







# Photoprotective Activity of Lichen Extracts and Isolated Compounds in *Parmotrema Tinctorum*

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**Abstract:** Lichens are an excellent source for the discovery and development of new photoprotective compounds. However, investigation on their bioactivities remains limited, especially in Vietnam. In this study, six lichen extracts (*Parmotrema poolii*, *P. sancti-angelii*, *P. tinctorum*, *Usnea baileyi*, *U. pulvinulata*, *U. undulata*) and five isolated compounds from commonly distributed species *P. tinctorum* (methyl  $\beta$ -orcinol carboxylate (1), atranorin (2), lecanorol (3), salazinic acid (4), 1 $\beta$ -acetoxyhopan-3 $\beta$ ,22-diol (5)) have been screened *in vitro* for their photoprotective activity. The photoprotective potential was investigated by determination of their absolute (UV-PF,  $\lambda_c$ , UVA-PF) and relative indexes (SUI, ISP). The structures of isolated compounds were elucidated by spectroscopic methods and confirmed by X-ray diffraction. The results showed that *P. poolii*, *P. sancti-angelii*, *P. tinctorum*, *U. baileyi*, *U. undulata*, and compounds of methyl  $\beta$ -orcinol carboxylate (1) and salazinic acid (4) acted as strict UVB filters, and their photoprotective activities are comparable to that of the commercial UVB filter (4-methylbenzylidene camphor). Meanwhile, *U. pulvinulata* acted as a UVA booster. Compound (3) presented characteristics of a UVA filter, (2) protected well from both UVA and UVB while (5) exhibited a weak photoprotective activity. For the first time, this work successfully extracted several highly potential solar photoprotective compounds from Vietnam's lichens.

**Keywords:** Lichen; *Parmotrema*; photoprotection; *Usnea*; UV filter.

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## 1. Introduction

The increase in solar UV radiation on the Earth's surface is now considered a major deleterious environmental factor to sun-exposed organisms [1,2]. Due to its harmful effects, the search for new sunscreens still needs to be actively continued. Recently, there is a considerable amount of study aiming to discover and develop new sunscreen metabolites from natural sources, including animals, plants, and lichens [3–5]. Lichens are stable, consistent, and identifiable mutualistic associations between algae and/or cyanobacteria (the photobiont) and fungi (the mycobiont) and dedicated bacterial communities [6,7]. By the year 2000, the number

of lichen species was recorded to be 13,500 to 30,000. With the expansion of investigation scope and development of sequencing methods, such number may increase reasonably [8]. Thanks to this association, lichens can survive under extreme ecological conditions, such as intense UV radiation, extreme temperatures, desiccation, etc. [9–12]. In terms of UV radiation, lichens have developed various photoprotect mechanisms such as screening, quenching, and repair mechanisms [13,14]. An important protective mechanism of lichens is the production of UV screening compounds, such as phenolic compounds (depsides, depsidones, diphenyl ethers), anthraquinones, xanthenes, or shikimic acid derivatives (calycin, mycosporines, scytonemin) [15–18]. Compounds isolated from lichens have been suggested as potential UV blockers due to their absorption on the UV region and their antioxidant activity and photostability [19–24]. Moreover, some isolates with complex structures required a long and complicated synthesis. Therefore, natural products are an important source for the discovery of new UV protectants. In this study, the photoprotective potential of six lichen extracts and five isolated metabolites were investigated by a simple and inexpensive method based on their absolute (UV-PF,  $\lambda_c$ , UVA-PF) and relative indexes (SUI, ISP), which were considered by cosmetic organizations. As a preliminary conclusion, Vietnamese lichens were confirmed as a promising source of photoprotective compounds.

## 2. Materials and Methods

### 2.1. Lichen material.

*Parmotrema poolii*, *P. sancti-angelii*, *P. tinctorum*, *Usnea baileyi*, *U. pulvinulata*, and *U. undulata* were collected in Lam Dong province, Vietnam, in December 2019. The identification was conducted by Dr. Kawinnat Buaruang (Lichen herbarium of Ramkhamhaeng University, Department of Biology, Faculty of Science, Ramkhamhaeng University, Thailand). The voucher specimens were deposited in the herbarium of the Department of Chemistry, Faculty of Science, Can Tho University of Medicine and Pharmacy, Can Tho City, Vietnam.

