Ashitaba (*Angelica keiskei*) leaves extract cream 8% inhibited the increase of melanin amount as effective as hydroquinone cream 4% and inhibited the increase of tyrosinase enzymes not as effective as hydroquinone cream 4% in the ultraviolet B-exposed Guinea pig (*Cavia porcellus*) skin.

### Abstract

**Background:** Excessive sun exposure results in increased activity of the tyrosinase enzyme and the amount of melanin in the skin which causes hyperpigmentation, a sign of premature aging. The purpose of this study was to prove that Ashitaba (*Angelica keiskei*) leaves extract cream 8% inhibited the increase of melanin and tyrosinase enzymes as effective as hydroquinone cream 4% in the ultraviolet B-exposed guinea pig (*Cavia porcellus*) skin.

**Methods:** This study used a randomized post-test only control group design. The subjects were 36 male guinea pigs (*Cavia porcellus*). Subjects were divided into two groups, the hydroquinone cream 4% group and the Ashitaba leaves extract cream 8% group exposed to UVB light. Skin samples were collected 48 hours after the last exposure to measure the tyrosinase enzyme levels using the ELISA method and the amount of melanin was examined by Masson-Fontana staining.

**Results:** The mean levels of tyrosinase in the hydroquinone cream 4% group was 19.51 ± 5.16 ng/L and the Ashitaba leaves extract cream 8% group was 23.76 ± 3.09 ng/L (p = 0.005). The mean amount of melanin in the hydroquinone cream 4% group was 2.98 ± 2.27% and the Ashitaba leaves extract cream 8% group was 4.77 ± 3.33% (p = 0.069).

**Conclusion:** The administration of Ashitaba (*Angelica keiskei*) leaves extract cream 8% inhibited the increase of melanin amount as effective as hydroquinone cream 4% and inhibited the increase of tyrosinase enzymes not as effective as hydroquinone cream 4% in the ultraviolet B-exposed guinea pig (*Cavia porcellus*) skin.

### Keywords

Ashitaba leaves extract, Hydroquinone, Melanin, Tyrosinase, Guinea pig, Ultraviolet B

### Introduction

Every organism on earth will undergo an aging process. Along with aging, various disease...
will arise. However, with the development of science and technology, humans found out the basis of the aging process which is very useful in an effort to slowing down and even preventing aging process. Anti-Aging Medicine (AAM) brings a new concept that the aging process can be equated with a disease that should be preventable and treatable.1

Indonesia is one of the tropical countries that is constantly exposed to the sun. Sunlight which has a wavelength of 100nm - 400nm is called the ultraviolet (UV) light.2 UV radiation will trigger the formation of free radicals such as reactive oxygen species (ROS) and can cause damage to DNA.3 Excessive sun exposure can lead to premature aging and cancer of the skin.4 The signs of skin aging are divided into 4 classifications, namely: wrinkles, loss of skin elasticity, vascular disorders and hyperpigmentation.5 Hyperpigmentation is characterized by increased activity of the tyrosinase enzyme and the amount of melanin in the skin4,6; which will cause black spots on the skin known as melasma.7,8 Physiologically, the skin will respond to these events by producing antioxidants to protect the skin against damage caused by exposure to UV rays.9 In support to this notion, study suggested that glutathione plasma has a strong negative correlation with the MASI score in person with melasma.8

Currently, several modalities of skin hyperpigmentation therapy are available. The topical therapy used as the gold standard for hyperpigmentation is hydroquinone. Topical therapy is often given in the form of a cream.10 Cosmetic products containing hydroquinone, potentially have side effects in the form of mutagenic potential, cause dermatitis, skin irritation, erythema, burning sensation, a prickling sensation, leukoderma, hypochromia and ochronosis.11,12 Therefore, it is necessary to find other therapies that are safe and effective for the skin. Ashitaba is a natural plant that contains flavonoids, saponins, alkaloids, triterpenoids and tannins.13 Several researchers have succeeded in proving in vitro that this plant can inhibit the tyrosinase activity which plays a role in causing hyperpigmentation.14,15 The effect of ashitaba leaves extract as tyrosinase inhibitor in vivo has been proen by Sugito et al., (2019) with a concentration of 8%.9 However, to date, there has not been a study to compare the effects of Ashitaba leaves extract cream 8% with hydroquinone cream 4% which is the gold standard of hyperpigmentation therapy.

