Photoprotector and anti-inflammatory roles of *Achatina fulica* snail slime extract in sunburn model mice

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Abstract
Problem. This study was conducted to find the antioxidant properties and its roles as photoprotector and anti-inflammatory agent in sunburn model mice. **Approach**. Biochemistry with spectrophotometer was conducted to trace the content of flavonoid, phenols, saponin, tannin, steroid (qualitative). The snail slime was divided into 4 concentration 20%, 50%, 70% and 100% that were applied onto the back of the mice 1hour prior UV radiation. This experimental study with test only control group design was conducted to study photoprotector and anti-inflammatory role a by using 75 *Mus musculus BALB/c* sunburn model mice were divided into 15 groups. The photoprotector effect was measured semi-quantitatively from the histopathologic changes 24 hours after radiation, meanwhile the anti-inflammatory effect was determined by using immunohistochemistry of anti-Interleukin-6 (IL-6) and leukocytes count of peripheral blood. **Findings.** The *A. fulica* snail slime extract was proven to contain flavonoid (+++), phenols (++), saponin (+++) and steroid (++). Snail slime application prior UV radiation showed significantly ($p<0,000$) prevent the histopathologic changes of the skin in sunburn effect from the lowest concentration 20%, it showed to prevent sunburn cell formation, crust formation, ulceration and dermal inflammation. The result of immunohistochemistry of anti-IL-6 also showed the snail slime also prevented the release of IL-6 in sunburn significantly ($p<0,01$) and decreased the leukocyte count of peripheral blood ($p<0,000$). **Conclusion.** The *A.fulica* snail slime contained antioxidant properties such as flavonoid, phenols, saponin and steroid, and potentially played role as photoprotector and anti-inflammatory agent in sunburn
Keywords: *Achatina fulica, antioxidant, IL-6, photoprotector, snail slime.*

Introduction

Sunburn is an acute inflammation of the skin caused by overexposed to ultraviolet (UV) radiation especially UVA and UVB. UVB radiation is the main caused of sunburn. The sign and symptoms of sunburn include redness (erythema), pain and tenderness, heat, swelling and blistering (Norval, 2015; Young, Claveau and Rossi, 2017).

The pathogenesis of sunburn involves several key steps in. DNA damage that leads to mutations in the DNA, inflammation induction by releasing of inflammatory mediators and cytokines such as Interleukin - 1 (IL-1), IL-6, IL-8, IL-10, and prostaglandin, also production of reactive oxygen species (ROS) (Young, Claveau and Rossi, 2017; McStay, 2017). Interleukin-6 (IL-6) is a cytokine that plays a crucial role in the immune response, particularly in inflammation including sunburn by recruit immune cells to the site of damage, such as neutrophils and macrophages, by increasing vascular permeability and mediating the expression of adhesion molecules (Young, Claveau and Rossi, 2017; Ansary *et al.*, 2021).

Ultraviolet radiation also changes the skin histology, caused by direct DNA damage nor inflammatory reaction. The hallmark of the sunburn reaction is sunburn cell formation. Sunburn cells (SBC) are apoptotic keratinocytes with pyknotic nuclei and eosinophilic cytoplasm that will reach the peak at 24

hours after UV radiation and will last for 72 hours. Sunburn cells mostly are induced by UVB radiation but UVA particularly also plays role in their formation. Other histopathologic changes that will be find in sunburn are crust formation, ulceration and dermal inflammation (Goettsch *et al.*, 1999; Young, Claveau and Rossi, 2017; McStay, 2017).

Many natural and chemical remedies are widely used to relief or to prevent sunburn, but the role of *Achatina fulica (A. fulica)* snail slime extract is not well recognized yet although it has gained popularity in the skincare industry for its potential benefits in skin hydration, repair, and anti-aging. Many studies have showed *A. fulica* snail slime contain allantoin, glycosaminogycans, anti-microbial peptides, glycolic acid, and lectin (Park et al., 2008; Gabriel, Mirela and Ionel, 2011; El Mubarak, Lamari and Kontoyannis, 2013; Thomas, 2013; Etim, Aleruchi and Obande, 2016), but the antioxidant properties were unclear.

