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Acute irritation of tetracyclic c-4-hydroxyphenylcalics [4] pyrogalolarene on skin of albino rabbits

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ABSTRACT

C-4-hydroxyphenylcalix[4] pyrogalolarene (Pg_4OH) is a synthetic chemical compound that shows strong antioxidant activity. It is potential to be developed as UV skin protector. For topical admission, an acute skin irritation test is being prerequisite to be fulfill. This study was conducted to investigate the irritation effect of Pg_4OH on the skin. *In vivo* study by quasi experiment with posttest only design was carried out by employing 3 adult male albino rabbits. The back hair was shaved at 24 h before the treatment. The Pg_4OH was smeared on the bare shaved-skin and left naturally within 4 h. The observation began at 24, 48 and 72 h after exposure. The erythema and edema scores were measured. Calculation by using primary irritation index (PII) was applied. Calculation was categorized with globally harmonized system (GHS) and international organization for standardization (ISO) parameters. As a result, among three rabbits, only one animal shown erythema and edema with very small size (score: 1), on 3 sites, at 72 h. Both the control site and the sample sites gained same score. Two other rabbits were obtained 0 value at each observation location. According to the PII, the observed data was obtained score 0.0093 (very light or negligible). It can be concluded that the tetracyclic compound of Pg_4OH does not irritate the skin.

ABSTRAK

Senyawa tetrasiklik C-4-hidroksifenilkaliks[4]pirogalolarena (Pg_4OH) merupakan senyawa hasil sintesis yang menunjukkan aktivitas antioksidan kuat. Senyawa ini potensial dikembangkan penangkal sinar UV. Untuk sediaan topikal, salah satu uji yang harus dilakukan adalah uji iritasi kulit akut. Penelitian ini bertujuan untuk mengkaji efek iritasi kulit senyawa Pg_4OH pada kelinci albino. Rancangan penelitian ini menggunakan eksperimen kuasi dengan rancangan percobaan *posttest only*. Pengujian ini di lakukan secara *in vivo* pada 3 kelinci albino jantan dewasa. Bulu punggung kelinci dicukur 24 jam sebelumnya sebelum diberi perlakuan. Selanjutnya dilakukan pemaparan senyawa Pg_4OH selama 4 jam. Pengamatan dilakukan pada jam ke-24, 48 dan 72 setelah paparan. Selanjutnya skor eritema dan edema kulit kelinci dinilai. Hasil pengamatan dihitung indeks iritasi primernya (IIP) yang kemudian hasil perhitungan dikategorikan berdasarkan kategori *globally harmonized system* (GHS) dan *international organization for standardization* (ISO). Dari ketiga kelinci hanya satu yang mengalami eritema dan sangat kecil pada jam ke-72 di 3 lokasi, yaitu 1 lokasi kontrol dan 2 lokasi sampel dengan masing-masing nilai 1, dua kelinci lainnya didapatkan nilai 0 pada setiap lokasi pengamatan. Hasil perhitungan IIP dari data pengamatan yang didapat yaitu 0,0093 termasuk ke dalam kategori sangat ringan atau dapat diabaikan. Dapat disimpulkan bahwa senyawa tetrasiklik Pg_4OH tidak mengakibatkan iritasi pada kulit.

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INTRODUCTION

Chronic exposure to UV radiation originating from sunlight is a determining factor in the development of melanoma and non-melanoma skin cancer (basal cell cancer and squamous cell cancer).[1-3] Reactive oxygen species (ROS) UV radiation can also react as an initiator and promoter of cancer by damaging oxidative biomolecules under oxidative stress conditions.[4-6] ROS can also trigger melanogenesis.[7] UV radiation, ROS, and free radicals produced in cytoplasm of the skin will be absorbed by the melanin pigment.[8] Overproduction and accumulation of melanin in the skin has a serious aesthetic effect which is the trigger for epidermal hyperpigmentation.[7] Topical antioxidants can be an alternative to prevent skin damage caused by exposure to acute and chronic UV sunlight, such as premature aging and skin cancer.[9]