Methods

This study was an experimental study using a randomized post test only control group design. This study was conducted in the Integrated Biomedical Laboratory Unit, Faculty of Medicine, Udayana University from June 2020 to January 2021. The samples used were 36 male guinea pigs (Cavia porcellus), local strain, aged 3-4 months, weighing 300-350 grams, one hybrid, brown fur, healthy, willing to eat and drink.

Subjects were divided into two groups, the hydroquinone cream 4% group and the Ashitaba leaves extract cream 8% group exposed to UVB rays. The hydroquinone cream was purchased from PT. Genero Pharmaceuticals. The Ashitaba leaves extract was prepared by using the maceration technique with ethanol 96% in the Laboratory of the Faculty of Agricultural Technology, Udayana University. The Ashitaba leaves extract cream 8% was prepared in the Integrated Biomedical Laboratory Unit, Faculty of Medicine, Udayana University according to previously reported study.9

The treatments were carried out on days without UVB radiation 2 times a day at 10.00.00 and 14.00.00 WITA (Vani, 2013). On the day of UVB exposure, topical ingredients were applied at 10:00:00 WITA (20 minutes before UVB exposure) and at 14:21:05 WITA (4 hours after UVB exposure). The cream treatments were applied at a dose of 0.4 mg on the back of shaved skin of guinea pig for 14 days. The UVB was exposed 3 times a week (Monday, Wednesday and Friday) at 10.20.00 WITA with a dose of 65 mJ/cm2 for 65 seconds each session. To avoid the effects of acute irradiation, skin samples were taken 48 hours after the last exposure. Animals were euthanized prior to sample biopsy with ketamine (100 mg/kgBW) intraperitoneally.

The tyrosinase enzyme levels was examined using the ELISA method and the amount of melanin was determined by Masson-Fontana staining according to previously described methods.9

Results

The mean of tyrosinase levels in the hydroquinone cream 4% group was 19.51 ± 5.16 ng/L while in the Ashitaba leaves extract cream 8% group was 23.76 ± 3.09 ng/L (p = 0.005). This means that the Ashitaba leaves extract cream 8% was less effective in reducing the tyrosinase levels in the ultraviolet B-exposed guinea pig (Cavia porcellus) skin compared to hydroquinone cream 4% with a significant mean difference. The mean amount of melanin in the hydroquinone cream 4% group was 2.98 ± 2.27% while in the Ashitaba leaves extract cream 8% group was 4.77 ± 3.33% (p = 0.069). This means that the Ashitaba leaves extract cream 8% was as effective as hydroquinone cream 4% in reducing the melanin levels in the ultraviolet B-exposed guinea pig (Cavia porcellus) skin.
Table 1. Tyrosinase levels and amount of melanin

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hydroquinone Cream (Mean±SD)</th>
<th>Ashitaba Leaves Extracts Cream (Mean±SD)</th>
<th>Difference (Mean±SD)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosinase (ng/L)</td>
<td>19.5±1.16</td>
<td>23.7±3.09</td>
<td>4.25±1.42</td>
<td>0.005</td>
</tr>
<tr>
<td>Melanin (% pixel melanin)</td>
<td>2.98±2.27</td>
<td>4.77±3.33</td>
<td>1.79±0.95</td>
<td>0.069</td>
</tr>
</tbody>
</table>

SD: Standard Deviation, p: Significance

Figure 1. Macroscopic appearance of Guinea pigs skin. (A) Hydroquinone cream 4% group before treatment. (B) Hydroquinone cream 4% group after 14 days of treatment. (C) Ashitaba leaves extract cream 8% group before treatment. (B) Ashitaba leaves extract cream 8% group after 14 days of treatment.

Figure 2. Microscopic appearance of the skin tissue with Masson-Fontana staining for the examination of melanin. (A) The hydroquinone cream 4% group showed a lower amount of melanin than the Ashitaba leaves extract cream 8% group. The arrows indicate the melanocyte cells that contain black melanin. (B) The Ashitaba leaves extract cream 8% treatment group showed a higher amount of melanin than the hydroquinone cream 4% group. The arrows indicate the melanocyte cells that contain black melanin.

Discussion

The effect of Hydroquinone Cream 4%

Skin hyperpigmentation, which is one of the characteristics of skin aging caused by exposure to UVB rays, can be inhibited by topical or oral therapy. Topical therapies for hyperpigmentation that are commonly used are hydroquinone, kojic acid, ascorbic acid, arbutin, azaleic acid, niacinamide, and glycolic acid. But the gold standard of topical therapy for hyperpigmentation is hydroquinone.