This study was conducted to identified the antioxidant properties in *A. fulica* snail slime extract and its role as photoprotector and anti-inflammatory agents against sunburn.

Methods

1. **Snail slime collection and antioxidant properties examination**

   The *A. fulica* snail slime was collected from 40 adult snails from Karangwangkal residence by modification of Staikou (2017) and Wiya (2020). The collected slime was centrifuged 5000rpm for 10 minutes twice, and then the supernatant was filtered. The supernatant was divided and added with distilled aqua volume : volume (v : v) to make 20%, 50%, 70% and 100% concentration (Young, Claveau and Rossi, 2017). These various snail slime concentrations were given topically on the back skin of the mice 1hour prior UV radiation.

   The examination of antioxidant properties was performed by using qualitative methods biochemistry reaction and spectrophotometer. We examined the content of flavonoid, phenols, saponin, tannin and steroid using method described by Harborne (1998).

2. **Experiment animal preparation**

   This study used 75 male, 8-11 weeks mice *Mus musculus BALB/c*, within weight 200-200g bought from animal housing unit of Department of Pharmacology Faculty of Medicine, Public Health and Nursing Gadjah Mada University. The mice were randomly divided into 15 groups of 5. One group as control group (XO), 2 groups of negative control received UV radiation (XA and XB), 4 groups of positive control received snail slime with various concentration 20%, 50%, 70% and 100% (X1, X2, X3, X4), 4 groups received snail slime in various concentration and UVA radiation (X1A, X2A, X3A, X4A), 4 groups received snail slime in various concentration and UVB radiation (X1B, X2B, X3B, X4B).

   All the mice were acclimated for 7 days prior the experiment. One day before treatment, their back skins were shaved about 2cm x 2 cm using depilatory cream and washed off.

3. **Sunburn induction using UVA and UVB lamp**

   Sunburn induction was performed by using fixated UVA and UVB lamps (Exoterra®) with the minimal erythema dose (MED) respectively 30 J/cm^2 and 200 mJ/cm^2 20 cm above the skin surface. The UV dose was measured using UV-meter Tenmars® (TM-213)

4. **Histopathologic changes evaluation**

   A skin biopsy was taken from each mouse 24 hours after UV radiation. The biopsy samples were done a tissue processing to make paraffin blocks before sliced 4mm using microtome, made several histopathologic preparations. The tissues were staining with haematoxylin eosin. The histopathologic changes were evaluated by two pathologist using a semi-qualitative measurement described by Goettsch (1999). Sunburn cells formation (SBC), crust formation, ulceration and dermal inflammation were observed and scored 0 to 5 (0= absent, 1=minimal, 2 =slight, 3 =moderate, 4 =marked, 5 =severe) and we took the average from 10 field on high magnification 400X.

5. **Immunohistochemistry anti-IL-6 examination**
The paraffin blocks that had been made were sliced 4mm using microtome and incubated with anti-IL-6 antibody and diaminobenzidine as chromogen and finally stained with haematoxylin eosin. The evaluation of immunohistochemistry by using Allred's score, accumulation between proportion score (0 – 5) and intensity score (0 – 3). Score 0-1 was considered as negative, 2 – 4 was considered as mild, 5 – 6 was considered as moderate and 7 – 8 was considered as strong (Allred et al., 1998; Novrial, Soebowo and Widjojo, 2020).

6. **Leukocyte counts evaluation**
Estimated leukocyte counts were performed by making thin blood smear preparation taken from mice's caudal venous and numbers of the leukocytes were counted from 10 field of high magnification. The average numbers were multiplied by 3000 to estimated leukocyte count per mm^3 (LOINC, 2022).

7. **Data analysis**
The data of the experimental results were analysed using one-way ANOVA and Kruskal-Wallis methods followed by post hoc Tukey with IBM SPSS Statistic 26 and GraphPad Prism 9. The role of antioxidant enzymes as photoprotector and anti-inflammatory properties was analysed with Spearman correlation test. The results were shown within tables and graphs.

Result

1. ***A. fulica* snail slime antioxidant properties**
This study revealed the antioxidant properties of *A. fulica* snail slime. The results were shown in table 1 and figure 1. This study showed the *A. fulica* snail slime contained high flavonoid (+++), phenols (++), saponin (+++) and steroid (++). No tannin activity was detected in *A. fulica* snail slime.

