

## Evaluation of Photoprotective of *Gyrinops versteegii* (Gilg.) Domke Leaves Extract with Different Solvents and Its Spatial Distribution

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**Abstract:** *Gyrinops versteegii* (Gilg.) Domke is one of the Thymelaeaceae family species with high economic value. The leaf synthesizes secondary metabolites (SMs) supporting herbal medicine, including a class of SMs that serve as sunscreen. However, the SMs are not equally distributed in the plant, depending on the organs, tissues or cell type. This research aimed to determine the spatial distribution of secondary metabolites in the leaves, especially in the epidermis and mesophyll tissues. The epidermis tissues were separated from the mesophyll using the *carborundum abrasion* (CA) technique, followed by the extraction of SMs using three different polarity solvents, namely methanol, hexane and chloroform. The photoprotective potential was evaluated using the SPF value with the spectrophotometry approach. Subsequently, the phytochemical analysis was done for the total tannin, flavonoid, and phenols content. The results showed that the highest SPF value was observed in the methanol epidermis extract with SPF value of 8.61, followed by methanol mesophyll extract (4.52), chloroform epidermis extract (3.97), chloroform mesophyll extract (2.52) and hexane epidermis extract (0.60) and hexane mesophyll extract (0.16). Total phenolic content (flavonoid, tannin and phenol) in epidermis extract with methanol solvent was higher ( $12.347 \pm 0.652$  mg GAE/g DW), followed by extract with chloroform and hexane solvents. The total phenolic content (flavonoid, tannin and phenol) of mesophyll extract with methanol solvent showed the highest content, followed by chloroform mesophyll and hexane extracts. The results of the histochemical analysis showed that photoprotective compounds accumulate in the epidermis and mesophyll tissue of the leaf of *G. versteegii*.

**Keywords:** *Gyrinops versteegii* (Gilg.) Domke; histochemical; photoprotective; SPF (Sun Protection Factor)

### 1. Introduction

The sun is a source of life for autotrophic organisms by utilizing it as an energy source. For humans, several processes, especially in the skin, involve solar radiation, such as vitamin D, skin color, and others (Ismail, 2013). Solar radiation benefits humans, negatively impacting skin health. Solar radiation consists of ultraviolet light (UV) that may have an adverse effect on prolonged exposure to human skin (Sineke, 2016). Solar radiation that reaches the earth's surface and impacts the skin is UV-A light (315-400 nm) and UV-B light (280-315 nm).

Excessive UV-A and UV-B exposure on the skin can cause erythema, inflammation, hyperpigmentation, wrinkling, hyperplasia, and photocarcinogenesis (Napagoda *et al.*, 2016; Lisnawati *et al.*, 2019). People apply skin protection to reduce the negative effect of sunlight in the form of sunscreen. Sunscreen assists the body's natural defense mechanism to protect against UV radiation. To prevent the impacts of excessive sun exposure, sunscreen products must have a wide absorbance range between 290-400 nm (Dutra *et al.*, 2004). In addition, sunscreen with SPF values contains synthetic chemicals such as titanium oxide and benzophenone, which were reported to affect the skin negatively (Fonseca & Rafaela, 2013).

Photoprotective can reduce skin damage due to ultraviolet radiation (UV) and skin cancer. One way to protect the skin from the sun is to use sunscreen. *Gyrinops versteegii* is one of the agarwood species with several groups of compounds such as phenolics, flavonoids, tannins, and terpenoids that have the potential as antioxidants cytotoxic activities (Nuringtyas *et al.*, 2018). Several secondary metabolites such as tannins, flavonoids, and phenolic compounds that can be sunscreen have also been reported (Ahsanti, 2016). The metabolites profiles of *G. versteegii* leaves have been reported using GC-MS (Wardana *et al.*, 2019) and the SMs variation due to the environment in different locations (Widayat *et al.*, 2021). The photoprotective capability of phenolic compounds consists of two mechanisms i.e., UV light absorption and obstructing reactive oxygen species formation (ROS) (Ilmiah *et al.*, 2018). In addition, according to Costa *et al.*, (2015), plant extracts rich in flavonoids can absorb ultraviolet light at the maximum peak of ultraviolet absorption in the UV-B and UV-A regions. The photoprotective study of agarwood was still little. Thus this study aimed to study the potency of agarwood *G. versteegii* leaf extract as a photoprotective source.