According to previous studies, the inhibitory effect of tyrosinase activity and the specific cell toxicity effect of melanocytes are the two main modalities in the development of depigmentation therapy. Hydroquinone is a hydroxyphenolic chemical compound that inhibits the conversion of DOPA to melanin by inhibiting tyrosinase enzyme activity. This can result in low tyrosinase levels. In vitro studies have shown that hydroquinone concentrations of 1, 10 and 100 µm can reduce tyrosinase levels which were examined by the immunoblotting method in melan-a cells. The mechanism of action of hydroquinone in addition to its tyrosinase inhibitor activity, is by inducing melanocyte cells toxicity and reversely inhibits cell metabolism. Because melanocytes produce melanin, hydroquinone toxicity cause a decrease in the amount of melanin.

Hydroquinone concentrations vary from 2% to 5% whereas higher concentrations are usually more irritating and have a greater risk of photoxicity. Hydroquinone 4% is the gold standard for melasma treatment to date. However, hydroquinone has a side effect in the form of mutagenic potential, cause dermatitis, skin irritation, erythema, burning sensation, a pricking sensation, leukoderma, hypochromia and ochronosis.

The effect of Ashitaba (Angelica keiskei) Leaves Extract Cream 8%

In this study, Ashitaba leaves extract cream 8% was used. Previously, Ashitaba leaves extract has been proven both in vitro and in vivo for hyperpigmentation therapy. In vitro, Arung et al., (2012) proved that the chalcone contained in ashitaba stems can inhibit melanin formation in B16 melanoma cells. Hyeong-U et al., (2011) reported in their research that the ethanol extract of Ashitaba with various concentrations could inhibit the activity of the tyrosinase enzyme, decrease the expression of the tyrosinase gene, tyrosinase-related protein-1, tyrosinase-related protein-2 and melanocyte-inducing transcription factor. This indicates that the action of Ashitaba leaves extract in inhibiting hyperpigmentation is not only limited to being a tyrosinase inhibitor but can also inhibit tyrosinase synthesis through transcriptional regulation.

In vivo, Sugito et al., (2019) have proven the effect of Ashitaba leaves extract in inhibiting the action of the tyrosinase enzyme and the amount of melanin in skin exposed to UVB light in experimental
animals using Ashitaba leaves extract with a concentration of 8%. The effectiveness of Ashitaba leaves extract in inhibiting the increase in tyrosinase and melanin due to the phytochemical content such as flavonoids of 41756.85 mg / 100g, the value of antioxidant capacity is 7093.34 mg/L, the IC50 value is 72.1261 mg/L.

Flavonoids can directly inhibit the activity of the tyrosinase enzyme in its ability as skin depigmentation in the melanogenesis process. The action of the tyrosinase enzyme can also be inhibited by flavonoids when it binds to copper. In addition, the work of the tyrosinase enzyme can also be inhibited by tannins and saponins so that after exposure to UVB rays, melanin formation will not occur because the process of melanin biosynthesis has been inhibited.

**Comparison of Hydroquinone Cream and Ashitaba Leaves Extract Cream**

Because there have been many proven effects of Ashitaba leaves extract cream in preventing hyperpigmentation, this study aimed to prove that Ashitaba (*Angelica keiskei*) leaves extract cream 8% inhibited the increased amount of melanin and tyrosinase enzymes as effective as hydroquinone cream 4% in the ultraviolet B-exposed guinea pig (*Cavia porcellus*) skin.

The results showed that the tyrosinase levels between the groups were significantly different and the tyrosinase levels were higher in the Ashitaba leaves extract cream group. So it can be concluded that the effect of Ashitaba (*Angelica keiskei*) leaves extract cream 8% inhibited the increase in tyrosinase levels was not as effective as hydroquinone cream 4%. The amount of melanin between groups was not significantly different. So it can be concluded that the effect of Ashitaba (*Angelica keiskei*) leaves extract cream 8% in inhibiting the increase in the amount of melanin was as effective as hydroquinone cream 4%.

In several previous studies, it was found that the average amount of melanin in guinea pig skin that had not been treated with UVB exposure and cream was 1%. On guinea pig skin that has been treated with exposure to UVB rays the average amount of melanin is 24.44%. Whereas in this study the guinea pig skin that had been exposed to UVB light and given 4% hydroquinone cream the average amount of melanin was 2.98% and the guinea pig skin that had been exposed to UVB light and giving Ashitaba leaves extract cream 8% the average amount of melanin was 4.77%.