The snail slime was also detected to contain antioxidant enzymes like SOD, CAT and GPX and was described in figure 2. The figure showed the antioxidant enzymes from *A. fulica* snail slime might maintain the level of antioxidant enzymes significantly ($p<0,05$) compared to control group and groups with UV radiation only. The snail slime with 100% concentration significantly had the best activities of antioxidant enzymes SOD, CAT and GPX in sunburn model mice.

2. **Histopathologic changes of the skin on sunburn model mice**
The histopathologic changes of sunburn were shown on figure 3 and 4. Sunburn cells The efficacy of *A. fulica* snail slime as photoprotector was shown on figure 5. It showed the snail slime affected a significant protection effect in histopathologic skin changes on UV radiation by inhibiting sunburn cell formation, crust formation, ulceration and dermal inflammation. This table also showed UVB radiation caused histopathologic changes severer than UVA radiation.

3. **Immunohistochemistry of Anti-IL-6 on sunburn mode mice**
The result of this study was to show the role of *A. fulica* snail slime as a photoprotector by suppressing releasement of IL-6 cytokine was shown on figurethe table 3. High level of IL-6 in skin tissue was represented by small Allred's score, in contrary low level of IL-6 was represented by high Allred's score. Allred's score was calculated by combining proportional score and intensity score of each sample. High Allred's score of anti-IL-6 antibody meaned, the production of IL-6 in the skin tissue was low in contrary the lower Allred's score showed higher IL-6 production in inflammation process.

The table 3 showed the lowest Allred's score was on group XB with UVB radiation only, while in UVA radiation only group (XA) the Allred's score was not significantly different ($p>0,05$) compared to control group (XO). Additional snail slime 100% before UV radiation showed no significant

difference compared to control group, in contrary other groups given with snail slime showed higher Allred's score compared to negative control groups but were not significant.

4. **Leukocytes count of peripheral blood.**
   The effect of snail slime to the leukocytes count was shown on the Figure 9. This figure showed, hyperleukocytosis occurred in all groups with UV radiation. The additional snail slime extract prior UV radiation showed to lower the number of leukocytes but statistically not significant ($p>0,05$) in UVA radiation groups and significant ($p<0,05$) compared to UVB radiation groups only.

5. **The role of antioxidant enzymes in *A. fulica* snail slime as photoprotector and anti-inflammatory agent.**
   Using Spearman's rho correlation analysis, the role of *A. fulica* snail slime as photoprotector and anti-inflammatory agent was shown in table 2. The table described *A. fulica* snail slime had strong negative correlation with coefficient coreelation ($0,40< r <0,69$) between antioxidant enzymes and histopathologic changes including SBC formation, crust formation, ulceration and dermal inflammation. This result also showed the antioxidant enzymes in *A. fulica* snail slime had no correlation with IL-6 releasement in sunburn, in contrary the enzymes had strong to very strong negative correlation with coefficient ($0,40< r <0,69$) and $r \geq 0,70$.)

