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Biochemistry

Fatty acid composition profiling in the dorsal skin of Sunda porcupine (*Hystrix javanica*)

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ABSTRACT. The fatty acid composition in the skin of Sunda porcupine (*Hystrix javanica*) is an interesting topic due to the special features of quills, especially in the dorsal region. Therefore, this study aims to analyze the composition of fatty acids in the dorsal region of Sunda porcupine skin. It was conducted using skin samples of the thoracodorsal and lumbosacral regions taken by biopsies and from frozen specimens. The skin lipid was extracted and then derivatized into fatty acid methyl ester before analyzing with gas chromatography mass spectrometry. The results showed that the skin is composed of up to 25 fatty acids ranging from C12 to C25 with various types but only 16 were found in both regions and sexes. Fatty acids with an antibacterial effect were found abundantly, such as oleic, palmitic, stearic, and linoleic acids. The total abundance in the thoracodorsal region was higher than lumbosacral, while the composition in male was higher than in female. Based on the results, the fatty acid composition in the dorsal skin region of Sunda porcupine consists of at least 16 types ranging from C12-C25. Additionally, the region and sex were observed to contribute significantly to the variation in skin fatty acid composition.

KEYWORDS: fatty acid, gas chromatography mass spectrometry, lumbosacral, sebaceous gland, thoracodorsal

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The skin as the outermost organ plays a role in protecting the body from various interferences. The epiderm is composed of several layers of keratinocytes that function to protect the skin against infectious agents [8]. For example, the stratum corneum layer has cellular components and structures that play a role in regulating acidity. This condition is one of the defense functions that act in suppressing the growth of bacterial populations on the surface of the skin [8].

Meanwhile, the Sunda porcupine (*Hystrix javanica*) is a species of rodent in the family Hystricidae that has quills as the main skin derivates apart from the hair. The quills which are well developed as the body defense structure are located in the dorsal areas of the body [17, 20, 32]. A previous study showed that the thorax and cranial part of the dorsal region consists of small, flexible, and flat quills while the lumbar to sacrum part comprises hard, stiffed and bigger quills. In addition, the surface skin characteristic in this region exhibits several differences in quill follicle density [24, 25]. Due to this special feature, this study was conducted to examine the characteristics of the Sunda porcupine skin. Previous studies have been conducted on the skin structure [24], bacterial population [25], alpha tocopherol in the skin [26], lectin binding in the sebaceous gland [27], immunoreaction of uncoupling protein 1 (UCP-1) antibody in sebaceous gland [28] and morphological evaluation of polysaccharide in wound healing [29].

The structure and chemical composition in the various parts of the skin are unique and determine the specific habitat for each type of microbe [11]. The skin surface of the Sunda porcupine especially in the dorsal region is the habitat of commensal bacteria, such as *Staphylococcus aureus*, *S. epidermidis*, and *Micrococccus* sp [25]. Previous studies exhibited a large-sized gland of the skin in each quill, compared to the hair follicle [25, 27]. Moreover, the density of quill follicle in the thoracodorsal region is higher compared to the lumbosacral, indicating a higher density of the sebaceous gland in the thoracodorsal region [25]. The sebaceous glands play a role in the transport of proteins, lipids, and glycerol to the skin surfaces for the hydration of the stratum corneum layer. The sebum produced from this gland is known to have components that act as antimicrobials [8]. Simple forms of lipids such as fatty acids and monoglycerides have antimicrobial activity and are capable of killing a wide range of microbes such as Gram-negative and positive

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bacteria, virus, yeast, and some parasite which infect the skin. Fatty acids and/or monoglycerides are known to have innate immune system properties against certain bacterias in skin infection [9, 15, 23].

However, the fatty acid composition in skin surface from the epidermis, superficial dermis, and sebaceous glands of Sunda porcupine skin has not been studied thoroughly. Therefore, this study aims to determine the composition of fatty acid in the dorsal region of Sunda porcupine skin. The fatty acids will be analyzed by their derivates form of fatty acid methyl ester (FAME). The identified components can be used as basic information about the potential fatty acid content affecting the skin physiology.

MATERIALS AND METHODS

Animals

This study used 6 adults Sunda porcupines consisting of 3 males and 3 females, weighing 6–8 kg as subjects and optimized by 2 frozen specimens namely 1 male with 1 female aged 2–4 years. The animals were bred in captivity for conservation purposes and to be released into the wild. All procedures were performed in accordance with the ethical approval of The Ethical Clearance Subcommittee of Life Science, Indonesian Institute of Sciences, No. B-12695/K/KS.02.04/XII/2017. All the animals were in healthy condition based on the veterinary routine examination. They were sampled for skin biopsy and the procedures were conducted under anesthesia using 10% HCl ketamine (Ilium Ketamil, Troy Laboratories, Glendenning, NSW, Australia) and 2% xylazine HCl (Ilium Xylazil, Troy Laboratories) at doses of 2.5 mg/kg body weight (BW) and 1 mg/kg BW. The animals were used mainly for natural wound healing in line with Prawira *et al*. [29]. Full thickness wounding was conducted by incision method to make an open wound with an area of approximately 11.3 ± 1.23 cm^2 in each region, i.e. Thoracodrosal (TD) and Lumbosacral (LS), while the skins obtained were used as the samples. Considering that the animals were to be used for natural wound healing evaluation, the medication was administered minimally. However, the analgesic in the form of dexamethasone and antibiotics namely penicillin and streptomycin were prepared for the unexpected responses. The vaginal swabbed was also performed on the females to predict the reproduction status based on the cytological observation, the overview of the animal data is shown in Table 1. Additionally, the frozen specimens were obtained from society donation as freshly dead animals to IPB University laboratorium specifically for research purposes, then the carcass and the skin were stored and frozen at −40°C.