## 2. Material and Methods

### 2.1. Materials

Materials used in this study were fresh leaves of *G. versteegii*, silicon carbide and *paper abrasive P240 CM* to separate epidermis dan mesophyll tissue of *G. versteegii* leaf, aquadest, chloroform, methanol, hexane, ethanol 96%, *Folin-Ciocalteu reagent*, *Folin-Denis reagent*, gallic acid, Na<sub>2</sub>CO<sub>3</sub> (*Sodium carbonate*), tannic acid, quercetin, AlCl<sub>3</sub> (*Aluminium chloride*), NaNO<sub>2</sub> (*Sodium Nitrite*), FeCl<sub>3</sub> (*Iron Chloride*) dan NaOH (*Sodium hydroxide*). Tools used in this study were slide microtome Reichert (Austria), binocular microscope (Boeco Germany), optical microscope (Advance), UV-Vis spectrophotometer (Beckman), vortex mixer (Vortex Genie 2<sup>TM</sup>), analytical balance (Libror AEL-200) and Erlenmeyer (Pyrex).

## 2.2. Methods

### 2.2.1. Sample preparation

The agarwood *G. versteegii* species leaves were collected from Jetis, Bantul, Yogyakarta. The leaves chosen were mature leaves with dark green color, with a length of 12-13 cm and a width of 3.5-4 cm. All leaves were immediately cleaned using running water and then air-dried. Subsequently, the leaves were weighed before undergoing further processing.

### 2.2.2. Epidermis and mesophyll extractions

The extraction process of *G. versteegii* leaves used the *carborundum abrasion* method as epidermis tissue separation technique with leaf mesophyll. The abrasion process of *G. versteegii* leaves was conducted using a modified method by Ilmiah *et al.*, (2018). Epidermis extraction used two materials, namely *silicon carbide* and *P240 CM abrasive paper*. Each leaf sample was weighed 50 g for each solvent used, i.e., methanol, chloroform and hexane. Each upper (adaxial) and lower (abaxial) epidermis of leaf lamina was removed by moderate pressure rubbing. Then, the eroded leaves were put into a 50 mL conical tube filled with either methanol, chloroform, or hexane solvents, followed by mixing in a vortex for 1 min. After that, the leaves were removed. Then, the supernatant was reused to extract 3–4 leaves by keeping 50 mL constant solvent volume so that the epidermis extract was produced in the solvent in the tube. Furthermore, the results of the epidermis extract were filtered into an Erlenmeyer and then transferred to a porcelain dish to be vaporized in an open space. Exfoliated leaves defined as mesophyll were blended until smooth and extracted using methanol, chloroform, and hexane using 1:5 ratio (w/v) maceration for 24 h with periodic stirring. The mesophyll extract was evaporated using the same method as the epidermis extract. The extract obtained in a paste was then weighed and stored in the refrigerator.

### 2.2.3. Total phenolic content (TPC) analysis

The TPC analysis followed a modified procedure from the Folin Ciocalteu method (Ilmiah *et al.*, 2018). A total of 5 mg of the dried extract was weighed and then diluted with 5 mL of methanol. The extract solution was aliquot 1 mL into a 10 mL volumetric flask and then added with 0.4 mL Folin Ciocalteu reagents, incubated for 5 min, subsequently, 4 mL of 7%  $\text{Na}_2\text{CO}_3$  and distilled water were added until it reached the volume of 10 mL. Further incubation time for two hours at room temperature was done prior to the absorbance measurement using a UV-Vis spectrophotometer at a wavelength of 745 nm. This study was conducted with three replications.

