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The Role of Achatina Fulica Snail Slime Extract Enzymatic Antioxidants as Photoprotector in Sunburn Model Mice

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ABSTRACT

Background. Achatina fulica (A. fulica) snail slime was well known having many advantages especially as moisturizer and antimicrobial, but enzymatic antioxidant properties have not been studied yet.
Objective. This study aimed to find the enzymatic antioxidant properties of snail slime extract and to find photoprotective potency in sunburn.
Method. This research was an experimental test using randomized post-test only control group design using 75 male BALB/c mice were divided into 15 groups. There were an untreated group, 4 groups were given various doses of snail slime extract topically, 5 groups were irradiated with UVA 30J/cm2 and 5 groups were irradiated with UVB 200mJ/cm2. Skin biopsy were performed 24 hours after irradiated to be analyzed for enzymatic antioxidants properties of SOD, CAT and GPX, and also histologic changes. Data analysis were conducted with one-way ANOVA and followed by post hoc LSD with SPSS 26.
Results. A. fulica snail slime contained enzymatic antioxidant properties of SOD, CAT and GPX respectively 40,59 ± 5,79U/mL; 13,73 ± 6,13 U/mL and 17,64 ± 3,84 mmol/L. These antioxidant properties showed role of photoprotector compared to groups that had been radiated with UVA nor UVB. Topical use of snail slime extract significantly increased the level of SOD, CAT and GPX in sunburn model mice ($p<0,05$). This study also showed snail slime extract could prevent skin damage caused by UVA nor UVB histologically.
Conclusion. Enzymatic antioxidants of A. fulica might have a photoprotector potency.

KEYWORDS: CAT, GPX, photoprotector, SOD, sunburn.

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INTRODUCTION

Ultraviolet (UV) A and UVB have been widely studied to cause acute nor chronic skin damages. Sunburn is one of acute skin damages caused by UV radiation. It is an inflammatory reaction characterized with erythema, pain, and oedema in severe condition [1], [2].

Reactive oxygen species (ROS) are known playing role in sunburn pathogenesis which cause oxidative stress that leads to skin damages [3]. The provision of exogenous antioxidants systemically and topically was expected to help endogenous antioxidants to balance the formation of free radicals and reduce the occurrence of oxidative stress so as to suppress the occurrence of damage to DNA, cells and skin organs due to sun exposure [4].

Achatina fulica (*A. fulica)* snail slime is widely used as ingredient in cosmetic products. It has been studied and proven to have many active compounds such as allantoin, glycolic acids, glycosaminoglycans, antimicrobial peptides and lectins that worked as moisturizer and wound healing assisting product [5]–[9]. It also was claimed to have antioxidant activities but it is not proven yet to have enzymatic antioxidant properties such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) as three major antioxidants that play role in protecting skin from damage [10].

This study aimed to find the enzymatic antioxidant properties of *A. fulica* snail slime and to find photoprotective potency in sunburn mice models.

EXPERIMENTAL

Preparation of A. fulica snail slime

Achatina fulica snails were collected and let them walk freely for 2 hours, and then scrubbed their pedals to collect the slime. The collected slime was then stirred for 1 hour and centrifuged at 5000 rpm for 10 minutes twice. Supernatant was collected and was divided into 20%, 50%, 70% and 100% concentration by adding distilled water (v/v) and vortexed homogenously. Two drops of snail slime (0,08mg) were given topically on the shaved area of mice back an hour prior UV induction.

Mice

Seventy-five male BALB/c mice, 8 – 11 old weeks with 20 – 25g weight, were collected from Experimental Animal Breeding Centre of Integrated Testing and Research Laboratory of Gajah Mada University. The mice were maintained under standard laboratory conditions (12 h light/darkness; at 25ºC ± 3ºC) with standard animal diet and water available *ad libitum*. All animal experiments were approved by the Medical and Health Research Ethics Committee Faculty of Medicine Universitas Jenderal Soedirman, with reference number Ref: 0110/KEPK/V/2020. The back of the mice were carefully shaved 2 x 2 cm, 1 day prior the experiment.

Experimental Design

This experiment was a randomized post-test only control group design with 75 mice was randomly divided into 15 experiment groups and treated as indicated in **table 1**.