### 2.2. General experimental procedures.

The NMR experiments were performed on Bruker DMX 300 and 500 spectrometers. UV spectra were recorded on a Hitachi U-2900 spectrophotometer.

Column chromatography was performed on normal phase silica gel (40-63  $\mu\text{m}$ , Kieselgel 60, Merck 7667). Thin-layer chromatography was performed on Kieselgel 60F254 plates (Merck), and spots were visualized under UV light or sprayed with vanillin (0.5 g vanillin in 80 mL sulfuric acid and 20 mL ethanol), and then heated at 120°C. All commercial UV filters were purchased from Sigma Aldrich. To prepare the O/W emulsion, double-distilled water was used. All solvents for extraction used were purchased from Chemsol, purity  $\geq 99.0\%$ .

X-ray structure determination was performed using a RIGAKU XtaLabPro diffractometer equipped with a Mo microfocus sealed tube generator coupled to a double-bounce confocal Max-Flux® multilayer optic and an HPAD PILATUS3 R 200K detector. Data collection and reduction were carried out using CrysaliPro. The structure was also solved by intrinsic phasing methods and refined by full-matrix least-squares methods on 140 parameters, weighted refinement:  $w = 1/[\sigma^2(F_o^2) + (0.0850P)^2]$  where  $P = [\max(F_o^2, 0) + 2F_c^2]/3$ .

### 2.3. Extraction and isolation.

To obtain the crude extracts, the ground air-dried thalli of each lichen (5 g) were extracted with 30 mL a mixture of acetone–dichloromethane (1:1) by maceration for 4 h at room temperature with sonication for 30 minutes. After evaporating the solvents under reduced pressure, the crude extracts were obtained.

To isolate the main secondary metabolites of *P. tinctorum*, 200 g of the dried, crushed material were successively and exhaustively extracted with 2 liters of a mixture of acetone–dichloromethane (1:1) by a hot Soxhlet to give acetone–dichloromethane extract. When the extract was evaporated under reduced pressure, a precipitate occurred and was filtered off (1.5 g). After re-crystallized, the precipitate was subjected to a silica gel column and eluted with *n*-hexane–ethyl acetate 95:5 to yield atranorin (2, 6.5 mg). The rest of the extract was then subjected to silica gel column chromatography and eluted by the solvent system of petroleum ether–ethyl acetate with increasing ethyl acetate ratios to obtain five fractions from Ac1 to Ac5. The fraction Ac3 was subjected to a silica gel column and eluted with *n*-hexane–ethyl acetate with increasing ethyl acetate ratios to yield methyl  $\beta$ -orcinol carboxylate (1, 5.9 mg), 1 $\beta$ -acetoxyhopan-3 $\beta$ ,22-diol (5, 4.5 mg), lecanorol (3, 7.1 mg) and salazinic acid (4, 8.3 mg).

Compound 1: colourless needles (acetone);  $^1\text{H NMR}$  ( $\text{CDCl}_3$ , 300 MHz)  $\delta_{\text{H}}$  (ppm) 2.10 (3H, *s*, 8-CH<sub>3</sub>), 2.46 (3H, *s*, 9-CH<sub>3</sub>), 3.92 (3H, *s*, -COOCH<sub>3</sub>), 5.09 (1H, *s*, 4-OH), 6.21 (1H, *s*, H-5), 12.05 (1H, *s*, 2-OH);  $^{13}\text{C NMR}$  ( $\text{CDCl}_3$ , 75 MHz)  $\delta_{\text{C}}$  (ppm) 105.4 (C-1), 158.1 (C-2), 108.6 (C-3), 163.3 (C-4), 110.7 (C-5), 140.3 (C-6), 172.7 (C-7), 7.8 (C-8), 24.2 (C-9), 52.0 (-COOCH<sub>3</sub>).