In terms of tyrosinase levels, the effect of Ashitaba (*Angelica keiskei*) leaves extract cream 8% was not as effective as hydroquinone cream. This was because hydroquinone cream is the gold standard for tyrosinase inhibitors. Research comparing hydroquinone and flavonoid activity against the catalytic ability of tyrosinase showed that the IC50 of hydroquinone was 15 μmol/L while some flavonoid derivatives such as flavones, flavonols, flavanones, flavonals, isoflavonoids, chalcones, and catechins have a higher IC50 so that their effectiveness against tyrosinase was lower.

However, regarding the amount of melanin, Ashitaba (*Angelica keiskei*) leaves extract cream 8% has the same effectiveness as hydroquinone cream 4%. This is interesting because Ashitaba leaves extract cream is not as effective as hydroquinone cream in reducing tyrosinase levels. However, this indicates that it is possible that the Ashitaba leaves extract cream could decrease the amount of melanin through other mechanisms, besides inhibiting the increase in tyrosinase activity. This may be related to its antioxidant activity.

Flavonoids and other active compounds can act as primary and secondary antioxidants. As primary antioxidants, flavonoids can reduce the negative effects of free radicals directly. Flavonoids are oxidized by radicals, resulting in more stable and less reactive radicals. In other words, flavonoids stabilize free radicals by reacting with reactive radical compounds. Due to the high reactivity of the hydroxyl groups of flavonoids, the radicals become inactive.

Flavonoids also can act as secondary antioxidants, which activate endogenous enzymatic antioxidants such as catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPx) through activation of Nuclear Factor Erythroid 2-related Factor 2 (Nrf2). Nrf2 is a key transcription factor that acts as a master regulator of the intracellular defense system against oxidative stress. Nrf2 belongs to the basic leucine zipper (bZIP) with a Cap 'n' Collar (CNC) structure. Nrf2 is ubiquitously expressed in various organs and tissues, including the skin. It is tightly regulated by a adaptor protein, Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm which acts as E3 ubiquitin ligase CUL3-RBX1 adaptor, which then plays an important role in Nrf2 degradation via the ubiquitin-proteasome pathway. In this context, Keap1 act as an electrophilic sensor through the sulfhydryl functional group of cysteine residues. Study showed that flavonoids can directly modify one of the seven reactive cysteines in Keap1 (Cys 151, 257, 273, 288, 297, 434, or 613), thereby activate Nrf2 to translocate to the nucleus, forming heterodimers with the Musculo Aponeurotic Fibromatosis (MAFs) family and binds to Antioxidant Responsive Elements (AREs) then activates the transcription of antioxidant genes such as SOD, GPx and Cat. These endogenous antioxidants can act as direct tyrosinase inhibition, shift melanogenesis
from the synthesis of eumelanin to pheomelanin through the reaction between the thiol and dopaquinone groups causing the formation of sulfhydryl-dopa conjugates, and eliminating the effects of oxidative stress in the skin tissue. In support to this notion, study suggested that glutathione plasma has a strong negative correlation with the MAS1 score in person with melasma. This can be the mechanisms underlying Ashitaba effect in melanin that increases due to exposure to UVB rays.

Because ROS due to UVB exposure can increase the amount of melanin through transcriptional activation of proopiomelanocortin (POMC) which is a precursor to melanocyte stimulating hormone (α-MSH), ROS can increase melanin production by melanocytes. Ashitaba leaves extract cream in this case can play a role in inhibiting the increase of α-MSH in addition to inhibiting melanin production by melanocytes, so that the effectiveness of Ashitaba leaves extract cream is comparable as hydroquinone cream.

**Conclusion**

It can be concluded that Ashitaba (*Angelica keiskei*) leaves extract cream 8% inhibited the increase of melanin amount as effective as hydroquinone cream 4% and inhibited the increase of tyrosinase enzymes not as effective as hydroquinone cream 4% in the ultraviolet B-exposed guinea pig (*Cavia porcellus*) skin. Further study is necessary to examine the toxicity of Ashitaba leaves extract cream on the skin, to determine the potential side effects such as irritation, both for short- and long-term application. Moreover, clinical trial is prerequisite before its application as an alternative therapy for hyperpigmentation in human.

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