Disscussion

1. **The *A. fulica* snail slime antioxidant properties**
   A. fulica snail slime was proven to have antioxidant properties such as flavonoids, phenols, saponin and also steroid. This result was aligned with a study conducted by Suhesti et al. (2021) that found the snail slime contained phenols and saponin. But this study did not detect the flavonoid and steroids. Their study also detected the tannin property in the snail slime, in contrary our study did not find any tannin activity.
   The *A. fulica* snail slime has been observed to possess significant antioxidant properties, as demonstrated by the quantification of several antioxidant enzymes. From our previous investigation, the mucus revealed superoxide dismutase (SOD) levels at an average of 40.59±5.79U/mL, catalase (CAT) at 12.73±6.13 U/mL, and glutathione peroxidase (GPX) at 17.64±3.84 mmol/L (Putranti, 2022). Other previous research conducted by Wahyuningsih (2016) which measured SOD levels in A. *fulica's* haemolymph-derived mucus, reported an average of 157.3 ± 94.63U/mL, suggesting that the haemolymph source presents higher SOD levels compared to the pedal-derived mucus examined in our study. Furthermore, a study by Brieva et al., (2008) on *H.aspersum* snail's pedal mucus indicated lower activities for both SOD and glutathione S-transferase (GST) when juxtaposed with *A. fulica's* mucus. Our findings underline the presence of not just SOD but also CAT and GPX enzymes in the pedal mucus of the *A. fulica* snail, highlighting its potential as an antioxidant resource.
   Many studies have confirmed that UVA and UVB radiation results in the formation of ROS in skin tissue (Fehér et al., 2016; Reis Mansur et al., 2016), thereby reducing levels of SOD, CAT and GPX which function to neutralize free radicals (Dunaway et al., 2018). Excessive ROS formation in the skin will cause oxidative stress which can trigger damage to the DNA of keratinocytes and fibroblasts (Young, 2016; de Jager, Cockrell and Du Plessis, 2017)..
   The potential activity of the antioxidant enzymes SOD, CAT and GPX of *A. fulica* snail slime which was administered topically to BALB/c mice model of sunburn induced by UVA and UVB rays, was seen by increasing the activity of SOD, CAT and GPX compared to the control group. This increase can be seen by administering a dose of snail slime starting at 20% compared to the negative control group, but in general a significant difference compared to that was found in the groups that received 100% snail mucus.
   The results of this study are in line with the research of Egoumenides et al., (2018) who assessed the effect of topical administration of melon concentrate on sunburn cell formation, MED and endogenous antioxidant levels. This research found that giving melon concentrate prevents the formation of sunburn cells, increased minimal erythematous dose (MED), so it can be said to have

photoprotective potential. SOD, CAT and GPX levels during topical administration of melon concentrate increased significantly compared to the negative control group and placebo group. These results indicate the possibility of restoration of SOD, CAT and GPX in skin irradiated by UVA and UVB rays in skin previously treated with melon concentrate topically (Egoumenides et al., 2018). This also shows that topical administration of *A. fulica* snail slime could restore SOD, CAT and GPX which decreased due to UVA and UVB radiation.

2. The role of *A. fulica* snail slime as photoprotector

The results of this study *A. fulica* snail slime had potency as photoprotector in sunburn by restoring the endogen antioxidant enzymes and inhibiting the histopathologic changes in sunburn model mice. The correlation between the level of antioxidant enzymes and histopathologic changes in sunburn showed a moderate to strong negative correlation. Higher level of antioxidant enzymes would supress the histopathologic changes in sunburn including SBC formation, crust formation, ulceration and dermal inflammation,

In accordance with findings presented by Egoumenides et al. (2018), a study employing melon extract on human skin to assess its photoprotective effects against UVB-induced damage revealed notable outcomes. Specifically, melon extract demonstrated an inhibitory effect on the formation of sunburn cells and further amplified the MED, signifying its potential as a protective agent against UV-induced erythema. Analogous findings were obtained in a study by Filip et al. (2011) where grape seed extracts were investigated for their photoprotective properties. The abundant antioxidants present in grape seeds were shown to effectively counteract the formation of sunburn cells when exposed to varying doses of UVB radiation. Such consistent results across diverse studies underscore the potential of natural extracts as potent agents in UV protection strategies.

In the present investigation, the application of *A. fulica* snail slime displayed a marked protective effect against the crust formation in the histopathological examination of mouse skin subjected to UVA and UVB irradiation, when juxtaposed with the negative control group. This finding suggested that beyond its known properties, snail slime possessed noteworthy anti-inflammatory capabilities. These observations were consistent with a study by Wiya, Nantarat and Saenphet (2020) where the administration of *A. fulica* snail slime was shown to mitigate granuloma formation in the ears of mice. Additionally, research by Adikwu and Alozie (2007) posited that the slime from *A. fulica* snails facilitated wound healing by augmenting the natural proliferation and regenerative processes of skin tissue, potentially through modulatory effects on the immune system. Given the smooth wound healing observed, it was conceivable to infer that snail slime might also offer potential therapeutic interventions against keloid formation.