Compound 2: colorless powder;  $^1\text{H NMR}$  ( $\text{DMSO-}d_6$ , 500 MHz)  $\delta_{\text{H}}$  (ppm) 10.52 (1H, *s*, 2-OH), 10.21 (1H, *s*, 3-CHO), 6.41 (1H, *s*, H-5), 2.35 (3H, *s*, H-8), 6.65 (1H, *s*, H-5'), 2.04 (3H, *s*, H-8'), 2.39 (3H, *s*, H-9'), 3.88 (3H, *s*, 7'-OCH<sub>3</sub>);  $^{13}\text{C NMR}$  ( $\text{DMSO-}d_6$ , 125 MHz)  $\delta_{\text{C}}$  (ppm) 107.9 (C-1), 163.6 (C-2), 109.0 (C-3), 161.7 (C-4), 115.2 (C-5), 151.4 (C-6), 164.5 (C-7), 193.8 (C-8), 20.1 (C-9), 110.6 (C-1'), 157.4 (C-2'), 116.3 (C-3'), 148.8 (C-4'), 115.7 (C-5'), 136.5 (C-6'), 169.7 (C-7'), 9.3 (C-8'), 21.1 (C-9'), 52.3 (7'-OCH<sub>3</sub>).

Compound 3: red needles (acetone);  $^1\text{H NMR}$  ( $\text{MeOD}$ , 300 MHz)  $\delta_{\text{H}}$  (ppm) 2.27 (3H, *s*, H-7'), 2.54 (3H, *s*, H-8), 6.19 (1H, *brs*, H-3); 6.26 (1H, *brs*, H-5), 6.40 (1H, *brs*, H-3'), 6.46 (1H, *brs*, H-5'), 6.53 (1H, *brs*, H-1');  $^{13}\text{C NMR}$  ( $\text{MeOD}$ , 75 MHz)  $\delta_{\text{C}}$  (ppm) 105.1 (C-1), 164.4 (C-2), 101.8 (C-3), 166.8 (C-4), 112.9 (C-5), 144.7 (C-6), 171.5 (C-7), 24.4 (C-8), 114.8 (C-1'), 159.4 (C-2'), 107.4 (C-3'), 152.3 (C-4'), 114.5 (C-5'), 141.6 (C-6'), 21.4 (C-7').

Compound 4: colourless needles (acetone);  $^1\text{H NMR}$  ( $\text{DMSO-}d_6$ , 300 MHz)  $\delta_{\text{H}}$  (ppm) 2.45 (3H, *s*, 9-CH<sub>3</sub>), 4.64 (2H, *s*, 8'-CH<sub>2</sub>-), 6.79 (1H, *brs*, H-9'), 6.88 (1H, *s*, H-5), 8.29 (1H, *brs*, 4-OH), 10.48 (1H, *s*, 8-CHO), 12.06 (1H, *brs*, 2'-OH);  $^{13}\text{C NMR}$  ( $\text{DMSO-}d_6$ , 75 MHz)  $\delta_{\text{C}}$  (ppm) 111.9 (C-1), 165.9 (C-2), 110.7 (C-3), 164.0 (C-4), 117.4 (C-5), 152.8 (C-6), 160.3 (C-7), 192.7 (C-8), 21.4 (C-9), 109.6 (C-1'), 152.2 (C-2'), 123.5 (C-3'), 148.2 (C-4'), 137.3 (C-5'), 138.1 (C-6'), 163.5 (C-7'), 52.6 (C-8'), 94.8 (C-9').

Compound 5: colorless powder; CCDC 1811397 contains the supplementary crystallographic data for this paper. The data can be obtained free of charge from The Cambridge Crystallographic Data Centre via [www.ccdc.cam.ac.uk/data\\_request/cif](http://www.ccdc.cam.ac.uk/data_request/cif).