The calixarene compounds have been successfully synthesized and evaluated their antioxidant properties. One among the type of this compounds is C-4-hydroxyphenylcalyx [4] pyrogalolarene (Pg_4OH). This compound exhibits a strong antioxidant with IC_{50} 88.2 µg/mL[10] and it is potential to be used as a topical antioxidant. However, so far there is not many studies conducted to evaluate the acute skin irritation of the Pg_4OH tetracyclic compounds. For the first time, an acute skin irritation test to investigate the effect of Pg_4OH was conducted in this study. Topical preparations by employing albino rabbits for skin irritation is applied.

MATERIALS AND METHODS

Compound and animal

C-4-hydroxyphenylcalyx [4] pyrogalolarene was synthesized by Salim in the Laboratory Organic Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Gadjah Mada under supervisor of Prof. Jumina.[10] The structure of this compound is presented in FIGURE 1.

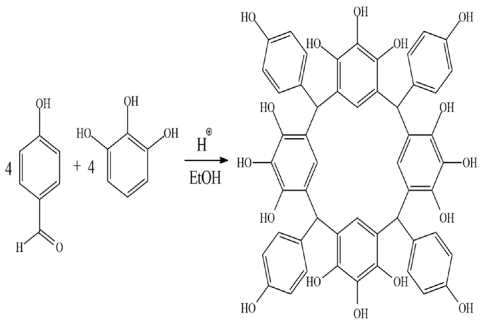

FIGURE 1. Structure of Pg_4OH

The acute irritation test was conducted in the Laboratory of Pharmacology and Therapy, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta. A quasi experimental with a posttest trial design only were employed. Three males albino rabbits (*Oryctolagus cuniculus*) species were used in this study. The rabbitts were verified as healthy, mature rabbits with age 4 months, and body weight of 2 kg.[11,12] Before the test begins, the rabbits were acclimatized in a room temperature for 5 days. Each rabbit was placed in an individual cage. At least 24 h before testing, the rabbit's back hair was shaved (10 x 15 cm width) to expose the samples.[12] Protocol of the study has been approved by the Medical and Health Research Ethics Committee, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta.

Procedure

The Pg_4OH solution preparation was prepared by dissolving of 0.5 g Pg_4OH in 1.25 mL distilled water in order to obtain the concentration of 0.4 g/mL.[12,13] The test preparation was the exposed by applying it to the area of the skin ± 6 (2 x 3) cm^2 with the exposure location, shown in FIGURE 2. The exposure location was covered with Hypafix® plaster in order to keep it from any allergen or dust.

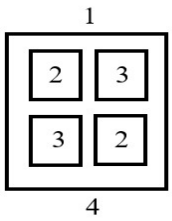

FIGURE 2. Scheme of rabbit back[14] (1. Head, 2. Location of the Pg4OH exposure, 3. Location of the control exposure, 4. Rabbit's tail)

Each rabbit was exposed with the Pg_4OH for 4 h period. The samples residue was immediately removed by using water after 4 h of exposure. For each exposure location, 2 types of responses were observed: erythema and edema (TABLE 1). The response-assessment was carried out at 1, 24, 48 and 72 h after the patch to be opened.

TABLE 1. Assessment score to the rabbit's skin.[12]

Formation of erythema	Score
• There is no erythema	0
• Very small erythema (almost indistinguishable)	1
• Erythema is clearly visible	2
• Moderate to severe erythema	3
• Severe erythema (red meat) until eschar formation which inhibits the assessment of erythema	4
Formation of edema	
• There is no edema	0
• Very small edema (almost indistinguishable)	1
• Small edema (visible boundary area, clearly)	2
• Middle-level edema (the area increases by about 1 mm)	3
• Severe edema (the area increases more than 1 mm and widens beyond the exposure area by the test preparation	4

Analysis

Primary irritation index (PII) was calculated using the following formula[14]:

$$PII = (A-B)/C$$

Where A is number of erythema score and edema of all sample (at 24, 48 and 72 h) divided by the number of observations, B is the number of erythema and edema values of all control (at 24, 48 and 72 h) divided by the number of observations and C is the number of animals. The PII was then categorized based on the GHS[15] criteria (TABLE 2) and ISO 10993-10[13] (TABLE 3).

