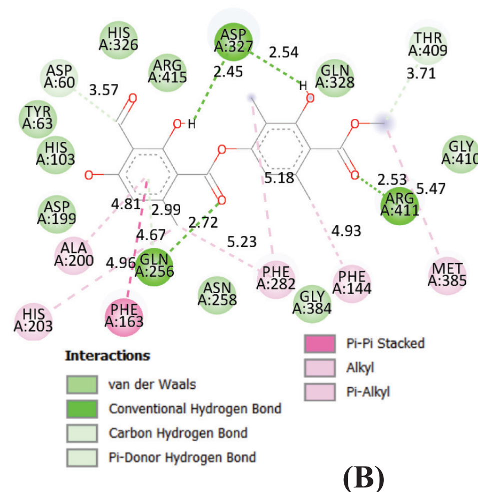
| Types of Interaction | Amino acid residues with a distance (Å) |
|---|---|
| H-bonding | **GLN256** (2.72), **ASP327** (2.45, 2.54), **ARG411** (2.53) |
| C-H bond, Pi-Donar Hydrogen bond, Alkyl, Pi-Alkyl, Pi-Pi stacked | **ASP60** (3.57), THR409 (3.71), PHE144 (4.93), ALA200 (4.81), HIS203 (4.96), PHE282 (5.23, 5.18), MET385 (5.47), PHE163 (4.67) |
| Van der Waals | **TYR63**, HIS103, **ASP199**, **ASN258**, **HIS326**, GLN328, GLN384, GLY410 |

### ADMET analysis

The hit candidate falls into toxicity class 4. Atranorin has an LD50 value of 950 mg/kg, and the compounds comply with Lipinski's rule of five. Additional properties are detailed in Table 3. Overall, studies indicate that atranorin is a promising hit candidate for further investigation.




(A)




(B)

**Figure 6:** 3D (A) and 2D (B) interaction of atranorin with Protein (PDB ID 5ZCC)

**Table 3:** ADMET properties of Atranorin

| Water solubility | Caco2 permeability | Intestinal absorption (human) | Skin permeability | BBB permeability | CNS permeability | CYP2D6 substrate |
|---|---|---|---|---|---|---|
| -3.356 | 0.176 | 81.015 | -2.745 | -1.202 | -3.22 | No |
| **CYP3A4 substrate** | **CYP1A2 inhibitor** | **CYP2C19 inhibitor** | **CYP2C9 inhibitor** | **CYP2D6 inhibitor** | **CYP3A4 inhibitor** | **Total Clearance** |
| No | No | No | No | No | No | 0.659 |
| **Renal OCT2 substrate** | **Hepatotoxicity** | **Carcinogenicity** | **Immunotoxicity** | **Mutagenicity** | **Cytotoxicity** | |
| No | No | No | No | No | No | |

## Conclusion

In the present study, analysis of free radical scavenging activity, total phenolic and flavonoid content showed that *Stereocaulon piluliferum* might be a potent source of natural antioxidants. Atranorin, which are found in all species of *Stereocaulon,* showed a high inhibition activity against alpha-glucosidase by showing binding affinity of -8.5 kcal/mol. However further investigations are required to isolate and characterize the active constituents from this lichen to evaluate their therapeutic role.

## Author Contributions

A Adhikari conceived the entire research project, while A Phunyal and D Karki authored all sections of the manuscript. The manuscript was subsequently reviewed and edited by A Adhikari. All authors have reviewed and consented to the final published version of the manuscript.

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