In this investigation, the incorporation of *A. fulica* snail slime demonstrated a protective role against skin ulceration induced by UVA and UVB radiation, evident across a dosage range of 20% to 100%. While there is a paucity of literature specifically connecting snail mucus extract to the prevention of UV-induced ulceration, several photobiological studies have explored the protective capabilities of natural constituents, including grape seeds, propolis, and melon extract. Notably, these ingredients have been identified to possess antioxidant enzymes like SOD, CAT, and GPX, which have been substantiated to confer protection against UV-induced skin tissue damage (Bolfa et al., 2013; Egoumenides et al., 2018). Consistent with these findings, our study reaffirmed that the presence of antioxidant enzymes SOD, CAT, and GPX in snail mucus extract correlates inversely, albeit with a weak to moderate relationship, with the propensity for ulceration under UVA and UVB exposure.

In our current investigation, dermal inflammation was significant difference in all groups (p=0,000), but the groups subjected to UVA radiation displayed not statistically significant differences slight dermal inflammation compared to UVA radiation only group, while those exposed to UVB radiation exhibited significant differences in additional 20%, 70% and 100% snail slimes groups compared to

UVB radiation only group. These observations resonated with existing literature in photobiology, which posits that both UVA and UVB radiations can precipitate dermal inflammation.

From a histopathological standpoint, sunburn-associated dermal inflammation is characterized by an infiltration of neutrophils and lymphocytes within the dermal layer. Regarding the dynamics of sunburn, vasodilation peaks approximately 24 hours post-radiation, succeeded by neutrophil infiltration persisting up to 48 hours. This is subsequently followed by lymphocyte infiltration, as delineated by (Nicolaou, Pilkington and Rhodes, 2011). The presence of neutrophil cells and lymphocytes in sunburned skin can be attributed to the secretion of cytokines, predominantly interleukin-8 (IL-8). This cytokine plays a pivotal role in facilitating leukocyte infiltration. Concurrently, there is a release of prostaglandin (PG) E2 and nitric oxide, both of which are instrumental in mediating vasodilation (Strickland et al., 1997; Rhodes et al., 2009).

3. The role of *A. fulica* snail slime as anti-inflammatory agent

In our study, anti-inflammatory activities were represented by immunohistochemistry of anti-IL-6 and peripheral blood leucocyte counts. The result of this study showed both variables had significant differences compared to al groups. There were no statistically significant differences in IL-6 releasement in almost all groups except the groups of additional 20% and 50% snail slime with UVB radiation. The Allred's scores significantly lower compared to normal group.

In the context of sunburn pathogenesis, IL-6 is upregulated in response to UV radiation-induced DNA and mitochondrial damage in epidermal keratinocytes, predominantly from UVB exposure. This cellular damage, coupled with the generation of reactive oxygen species (ROS), activates a cascade of pro-inflammatory cytokines including IL-1, IL-3, IL-8, GM-CSF, M-CSF, G-CSF, TGF-α, TGF-β, TNF-α, and PDGF. Beyond epidermal keratinocytes, chronic UV exposure also exerts detrimental effects on dermal fibroblasts, prompting the release of matrix metalloproteinases (MMP)-1, MMP-2, MMP-3, MMP-9, MMP-11, MMP-17, and MMP-27. Elevated concentrations of IL-6, along with other pro-inflammatory cytokines, are consistent with the inflammatory responses observed in sunburn cases (Ansary et al., 2021).

Our findings corroborated the study executed by Wiya et al. (2020), which delved into the anti-inflammatory properties of *A. fulica* snail slime. Wiya assessed the efficacy of this mucus in ameliorating oedema in mouse ears induced by ethyl phenyl propiolate (EPP) and in attenuating granuloma formation triggered by cotton ball implants. The data posited that *A. fulica* snail slime served as a potent anti-inflammatory agent, demonstrating therapeutic potential during both acute and chronic inflammatory phases.

In the present investigation, exposure to ultraviolet (UV) radiation, encompassing both UVA and UVB spectra, induced a systemic augmentation in the leukocyte count of murine subjects. Upon administration of snail slime prior UVA radiation, no significant alteration in leukocyte count was discernible. Conversely, in the subset exposed to UVB radiation, a notable decrement in leukocyte numbers was observed significantly.