### 2.4. Determination of photoprotective activity.

The photoprotective activity was determined according to the method of Legouin [25] with some modifications. Briefly, the oil/water (O/W) emulsion was prepared by dissolving 10

g of a sodium dodecyl sulfate solution (SDS) 10% in 20 g of distilled water. After vigorous mixing in a blender, 10 g of liquid paraffin was incorporated to obtain a homogeneous emulsion. The test samples were added to O/W emulsion at 10% (w/w) concentration. A final test sample concentration of 20 µg/mL was observed by dilution in ethanol. Three commercial UV filters (4-methylbenzylidene camphor (4-MBC)), Avobenzone, and Octocrylene were used as positive controls. Absorbance ( $A_\lambda$ ) measurements between 290 and 400 nm corresponding to UV region were recorded. The experimental data were transferred into an Excel spreadsheet specially arranged for calculation. Transmittance ( $T_\lambda$ ) was calculated from absorbance. The experimental values resulted directly in both UV-PF and UVA-PF values, the critical wavelength ( $\lambda_c$ ) [26], the UVA-PF/UV-PF ratio, the Spectral Uniformity Index (SUI) [27] and the Ideal Spectral Profile (ISP) [28]. UV-PF values are the means of different values calculated from ( $E_\lambda \times I_\lambda$ ) relative to Mexico, Melbourne, COLIPA International 2006, and COLIPA UVA 2007. All tests were done in duplicate, and the results averaged.

$$A(\lambda) = 10^{-T} \tag{1}$$

$$UV - PF = \frac{\sum_{290}^{400} E_\lambda I_\lambda A_\lambda}{\sum_{290}^{400} E_\lambda I_\lambda T_\lambda A_\lambda} \tag{2}$$

$$UVA - PF = \frac{\sum_{320}^{400} E_\lambda I_\lambda A_\lambda}{\sum_{320}^{400} E_\lambda I_\lambda T_\lambda A_\lambda} \tag{3}$$

$E_\lambda$  is the spectral irradiation of terrestrial sunlight at  $\lambda$ ,  $I_\lambda$  is the erythemal action spectrum at  $\lambda$  and  $T_\lambda$  is the spectral transmittance of the sample at  $\lambda$ .

$$\lambda_c = 0.9 \int A(\lambda) d\lambda \tag{4}$$

$$UVA/UVB \text{ ratio} = \frac{\int A(\lambda) d\lambda / \int d\lambda}{\int A(\lambda) d\lambda / \int d\lambda} \tag{5}$$

$$SUI = \frac{\sum_{290}^{380} A_\lambda}{\sum_{290}^{380} |A_\lambda - \bar{A}|} \tag{6}$$

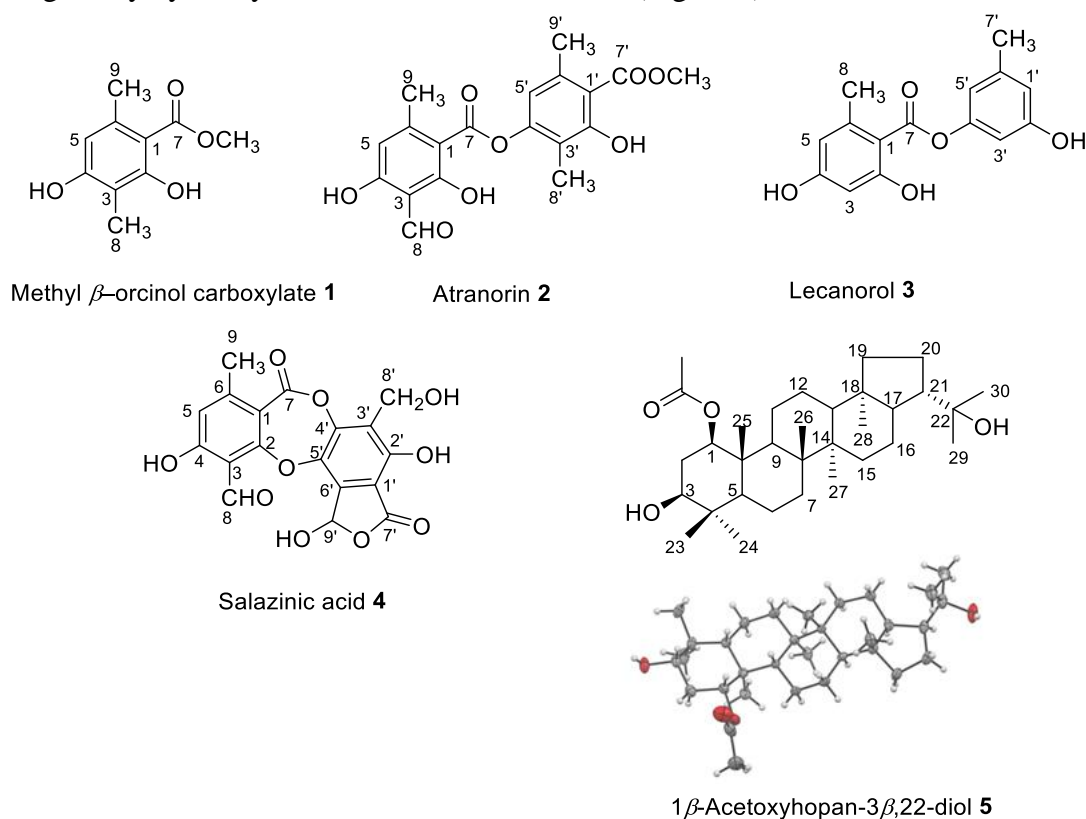
$$ISP = \frac{\sum_{290}^{400} |A_\lambda - \hat{A}_\lambda|}{\sum_{290}^{400} A_\lambda} \times 100 \tag{7}$$