Sunburn Induction

Exo-terra UVA Intense Basking Lamp 75 watt and Exo-terra UVB Solar Glo Lamp 160 watt were used as solar simulator. These lamps were fixed in a box 20cm above the mice. The dosimetry was calculated by using UV-meter (T-213,Tenmars), with doses of UVA and UVB 30J/cm2 and 200mJ/cm2 respectively [2], [11].

Evaluation of Enzymatic Antioxidants Activity

Evaluation of activity and levels of SOD, CAT and GPX were carried out on *A. fulica* snail mucus and mice skin treated by colorimetric method with a spectrophotometer. Snail slime samples were mixed with 0.1M Tris/HCL (pH 7.4) in cold conditions on ice. The mixture was then centrifuged at 14,000 x g for 5 min at 4ºC. The separated supernatant was then transferred to a clean tube and then stored on ice. Mice skin tissue was prepared to weigh 10mg, then perfused with PBS/150mM KCl to remove red blood cells. The mixture was homogenized at a cold temperature on ice with 0.1M Tris/HCl pH 7.4, then centrifuged at 14,000 x g for 5 minutes at 4ºC. The supernatant was separated and transferred to a clean tube, then stored on ice.

Table 1. Experimental design

Group	Treatment
Group 1	Normal Control (X0)
Group 2	UVA induced sunburn (XA)
Group 3	UVB induced sunburn (XB)
Group 4	Normal + Snail slime 20% (X1)
Group 5	Normal + Snail slime 50% (X2)
Group 6	Normal + Snail slime 70% (X3)
Group 7	Normal + Snail slime 100% (X4)
Group 8	Snail slime 20% + UVA induce sunburn (X1A)
Group 9	Snail slime 50% + UVA induce sunburn (X2A)
Group 10	Snail slime 70% + UVA induce sunburn (X3A)
Group 11	Snail slime 100% + UVA induce sunburn (X4A)
Group 12	Snail slime 20% + UVB induce sunburn (X1B)
Group 13	Snail slime 50% + UVB induce sunburn (X2B)
Group 14	Snail slime 70% + UVB induce sunburn (X3B)
Group 15	Snail slime 100% + UVB induce sunburn (X4B)

Antioxidant Activities

SOD activity. Each sample, standard and RANSOD buffer of 20 µL were mixed with 1000 L of RANSOD R1 reagent and 100 µL of R2 reagent. The inhibition of reducing activity by SOD was measured by colorimetric method at a wavelength of 546nm.

CAT activity. Examination of snail slime and mouse skin using Catalase Assay Kit (CAT100) with a colorimetric method. This examination is based on the measurement of H2O2 levels which are arranged after the catalysis process occurs. CAT activity can be seen and assessed quantitatively by adding H_2O_2 and its absorbance is seen at a wavelength of 240nm.

GPX activity. GPX levels in snail mucus and mouse skin tissue can be tested by using colorimetry using Abcam (ab102530 - Glutathione Peroxidase Assay Kit) protocol. The absorbance was read at 340nm in the first minute (A1) and second minute (A2).

Histological Examination.

Skin histological examination was carried out by taking skin tissue from the back of mice using a 5mm diameter punch biopsy and stored in a 10% formalin tube and put the label according to the group and sequence, then tissue processing was carried out. Tissue processing includes fixation, dehydration, clearing and paraffin infiltration and embedding. Tissue block was sectioned with microtome 5mm and stained with hematoxylin-eosin (HE) [12]. Four parameters were scored: dermal inflammation, crust formation, ulceration (absence of epidermis with reaction in the dermis, accompanied by dermal tissue loss and sunburn cells (epidermal single cell death). All parameters were examined semi-quantitatively [0=absent, 1=minimal, 2=slight, 3=moderate, 4=marked, 5=severe] [11].

Data Analysis

The data were analysed using one-way ANOVA test because there were 15 research groups with the dependent variables being SOD, CAT, and GPX levels. The one-way ANOVA test will be followed by a post hoc LSD test to determine the differences between the pairs of the study groups. Before the one-way ANOVA test was performed, the Saphiro-Wilk and Levene test were carried out to show data normality and homogeneity of variances. Data analysis will be assisted by using SPSS version 26 software with a significance level of p <0,05. For the histologic examination, data was categorical. We performed Kurskal-Wallis and Spearman test to find the correlation between enzymatic antioxidant activities with histologic changes in sunburn [13].

RESULTS

SOD, CAT and GPX Activity in A. fulica snail slime extract

SOD, CAT and GPX activities were found in *A. fulica* snail slime extract. The level of SOD, CAT and GPX were shown in table 2.