#### 2.2.4. Total flavonoid content (TFC) analysis

An amount of 5 mg of epidermis or mesophyll extracts dissolved in 5 mL of 96% ethanol. Then 1 mL of the extract solution was aliquoted into a clean tube and added with 1.8 mL of 10% AlCl<sub>3</sub> and 0.2 mL of 5% NaNO<sub>2</sub> and 2 ml of distilled water. The Sample was incubated for an hour. Finally, the absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 405 nm. We used three replicates for each epidermis or mesophyll extract.

#### 2.2.5. Tannin content analysis

A total of 5 mg of the extract was weighed and then dissolved with 5 mL of methanol. Subsequently, 1 mL of the extract was transferred to a test tube, followed by adding 0.5 mL of Follin Denis, 1 mL of 7% Na<sub>2</sub>CO<sub>3</sub> and 7.5 mL of distilled water. Then, the mixture was incubated for an hour and the absorbance was measured at a wavelength of 760 nm. Finally, three replicates were conducted for each type of extract.

#### 2.2.6. SPF (Sun Protection Factor) value measurement

Around 5 mg of the extract was dissolved in 25 mL of 96% ethanol. The SPF value was determined by measuring the absorbance of the extract using a UV-Vis spectrophotometer at a wavelength of 290-320 nm with a 5 nm interval for each wavelength (Widyawati *et al.*, 2019). The SPF value was calculated by entering the absorbance value of each extracted sample using the Mansur equation (Ng'etich *et al.*, 2014) (Equation 1). The value of EE (λ) × I (λ) was a constant value that has been determined as mentioned in Table 1 (Malsawmtluangi *et al.*, 2013).

$$SPF = CF \times \sum_{320}^{290} EE(\lambda) \times I(\lambda) \times Abs(\lambda)$$

**Equation 1.** SPF value determined by measuring the absorbance of the extract using a UV-Vis spectrophotometer at a wavelength of 290-320 nm with a 5 nm interval for each wavelength. Description: CF = Correction Factor (10); EE = Erythema Effect Spectrum; I = Solar Intensity Spectrum; Abs = Absorbance (λ).

**Table 1.** Product function constants used in the SPF calculation (Malsawmtluangi *et al.*, 2013).

Wavelength (λ)	Function Constant (EE×I)
290	0.0150
295	0.0817
300	0.2874
305	0.3278
310	0.1864
315	0.0839
230	0.0839

#### 2.2.7. Histochemical assay

Phenolic detection analysis used FeCl<sub>3</sub> 10% and Na<sub>2</sub>CO<sub>3</sub> as reagents. Fresh leaves cross-section of *G. versteegii* was soaked for 15 mins in a respected reagent. The sample was observed under a light microscope at the end of the incubation. Phenol in the tissue was characterized by

a change in color from green to black. Flavonoid analysis used NaOH 5% as a reagent. The leaf cross-section of *G. versteegii* was soaked for 15 min. The formation of yellowish color development indicated the presence of flavonoids in the tissue. FeCl<sub>3</sub> as a reagent was used to detect the presence of tannin. The fresh leaf cross-section *G. versteegii* was soaked for 15 mins in 10% FeCl<sub>3</sub> followed by observation under a light microscope at 400 × magnification to detect the presence of tannin indicated by the formation of black, blue, or green color in the tissue.

### 2.2.8. Data analysis

Statistical analysis on the calculation of total flavonoid, total phenolic, total tannin, and SPF value obtained from each treatment of epidermis and mesophyll extract of *G. versteegii* leaves was conducted by two-way ANOVA and continues using LSD posthoc when a significant difference result at  $\alpha < 0.05$  was observed. Furthermore, the SPF value obtained from each extract was evaluated following the SPF value according to Table 2.