where  $A_\lambda$  is the spectral absorbance at  $\lambda$  and  $\hat{A}_\lambda$  is the ideal spectral absorbance at  $\lambda$ .  $\hat{A}_\lambda$  is equal to the mean absorbance between 290 and 385 nm for all wavelengths in this spectral interval. Between 385 and 400 nm,  $\hat{A}_\lambda$  is given as:

$$\hat{A}_\lambda = A_{385} \times \frac{400 - \lambda}{15} \tag{8}$$

### 3. Results and Discussion

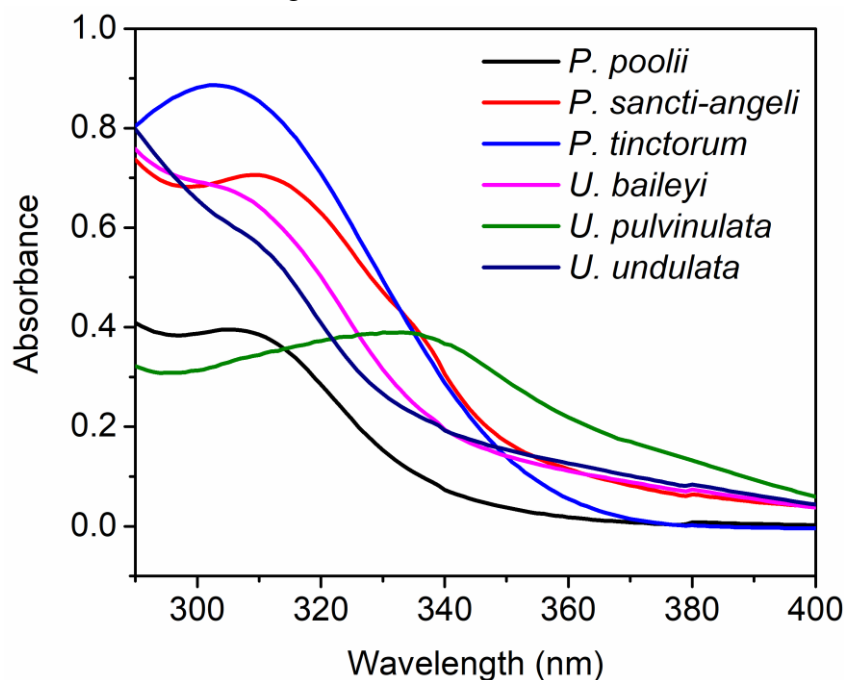
General extraction yields varied between 7.2 % and 12.4 %, in which *P. tinctorum* possessed the highest one. With an extractable yield higher than 10% (w/w), such species were interested in industrial use. In this study, a mixture of acetone–dichloromethane (1:1) was used to extract a great variety of metabolites, which have been considered clearly as lichen UV-protectant metabolites [29]. The results suggested that *P. tinctorum* contained more photoprotective ingredients than five other species. Besides collection with suitable quantity, a study on phytochemical constituents of *P. tinctorum* was conducted to obtain compounds involved in the photoprotective activity. From acetone–dichloromethane extract, five compounds were isolated and determined structures, including one monocyclic aromatic compound (methyl  $\beta$ -orcinol carboxylate 1), two depsides (atranorin 2, lecanorol 3), one depsidone (salazinic acid 4) and one triterpenoid (1 $\beta$ -acetoxyhopan-3 $\beta$ ,22-diol 5). Their structures were elucidated by spectroscopic data and compared with those in references [30–32]. The absolute configuration of 5 was confirmed as C1 *R*, C3 *S*, C5 *S*, C8 *R*, C9 *S*, C10 *R*, C13 *R*, C14 *R*, C17 *S*, C18 *S*, C21 *S*. Here, the absolute configuration of (5) was elucidated unambiguously by X-ray diffraction for the first time (Figure 1).