TABLE 2. Criteria for classifying corrosive / irritant test preparations for the skin.[15]

Categories		Criteria
Category 1 (Corrosive)	1A	Corrosive response occurred on exposure for ≤ 3 min, observing for ≤ 1 h at ≥ 1 of 3 subjects
	1B	Corrosive response occurred on exposure for > 3 min to ≤ 1 h, observing for ≤ 14 d at ≥ 1 of 3 subjects
	1C	Corrosive response occurs on exposure for > 1 h to ≤ 4 h, observation for ≤ 14 d at ≥ 1 of 3 subjects

Category 2 (Irritant)	i. The average score for erythema/edema is 3 2,3 to ≤ 4, after exposure for 4 h, observation for 3 days, ii. Inflammation does not heal until day 14 at least 2 subjects, alopecia occurs in certain areas, hyperplasia, scaling or iii. There is a clear effect of erythema / edema on 1 test animal even though it does not meet the criteria above
Category 3 (Mild irritation)	Mean values for erythema/edema are ≥ 1.5 to 3 2.3 after 4 h of exposure, observed for 3 days after the skin reaction but not included in the above categories, at least 2 of 3 subjects

TABLE 3. Categories of irritant responses to rabbits.[13]

Mean value	Response categories
0.0 - 0.4	Very light (negligible)
0.5 - 1.9	Mild irritant
2.0 - 4.9	Moderate irritant
5.0 - 8.0	Strong irritant (severe)

RESULTS

The acute skin irritation test results after exposed of Pg_4OH solution preparation on albino rabbits can be observed in TABLE 4. The pH of Pg_4OH solution preparation was 6.37, while the pH aquadest used in this experiment was 6.83. No reaction occurred during the first until the 48 h observation. At the 72 h observation only a tiny erythema with the score of one arose on rabbit-3, both from the samples and control locations. No reaction occurred in rabbit-1 and rabbit-2. The PII obtained was 0.0093.

TABLE 4. Observation results

Subjects	Treatment	Degree of lesion every duration								Total
		1 h		24 h		48 h		72 h		
		E	U	E	U	E	U	E	U	
Rabbit 1	Control	0	0	0	0	0	0	0	0	0
	Sample	0	0	0	0	0	0	0	0	0
Rabbit 2	Control	0	0	0	0	0	0	0	0	0
	Sample	0	0	0	0	0	0	0	0	0
Rabbit 3	Control	0	0	0	0	0	0	0	0	1
	Sample	0	0	0	0	0	0	1	1	2
Total score of control (rabbit 1+rabbit 2+rabbit 3)										1
Total score of control (rabbit 1+rabbit 2+rabbit 3)										2
pH control (aquadest): 6.83; pH sample (Pg_4OH): 6.37										

DISCUSSION

The irritation test was conducted using a closed patch test method. Males albino rabbits (Ory*ctolagus cuniculus*) were chosen as the subjects due to it has a fairly wide skin surface compared to rat or mice. The number of subjects used were three males albino rabbits as recommended by the ISO[13], BPOM[14], GHS[15], and standards.