Baseline leukocyte counts in murine circulatory systems typically range between 2,000 – 11,000 cells per microliter. The leukocyte profile predominantly consists of lymphocytes (70 – 80%), followed by neutrophils (20 – 30%), eosinophils (0 – 7%), and minimal proportions of basophils and monocytes (< 2%). The onset of stressors or acute inflammatory stimuli can precipitate a decline in lymphocyte populations while concurrently escalating neutrophil counts. Such inflammatory perturbations stimulate the upregulation of neutrophilic growth factors, chiefly granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF), accompanied by the secretion of chemo-attractive cytokines, namely IL-6 and IL-8 (Bolliger and Everds, 2012; O'Connell et al., 2015).

Pertinently, within the scope of this research, the Allred anti-IL-6 score exhibited a low negative correlation (r=-0.234) with elevated leukocyte levels in murine subjects, though detailed data remains undisclosed. This study also conducted a correlation analysis between antioxidant enzymes and anti-IL-6 Allred's score and leukocyte counts. The results showed no correlation between antioxidant enzymes with Allred's score of anti-IL-6, in contrary there were high negative correlation between antioxidant enzymes with peripheral leukocyte counts ($0,6 \leq r \leq 0,79$).

The findings presented herein deviate from prior studies examining the interplay between antioxidants and IL-6 activity, but aligned from peripheral leukocyte counts point of view. During the inflammatory response triggered by UVB irradiation, there ensues a surge in the release of pro-inflammatory cytokines, such as TNF-α, IL-1, IL-6, and IL-8. These cytokines are pivotal in orchestrating leukocyte mobilization from the vasculature, functioning both as chemo-attractants and as stimulants for the upregulation of endothelial cell adhesion molecules (ECAM). Such modulation promotes the trans-endothelial migration of cells through the capillary walls (Kochevar, Taylor and Krutmann, 2012). The introduction of antioxidants has been proposed to mitigate the ROS-mediated inflammatory responses elicited by UV radiation, encompassing modulation of IL-6 activity (Dunaway et al., 2018; Ansary et al., 2021). It was plausible that the observed outcomes stem from the multifaceted nature of the inflammatory reaction in sunburn, which may be modulated by not solely IL-6 but an array of the aforementioned pro-inflammatory mediators.

Conclusion

In our investigation, we discerned that the mucus derived from the A. fulica snail exhibited pronounced antioxidant capacities, encompassing specific antioxidant enzymes. These components may function synergistically as photoprotective and anti-inflammatory agents in mitigating sunburn-associated damage. Notwithstanding these findings, subsequent research is warranted to substantiate these roles from diverse analytical perspectives and to further elucidate the underlying mechanisms.

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Conflict of Interest

We declared no conflict of interest in this study.

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Table 1. Antioxidant properties of *A. fulica* snail slime

Antioxidant	Reagens	Result
Flavonoids	Mg ribbon, HCl	+
Phenols	FeCl3 5%	+
Saponin	Hot water and HCl	+++
Tannin	FeCl3 1%	-
Steroids	Glacial acetic acid, H2SO4	+

Table 2. Spearman's correlation of the role antioxidant enzymes in *A. fulica* snail slime as photoprotector in histopathologic changes and anti-inflammatory agent in reducing IL-6 releasement and leukocytes count

			Dermal Inflammation	Crust	Ulceration	SBC	Allred's score	Leukocyte Counts
Spearman's rho	SOD	Correlation Coefficient	-,497**	-,486**	-,415**	-,490**	,094	-,647**
		Sig. (2-tailed)	,000	,000	,000	,000	,430	,000
		N	72	72	72	72	72	72
	CAT	Correlation Coefficient	-,577**	-,589**	-,456**	-,501**	,157	-,735**
		Sig. (2-tailed)	,000	,000	,000	,000	,188	,000
		N	72	72	72	72	72	72
	GPX	Correlation Coefficient	-,577**	-,576**	-,450**	-,488**	,114	-,696**
		Sig. (2-tailed)	,000	,000	,000	,000	,340	,000
		N	72	72	72	72	72	72

** Correlation is significant at the 0,01 level (2-tailed)

* Correlation is significant at the 0,05 level (2-tailed)