Table 2. Level of SOD, CAT and GPX in *A. fulica* snails snail slime extract

Level of Antioxidant	SOD (U/mL)	CAT (U/mL)	GPX (mmol/L)
Mean ± SD	40,59 ± 5,79	13,73 ± 6,13	17,64 ± 3,84

SOD, CAT and GPX Levels of Mice Skin with Additional Topical A. fulica Snail Slime Extract.

The result of SOD, CAT and GPX levels of mice skin with additional various concentrations of topical *A. fulica* snail slime extract were shown in table 3.

Table 3. SOD, CAT and GPX Levels of Mice Skin with Additional Topical *A. fulica* Snail Slime and Sunburn Induction

Groups	**Mean ± SD; CI 95%**		
	SOD (U/mL)	**CAT (U/mL)**	**GPX (mmol/L)**
1 (X0)	41,71 ± 3,229	11,72 ± 0,606	12,40 ± 0,625
4 (X1)	43,23 ± 3,014	11,92 ± 0,817	11,99 ± 0,58
5 (X2)	42,48 ± 2,724	11,94 ± 0,814	13,38 ± 0,607
6 (X3)	48,53 ± 2,391	14,11 ± 0,662*	15,09 ± 0,621*
7 (X4)	60,36 ± 7,12*	15,05 ± 0,447*	18,38 ± 1,227*
One-way ANOVA (Sig. p<0,05)	0,000	0,000	0,000
1 (X0)	41,71 + 3,229*	11,72 + 0,606*	12,40 + 0,625*
2(XA)	20,58 + 4,112	2,76 + 0,584	4,36 + 0,570
3(XB)	11,20 + 2,622	1,84 + 0,466	3,91 + 0,404
One-way ANOVA (Sig. p<0,05)	0,000	0,000	0,000

Additional of 75% concentration of *A. fulica* snails slime topically might increase the level of CAT and GPX significantly compare to control group (p<0,05), while 100% concentration significantly increased SOD, CAT and GPX (p<0,05).

UVA and UVB showed decrease level of SOD, CAT and GPX significantly compare to control groups (p < 0,05), but only SOD level showed significantly different between UVA and UVB induction group (p<0,05).

SOD, CAT and GPX Levels on UVA and UVB Sunburn Induction with Addition of Various Concentration Topical Snail Slime Extract.

The result of SOD, CAT and GPX levels on mice skin with additional various concentration of snail slime topically before UVA and UVB sunburn induction were shown in table 4.

Additional of 100% snail slime significantly increased the level of SOD and GPX compared to normal group on the mice skin after UVA sunburn induction, while CAT level showed no difference compared to normal groups. But after all, additional

various concentration of snail slime topically to the mice skin before UVA induction showed significant differences of increasement level of SOD, CAT and GPX compared to UVA negative control group (XA) with p<0,05.

Table 4. SOD, CAT and GPX Levels on UVA Sunburn Induction with Topically Addition of Various Concentration of Snail Slime

Groups	Mean ± SD; CI 95%		
	SOD (U/mL)	CAT (U/mL)	GPX (mmol/L)
1 (X0)	41,71 ± 3,229	11,72 ± 0,606	12,40 ± 0,625
2(XA)	20,58 ± 4,112	2,76 ± 0,584	4,36 ± 0,570
8(X1A)	33,23 ± 2,397	4,79 ± 0,405	7,27 ± 0,620
9(X2A)	42,28 ± 3,606	6,29 ± 0,891	9,05 ± 0,925
10(X3A)	44,22 ± 2,849	10,33 ± 1,426	12,10 ± 0,437
11(X4A)	52,52 ± 7,232*	11,94 ± 0,891	14,29 ± 0,377*
3 (XB)	11,20 ± 2,622	1,86 ± 0,466	3,90 ± 0,404
12 (X1B)	17,93 ± 3,226	4,65 ± 0,532	6,23 ± 0,538
13 (X2B)	24,02 ± 10,960	5,45 ± 0,411	8,56 ± 0,505
14 (X3B)	36,02 ± 2,571	9,73 ± 1,140	11,54 ± 0,577
15 (X4B)	34,30 ± 2,357	10,90 ± 1,140	13,95 ± 0,387*
One-way ANOVA (Sig. p<0,05)	0,000	0,000	0,000

Snail slime addition with 100% concentration significantly increased the level of GPX (p<0,05) in UVB sunburn induction compared to normal group but not in SOD and CAT levels. However, it showed snail slime might maintain the level of SOD and CAT closely to normal level in mice skin.