**Table 2.** Protection categories based on SPF value from each extract of *G. versteegii* leaves (Widyawati *et al.*, 2019).

SPF value	Protection category
1 < 4	Minimal
4 < 6	Medium
6 < 8	Extra
8 < 15	Maximal
>15	Ultra

## 3. Results and Discussion

### 3.1. Epidermis and mesophyll extraction of *Gyrinops versteegii* (Gilg.) domke leaves

The carborundum abrasion technique was successfully conducted using silicon carbide modified from Nuringtyas *et al.*, (2012) to isolate the epidermis of *G. versteegii* leaf. In this research, epidermis and mesophyll separation were done by additional application of P240 CM abrasive paper, rubbed on the adaxial and abaxial surface of *G. versteegii* leaf slowly and with moderate pressure. Figure 1 shows the comparison of *G. versteegii* leaves before and after abrasion was conducted.



**Figure 1.** Cross-section of fresh *G. versteegii* leaf with magnification 10×40. Description: A = Before abrasion; B = After abrasion, scale bar 30 µm.

Epidermis and mesophyll tissues of *G. versteegii* leaves were extracted using three different polarity solvents, methanol, chloroform and hexane. The order of the polarity of the solvent from non-polar to polar is hexane, chloroform and methanol (Abubakar & Haque, 2020). The results showed differences in weight, color and the physical properties of the extracts produced in each of the solvents used. The mesophyll tissue consistently produced more extract than the epidermis part. This is understandable because the epidermis is much less than the mesophyll. The highest result was obtained from mesophyll methanolic extract with a value of 2.305 g, followed by the chloroform and then hexane of the mesophyll. The same pattern was observed in the epidermis extract, where methanol gave the highest yield of 0.868 g, followed by chloroform and hexane. Both methanol and chloroform produced comparable dark green color paste, while hexane extracts were blackish-dark green (Table 3). Methanol is the most widely used solvent in the isolation process of organic compounds from natural materials because it can attract polar and non-polar compounds (Le *et al.*, 2018; Manurung *et al.*, 2021). Methanol consistently produced the most extracts in the previous study that extracted *G. versteegii* leaves using three different solvents with different polarities (Nuringtyas *et al.*, 2018; Manurung *et al.*, 2021). Prihantini & Rizqiani, (2019) reported that *G. versteegii* contained a high amount of phenolic and terpenoids, which explains why methanol extracts are usually higher than others.

**Table 3.** Result of epidermis and mesophyll of *Gyrinops versteegii* (Gilg.) domke leaf extraction.

Solvent	Extract	Weight (g)	Colors
Methanol	Epidermis	0.868	Dark green
	Mesophyll	2.305	Dark green
Chloroform	Epidermis	0.354	Dark green
	Mesophyll	0.475	Dark green
Hexane	Epidermis	0.241	Dark green- blackish
	Mesophyll	0.363	Dark green- blackish

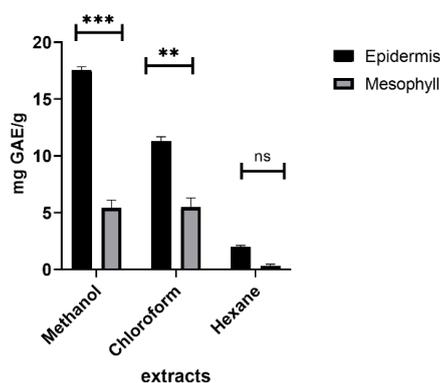
### 3.2. Total phenolic content

Phenolic compounds are one of the secondary metabolites that are abundant in plants. Phenolics have a diverse structure categorized into hydrolyzable tannins (HTs), proanthocyanidins (PAs; syn. condensed tannins), flavonoids, and hydroxycinnamic acid derivatives. Phenolics play a vital role in the interaction of plants with their environment. Its role is diverse, including signaling compounds and antioxidants to survive biotic and abiotic stresses (Hutzler *et al.*, 1998; Kim *et al.*, 2020). To survive abiotic stress, phenolic functions neutralize a prolonged increase in ROS due to plant response to oxidative stress.