Skin irritation might arise caused by inflammation reaction due to the release of proinflammatory cytokines from keratinocytes. It is usually occurred as the responses to a chemical stimuli. The chemical stimuli can caused skin barrier disorders, changes in epidermal cells, and the release of cytokines. Symptoms that arise in the acute phase are erythema, edema, hardening of the skin, tenderness, vesicles, or pustules.[16]

We began this experiment by checking pH measurement, both from the Pg_4OH and from the aquadest in order to ensure that the pH value of the two test materials is skin tolerable (≥2 or ≤11.5). Hence it does not caused corrosive effect on the skin animal. If the compound is suspected to be corrosive, the experiment must be stopped.[14] The pH value is one of the things that need to be considered in this test. Higher or lower pH is one of the causes of side effects on the skin such as erythema and edema.[17] According to the experiment the pH of aquadest (control) was 6.83 while the pH of the sample was 6.37.

The PII score obtained was 0.0093. Based on the ISO 10993-10[13], this result belongs to negligible category (with a value of 0.0-0.4). Considering GHS[15] criteria, these results can not be specifically categorized since the lowest score for GHS is 1.5-2.3. It is logic that the Pg_4OH did not cause skin irritation.

The drug development phase begins with the synthesis or isolation process by studying the relationship between the structure of the drug and its activity. In the previous study, it was reported that IC_{50} of Pg_4OH was 88.2 µg/mL.[10] The IC_{50} is a parameter in determining antioxidant activity, in the form of effective concentrations of antioxidant compounds to inhibit 50% of DPPH free radical activity.[18] According to Armala[19], the level of antioxidant activity can be classified as very strong (IC_{50}: <50 µg/mL), strong (IC_{50}: 50-100 µg/mL), moderate (IC_{50}:101-150 µg/mL), and weak (IC_{50}: 250-500 µg/mL). It was indicated that Pg_4OH compounds were classified as strong antioxidants.

Topical antioxidants can prevent skin damage such as premature aging and skin cancer caused by the formation of ROS due to exposure to acute or chronic UV light (both UV-B and UV-A).[9] The formation of ROS such as OH, RO_2, RO, $^{\bullet}O^{-}_2$, H_2O_2 and ROOH can be the causes of tumors by oxidatively damaging biomolecules under oxidative stress conditions: as in the formation of 8-hydroxy-2-deoxiguanosine (8-OHdG) and lipid membranes in DNA.[5,6,20-22] Protection towards the negative effect of solar UV radiation by the melanin pigment absorbing UV radiation, ROS, and free radicals produced by the cytoplasm.[8,23] However melanin overproduction and accumulation in the skin triggers a serious aesthetic problem, that is epidermal hyperpigmentation.[7,24]

The use of sunscreen prevent skin damage due to sunlight is the gold standard in many countries.[9] However, recent developments have found that the limitations of the efficiency of sunscreens include 1). It only efficient as a UV-B filter (290-320 nm), while there is not a single product able to provides good protection in the entire UV-A range (320-400 nm); 2). SPF (sun protective factor) is determined in the laboratory as appropriate when used 2 mg/cm^2, but controlled studies show that most people use sunscreens at concentrations of 0.5 mg/cm^2 or smaller; 3). Many formulations of organic and inorganic sunscreens can trigger the formation of ROS.

The use of oral or topical antioxidants in the treatment of dermatoses aims to neutralize excess free radicals, reduce or prevent attacks on cellular structures. Because maintaining or reshaping the redox balance is a goal in this situation, the use of antioxidants must always be in

line with other precautions.[25]

In this study experimental by using animals was conducted.[26] All observations on animals determine whether they can be continued to be test on humans. Toxicity tests were divided into acute, subchronic, chronic toxicity tests and special toxicity tests which included the teratogenicity, mutagenicity, and carcinogenicity tests[14]. Skin sensitization, eye irritation, acute dermal irritation, vaginal mucosal irritation, acute dermal toxicity, and dermal subchronic toxicity tests ought to be applied in order to determine the safety level of a chemical preparation for skin. The selection of this test depends on the purpose of using the substance[14]. Acute skin irritation test being chosen in this study since the Pg_4OH is being used as topical antibiotic compounds that work directly on the skin.

CONCLUSION

In conclusion, the tetracyclic Pg_4OH compound does not cause skin irritation on albino rabbits.

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