**Figure 1.** Structures of isolated compounds in *P. tinctorum*.

The photoprotective effects of lichen extracts and isolated compounds were evaluated on absolute (UV-PF,  $\lambda_c$ , UVA-PF) and relative indexes (SUI, ISP), which were mentioned by cosmetic organizations. The absorbance was recorded between 290–400 nm, corresponding to the UVB and UVA regions. Based on the values obtained with commercial organic filters, a decision tree was created to classify the filters. Figure 2 presented that the extracts of *P. poolii*, *P. sancti-angelii*, *P. tinctorum*, *U. baileyi*, and *U. undulata* exhibited a maximal absorbance in the region of UVB (290–320 nm) similar to the reference compound 4-MBC, while *U.*

*pulvinulata* showed a maximal absorbance in the region of UVA (320–400 nm) similar to positive UVA filter Avobenzone (Figure 2).



**Figure 2.** The UV absorption profiles of six lichen extracts.

According to the values obtained in Table 1 and decision tree for predicting the UV protection range in Figure 3 [25], the extracts of *P. poolii*, *P. sancti-angelii*, *P. tinctorum*, *U. baileyi*, *U. undulata*, and compounds (1), (4) were suggested as strict UVB filters. With the values  $UV\text{-}PF > 1.5$ ,  $\lambda_c > 370$  nm,  $UVA\text{-}PF < 2$ , *U. pulvinulata* was suggested as UVA booster. Compound (3) exhibited as a UVA blocker, while compound (2) was categorized as (UVA + UVB) blocker. Compound (5) with the triterpenoid skeleton was classified in the rejected group due to the value  $UV\text{-}PF < 1.5$ . The results completely agreed with natural UV protectants' chemical features as the normal presence of unsaturated bonds in conjugated systems [15].

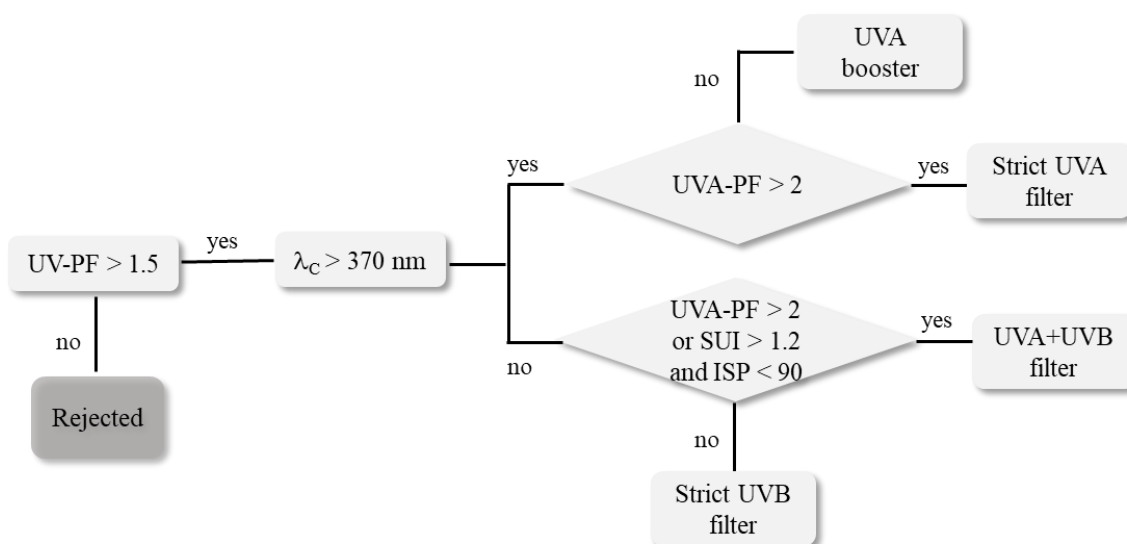
**Table 1.** Absolute, relative indexes of the extracts and compounds compared to three commercialized UV filters.