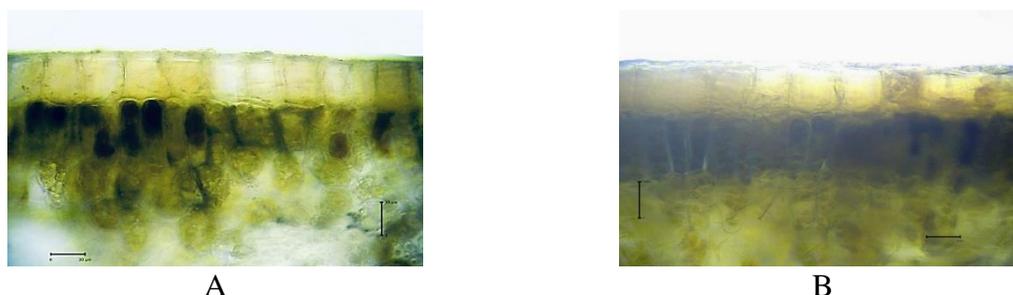
In this study, it was found that the high phenolic content in the leaf epidermis of *G. versteegii*. These results are consistent for extraction with solvents with different polarities. The

highest content observed in methanol extract was  $12.347 \pm 0.652$  mg GAE/g DW followed by a comparable amount on mesophyll methanol extract and epidermis chloroform extract. Extract hexane contained a similar amount of TPC both in the epidermis and mesophyll (Figure 2).

Higher accumulation levels of phenolic content in the epidermis as the outer layer of the leaf compared to mesophyll might be related to their function in protecting plants from damage due to UV-radiation. Cao *et al.*, (2019) also reported that radiation exposure could stimulate the synthesis and accumulation of protective compounds. Moreover, Karabourniotis (1995) supported that finding in his study, showing that phenolic compounds were accumulated in epidermal cells responsible for filtering UV-B radiation. In this study, we observed that in the histochemical assay, the phenolic compounds in the leaf of *G. versteegii* were detected in both epidermis and mesophyll tissue (Figure 3).



**Figure 2.** Total phenolic content of epidermis and mesophyll extracts of *G. versteegii* leaves. Description: \*\*\*=  $p < 0.0001$ , \*\*=  $p < 0.01$ , NS = not significantly different. Statistic analysis was conducted using two-way ANOVA.

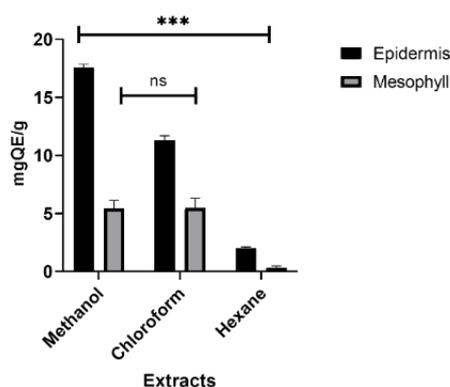


**Figure 3.** The phenolics histochemical assay of *G. versteegii* leaf cross-section (10×40 magnification). Description: A = Phenolic compounds in mesophyll (Palisade) tissue, B = Phenolic compounds in epidermis. Scale Bar 30  $\mu\text{m}$ .

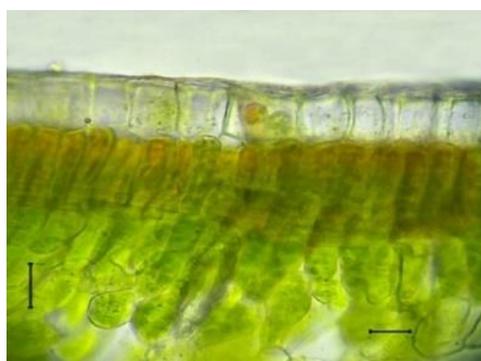
### 3.3. Total flavonoid content

Total flavonoid content analysis showed that the highest flavonoid was observed in the epidermis methanolic extract of  $17.583 \pm 0.27$  mgQE/g DW followed by epidermis chloroform extract of  $11.280 \pm 0.41$  mgQE/g DW. Mesophyll of methanol and chloroform extract was not significantly different. Hexane generally produced a minimal amount of extract for epidermis and mesophyll compared to the other solvents (Figure 4).