Achatina fulica Snail Slime Extract Effects on Histologic Changes of the Skin

The effects of snail slime extract addition to skin of the sunburn model mice histologically were showed in table 5. Based on the results on table 5, additional *A. fulica* snail slime extract topically also reduced the skin damages induced by UV radiation. Overall, in UVA radiation groups, snail slime gave significant results on crust formation and ulceration. Sunburn cells and dermal inflammation also gave a good result but not significantly different. The snail slime also gave significant results in all skin changes parameters in UVB radiation groups including sunburn cells, crust formation, ulceration and dermal inflammation (figure 1).

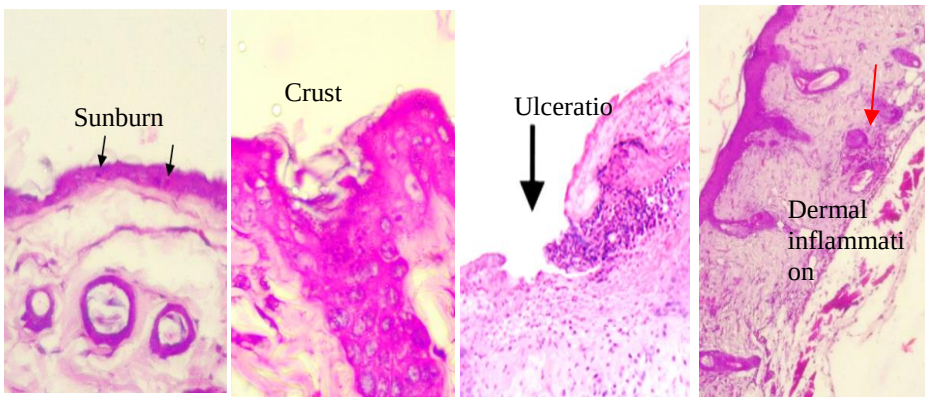

Figure 1. Histologic changes caused by UV radiation

Table 5. Histologic Changes in Sunburn Model Mice induced by UVA and UVB

Groups	Sunburn cells	Crust Formation	Ulceration	Dermal Inflammation
1 (X0)	None (0)	None (0)	None (0)	None (0)
2(XA)	Minimal (0,60)*	Slight(2,20)*	Minimal (0,60)*	Minimal (1,4)*
8(X1A)	None (0,20)	None (0,20)	None (0)	Minimal (0,6)
9(X2A)	None (0)	None (0,25)	None (0)	Minimal (0,6)
10(X3A)	None (0)	Minimal (0,5)	None (0)	Minimal (0,5)
11(X4A)	None (0)	None (0)	None (0)	None (0)

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Kruskal-Wallis Test (Sig. p<0,05)	0,223	0,003	0,011	0,070
1 (XO)	None (0)	None (0)	None (0)	None (0)
3 (XB)	Slight (1,80)*	Moderate (3,20)*	Slight (2.40)*	Slight (1,8)*
12 (X1B)	None (0,20)	None (0,20)	None (0)	None (0,4)
13 (X2B)	None (0)	None (0)	None (0)	Minimal (1)
14 (X3B)	None (0)	None (0)	None (0)	None (0)
15 (X4B)	None (0)	None (0)	None (0)	None (0)
Kruskal-Wallis Test (Sig. p<0,05)	0,002	0,002	0,007	0,004

To show the correlation between antioxidant enzymes and the histologic changes of the skin, we performed data analysis using Spearman correlation test. The result was shown on the table 6 below.

Table 6. Correlation between level of antioxidant enzymes with skin histologic changes in sunburn model mice

Enzymatic Antioxidants	**Correlation Coefficient (Spearman's rho - r)**				**Significancy p < 0,05 **(p < 0,01)**
	Sunburn Cells	**Crust Formation**	**Ulceration**	**Dermal Inflammation**	
SOD	-0,536**	-0,509**	-0,449**	-0,501**	0,000
CAT	-0,552**	-0,622**	-0,513**	-0,556**	0,000
GPX	-0,531**	-0,603**	-0,503**	-0,538**	0,000

The data showed that enzymatic antioxidants had negative correlation with histologic changes in sunburn model mice significantly, with correlation coefficient from moderate (r = 0,40-0,599) to strong (r = 0,60-0,799). The higher enzymatic antioxidant level would show lower skin damages in sunburn model mice.