Extracts/Compounds	UV-PF	$\lambda_c$	UVA-PF	SUI	ISP
<i>P. poolii</i>	3.0	337	1.5	1.3	100.4
<i>P. sancti-angeli</i>	4.1	353	1.9	1.7	99.7
<i>P. tinctorum</i>	2.1	335	1.2	1.2	99.4
<i>U. baileyi</i>	3.6	358	1.6	1.5	98.3
<i>U. pulvinulata</i>	2.2	374	1.9	1.4	94
<i>U. undulata</i>	3.1	363	1.6	1.6	97.1
Methyl $\beta$ -orcinol carboxylate (1)	4.0	320	1.1	0.9	110.5
Atranorin (2)	1.7	376	1.2	1.6	87.9
Lecanorol (3)	4.0	382	2.6	3.3	92.7
Salazinic acid (4)	2.7	338	1.4	1.3	103.9
1 $\beta$ -Acetoxyhopan-3 $\beta$ ,22-diol (5)	1.0	392	0.9	-0.6	-668
4-MBC <sup>a</sup>	13.1	330	1.7	0.9	105.0
Avobenzone <sup>b</sup>	9.6	379	107.5	3.0	99.8
Octocrylene <sup>c</sup>	5.0	339	1.8	1.4	89.0

UV-PF: UV Protection Factor, UVA-PF: UVA Protection Factor,  $\lambda_c$ : critical wavelength.

SUI: Spectral Uniformity Index, ISP: Ideal Spectral Profile

<sup>a, b, and c</sup>: commercial UVB, UVA, and (UVA+UVB) filters.



**Figure 3.** Decision tree for predicting the UV protection range. Reproduced from Ref. [25] (CC BY).

Here, the *in vitro* method for screening samples' photoprotective activity overcomes some disadvantages due to its simplicity and inexpensiveness. Moreover, all these *in vitro* methods so far required an amount of active raw material generally than 100 mg and prevented the evaluation of samples in minute amounts [25]. This method appeared to be a better choice for screening the photoprotective effect of natural products that were usually isolated in small quantities.

So far, UVB protection is urgent, and UVA protection is now considered to be equally essential. Our study confirmed that lecanorol could be a good UVA filter candidate. Besides, atranorin has been reported to act as UV filters. An increasing accumulation of such metabolite in *Cladonia rangiferina* has been observed under high light intensities supplemented by UVA and UVB [15]. Although salazinic acid had no high SPF value, this depsidone was used as a UVA booster [29]. Its strong absorption could explain the accumulation of these compounds in *P. tinctorum* extract in the UV region.

#### 4. Conclusions

This work showed a new solar photoprotective approach of Vietnamese lichens. The extracts of *P. poolii*, *P. sancti-angelii*, *P. tinctorum*, *U. baileyi*, and *U. undulata* were categorized as UVB blockers, while *U. pulvinulata* was suggested as UVA booster. The presence of methyl  $\beta$ -orcinol carboxylate, atranorin, lecanorol, and salazinic acid explained partly higher UV absorption of *P. tinctorum*. Further studies on photostability and phototoxicity of extracts and isolates should be continued for potential cosmetic application as sunscreen.

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## Conflicts of Interest

The authors declare no conflict of interest.

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