The highest flavonoid content of *G. versteegii* was shown in the epidermis methanol extract, followed by methanol mesophyll extract, chloroform epidermis extract, chloroform mesophyll extract, hexane epidermis extract and hexane mesophyll extract. The difference in flavonoid content was significantly different at a significant level of 0.05 in each epidermis and mesophyll extract in each solvent. It showed that more flavonoid compounds accumulated in the epidermal tissue than in the mesophyll tissue of *G. versteegii* leaves. Previous research also reported that flavonoids were found in very high concentrations in the epidermis, but they also accumulate in the leaves mesophyll because of acclimated to high solar irradiance (Gori *et al.*, 2021). The histochemical result confirmed that the epidermis and mesophyll tissue contained flavonoids (Figure 5).



**Figure 4.** Total flavonoid content of epidermis and mesophyll extracts of *G. versteegii* leaves. Description: \*\*\* =  $p < 0.0001$  significant different analyzed using two way ANOVA, Ns = not significantly different .



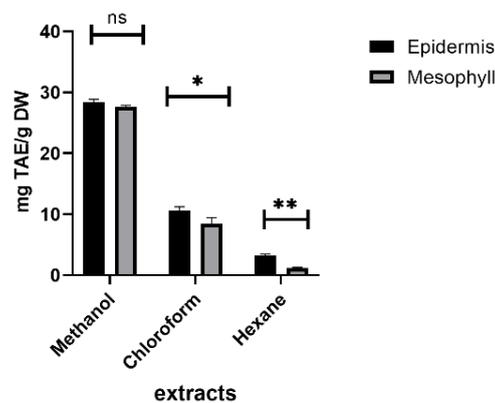
**Figure 5.** The flavonoid histochemical assay of *G. versteegii* leaf cross-section (10x 40). Scale Bar 30  $\mu$ m.

This ability of methanol to extract flavonoids is related to the character of flavonoids which tend to be semi-polar. In fact, methanol is one of the universal solvents widely used to extract phenolic and its derivatives from natural material (Suryanto *et al.*, 2009). This may explain the higher flavonoid content in methanolic extracts regardless of the tissue origin. Flavonoid plays many essential roles in the plant, especially close to its character as a potent natural antioxidant. Flavonoid is believed to play a significant role in protecting plants against

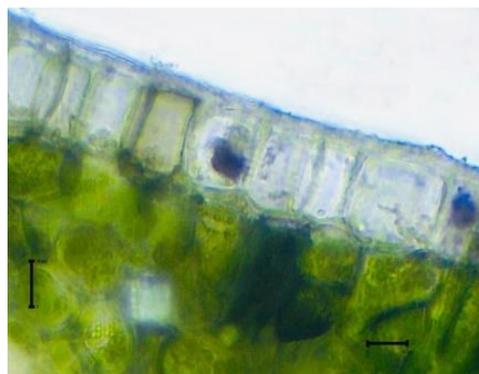
biotic stress i.e., pathogen, insect herbivore and abiotic stress such as UV radiation. Furthermore, as a potent antioxidant, flavonoids may become one of the early defenses against oxidative stress by scavenging ROS generated from the stress (Mierziak *et al.*, 2014; Saewan & Jimtaisong, 2013). Two maximum absorption peaks indicated the flavonoid's activity in protecting against UV radiation at 240-280 nm and 300-550 nm, which may be developed as photoprotective sunscreen for UV B and UV A (Nunes *et al.*, 2018).