DISCUSSION

This study showed *A. fulica* snails slime extract had enzymatic antioxidant properties such as SOD, CAT and GPX beside other active components previously found by other researchers like allantoin, glycolic acids, glycosaminoglycans, lectins, antimicrobial peptides [5], [6], [9]. These enzymatic antioxidants were not only detectable but also had potency to be used as topical antioxidant which in this study we used UVA and UVB radiation to induce sunburn.

In the pathogenesis of sunburn, ROS plays important role in acute UV radiation causes skin damages. UVB will damage directly to keratinocytes' DNA and releases ROS that cause oxidative stress, while UVA penetrates deeper to the dermis trans-UCA will be converted to cis-UCA and release ROS. Excessive ROS formation in the skin will cause oxidative stress that can trigger damage to the DNA of keratinocytes and fibroblasts [2], [3], [14]. Oxidative stress caused by the imbalance of free radicals and antioxidants. Many studies have confirmed that UVA and UVB radiation results in the formation of ROS in skin tissue,[15], [16] so that it will reduce levels of SOD, CAT and GPX which function to neutralize free radicals [17]. This pathogenesis became the base of rules the use of antioxidant for skin photodamage.

This study found that *A. fulica* snail slime extract in various concentration might increase the level of SOD, CAT and GPX compared to negative control groups by UVA and UVB sunburn models. In UVA induction groups, topical snail slime increased the level of SOD, CAT and GPX significantly compare to negative control groups, but only 100% concentration gave significant difference to normal group. Different results found in UVB induction groups, which additional snail slime did not show significant differences in SOD and CAT compared to normal group, but it significantly increased in GPX level (p<0,05). Compared to UVB negative control, additional snail slime showed significant differences in the level of SOD, CAT and GPX.

The results of this study were in line with the research which assessed the effect of topically administered melon concentrate on sunburn cell formation, MED and endogenous antioxidant levels [18]. The study found that giving melon concentrate prevents the formation of sunburn cells, increases MED, so it could have photoprotective potential. The levels of SOD, CAT and GPX in the topical administration of melon concentrate increased significantly compared to the negative control group and the placebo group. These results indicated the possibility of restoration of SOD, CAT and GPX on skin irradiated by UVA and UVB rays on skin previously treated with melon concentrate topically. This also shows that topical application of *A. fulica* snail slime could restore the level of SOD, CAT and GPX which decreased due to UVA and UVB radiation.

The presence of antioxidant enzymes SOD, CAT and GPX in *A. fulica* snail slime could increase or restore the levels of these antioxidant enzymes in the mice skin due to UVA and UVB radiation, so it may propose that these antioxidant enzymes

would also provide a photoprotective effect on the skin of mice histologically. This study showed that snail slime could reduce skin damages caused by UV radiation such as sunburn cell formation, crust formation, ulceration and dermal inflammation. The histologic changes related to the level of enzymatic antioxidant properties in snail slime with the relation coefficients were moderate to strong.

This study showed the UVA radiation significantly induced crust formation and ulceration while sunburn cell formation and dermal inflammation not different significantly, while the UVB radiation significantly induced all the skin changes histologically, especially on sunburn formation. Sunburn cell formation was found to occur in UVB radiation (moderate) than UVA (minimal). This finding aligned to other studies that used the sunburn cell formation as a parameter. In those studies were stated that UVB induced sunburn cell formation than UVA because UVB directly damaged keratinocytes' DNA and it would lose their ability to repair the damages adequately or as a result of lysosomal breakdown [19], [20].

CONCLUSIONS

This study concluded that *A. fulica* snail slime extract had enzymatic antioxidant properties that might work as photoprotector against sunburn and other skin damages induced by UV radiation. It also showed the anti-inflammatory activities against UV radiation that will be studied much further.

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Author Contributions

Putranti, I.O., first author, head of project study of disertation.
Sutrisna, E.; Ekowati, N.; Mawardi, P., reviewers for contents and analysis.
Novrial, D., histopathologist of this study.

Ethical Clearance (optional)

This project has been passed the ethical clearance conducted by Ethical for Health Research Commission of Faculty of Medicine, Universitas Jenderal Soedirman Ref: 0110/KEPK/V/2020).

Conflict of Interest

We declare there is no conflict of interest in this study.

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