### 3.4. Tannin

Tannins are part of the polyphenolics commonly found in plants. Ecologically, tannins strengthen plant defenses against caterpillars and pathogens, modulate dispersal in fruits and seeds and regulators of nutrient cycling and abiotic stress tolerance (Bukharina *et al.*, 2018). The tannin content in *G. versteegii* leaves was comparable in epidermis and mesophyll tissues (Figure 6). This may represent its function for defense against chewing insects, especially in reducing nutrient digestibility. A study by Yuan *et al.*, (2020) proved that tannic acid significantly affected the digestive capacity and food utilization rate of *Hyphantria cunea* larvae.



**Figure 6.** The total tannin content of epidermis and mesophyll extracts of *G. versteegii* leaves. Description: \*\*=  $p < 0.001$ , \*=  $p < 0.05$  and NS = not significantly different analyzed using two-way ANOVA.



**Figure 7.** The tannin histochemical assay of *G. versteegii* leaf cross-section (10× 40). Scale Bar 30  $\mu\text{m}$ .

Concerning its role protects plants from UV-B radiation, a study by Dare *et al.*, (2020) explained that tannin can inhibit collagenase and elastase enzymes activated by UV-B radiation. In addition, tannin presents a wider UV absorption profile, preventing the extension of UV photons from interacting with cellular components. In secondary metabolites observation, it was shown that tannin was accumulated particularly in upper epidermal vacuoles and palisade parenchyma (Halarewicz, 2011). This may explain the high tannin concentration in epidermis tissue of *G. versteegii* leaves (Figure 7).

### 3.4. Sun protection factor (SPF) value

Secondary metabolite compounds such as phenolics, flavonoids and tannins are known to have antioxidant abilities and play a role in protecting plants from UV radiation. In this study, the potency of each extract collected as a photo protectant was evaluated using in vitro sun protection factor (SPF) value. The higher the SPF value, the more sunscreen protection against UV light. SPF has the ability to absorb, reflect or scatter solar radiation (Mulenga *et al.*, 2014). The SPF value of the epidermis and mesophyll extracts of *G. versteegii* leaves can be seen in Table 4.

**Table 4.** Comparison of sun protection factor (SPF) epidermis and mesophyll extracts of *Gyrinops vesteegii* leaf with different solvents.

Solvent	Extracts	SPF value	Protectioncategory
Methanol	Epidermis	8.61	Extra
	Mesofil	4.25	Medium
Chloroform	Epidermis	3.97	Minimal
	Mesofil	2.52	Minimal
Hexane	Epidermis	0.60	-
	Mesofil	0.16	-

The highest SPF value was observed in the epidermis methanol extract with a value of 8.61, followed by methanol mesophyll extract, chloroform epidermis, mesophyll chloroform epidermis hexane and hexane mesophyll, respectively. The SPF value of the epidermal methanol extract was included in the extra category, while the mesophyll methanolic extract, including medium and chloroform, has minimal SPF protection (Table 4). Many factors affected the determination of the SPF value, including the secondary metabolites content that served as photoprotection and antioxidant. The high SPF value of epidermis methanol extract is in line with the high total phenolics, total flavonoids and total tannin content. A related study on the potency of *G. versteegii* leaves as sunscreen was conducted by Wahyuningrum *et al.*, (2018). The study results showed that the high chromophore group in the extract helped the absorption capacity of high-energy ultraviolet light and release energy in the form of low energy light.

#### 4. Conclusion

According to the findings of this study, photoprotective chemicals (phenolic, flavonoids, and tannins) were found to be higher in the epidermis methanol extract than in the mesophyll extract of the *G. versteegii* leaf. The epidermis methanol extract had the highest SPF value. The histochemical assay results showed that photoprotective compounds accumulated in the epidermis and were only distributed in the palisade of mesophyll tissue in *G. versteegii* leaf.

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#### Conflict of Interest

The authors declare no conflicts of interest.

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