The skin samples obtained from the biopsy and frozen specimens were then frozen and sectioned to separate the skin surface namely the epidermis, superficial dermis, and sebaceous gland from other parts. Next, samples were divided into 4 groups, as male TD, male LS, female TD, and female LS with each group weighing 4–4.5 g which was divided into 2–2.25 g each for total lipid percentage and fatty acid composition.

Total lipid percentage

The Total lipid in the skin was quantified using the Soxhlet method, the skin of each group was weighted (W1), dried using evaporator at 60°C overnight, and then grinded. The clean cups were weighed (W2) and the sample was put in the individual cup, then the Petroleum benzene and solvent were mixed with the samples, and then proceeded into the Soxhlet extractor (Soxtec System HT6 1043, Foss Tecator, Eden Prairie, MN, USA) at 105°C temperature for 20 min. Cooling was carried out while the solvent was rinsed from the extractor hull to the extraction cup for 35 min. The mixture was then evaporated, dried, and weighed as the final weight of samples (W3). The difference between the final (W3) and clean cup weight (W2) divided by sample weight (W1) is the percentage of total lipid.

Lipid extraction, FAME derivatization and Analysis by Gas Chromatography Mass Spectrometry (GCMS)

Samples were extracted according to the method of Bligh and Dyer [3] to obtain the total lipid extract. This was carried out by mixing 2 mg of tissue samples with a mixture of 4 mL methanol and 2 mL chloroform, followed by homogenization, the addition of 2 mL double distilled water (DDW), and homogenization. The formed solution was separated into two phases with alcohol at the top and chloroform at the bottom. Subsequently, 1 mL of chloroform phase was derivatized into fatty acid methyl ester or (FAME) using 0.5 N NaOH and BF_3 in methanol. Three mL of hexane was added followed by mixing using vortex and heating for 5 min at 60°C and then cooling. The formed solution is divided into 2 layers, the top or hexane layer was taken, filtered with Na_2SO_4, and injected (1 µL) into the GCMS (Shimadzu QP2010, Shimadzu Corp., Tokyo, Japan) with column DB-5MS having a length of 30 m, diameter 0.25 mm, and thickness 0.25 µm (122-5532UI, Agilent Technologies, Santa Clara, CA, USA). The GCMS was operated at temperature 60–250°C (7°C/min) with 57.4 kPa of pressure and flow rate 104 mL/min, while the flow of the carrier gas, Helium was

Table 1. Animals data

Animal	Weight (kg)	Sex	Age (Year)	Reproduction status
1	7.20	Male	4	-
2	8.05	Male	4	-
3	6.30	Male	2	-
4	8.10	Female	4	Estrus
5	7.20	Female	2	Estrus
6	8.60	Female	4	Early diestrus

1 mL⁄min. The FAMEs were identified by comparing the retention time of authentic compounds (peaks) along with Library.Wiley7 and NIST 147 mass spectral data bank.

Analysis of data

The percentage and area of each peak in chromatogram were analyzed statistically with ANOVA single factor, while the difference of each identified fatty acid between groups was compared using Duncan's multiple range test and student's *t* test at $P<0.05$, all analysis was conducted using the XLStat 2017 in Microsoft Excel.

RESULTS

An overview of the living animals' data is shown in Table 1, the smallest male weighed 6.30 kg and aged 2 years, while the smallest female weight was 7.20 kg with a similar age. Based on the reproduction status, the females were in estrus and early diestrus when the biopsy procedure was performed.

The total lipid percentage of each group varied from 1.8% to 8.23% as demonstrated in Table 2. The highest percentage was found in male TD, while the lowest was in female TD. However, the total lipid in TD region namely 10.5% was higher than in LS region 5.18%, while the total lipid in male 11.5% was higher than in females at 3.73%.

The GCMS examination results on the skin of the epidermis and superficial dermis showed fatty acids with C12-C25 carbon chains (Fig. 1). A total of 16 types of fatty acids (C12-C24) were identified in all group, namely lauric (C12:0), myristic (C14:0), pentadecanoic (C15:0), palmitoleic (C16:1), palmitic (C16:0), 14-methylhexadecanoic (C17:0), linoleic (C18:2), oleic (C18:1), isooleic (C18:1), stearic (C18:0), arachidonate (C20:4), eicosenoic (C20:1), arachidic (C20:0), behenic (C22:0), tricosanoic (C23:0), and lignoceric acid (C24:0) (Table 3). In the male TD and LS regions, 22 types of fatty acids with carbon chains of C12-C25 were identified, while 2 were identified only in the male LS group, namely 2-hexylcyclopropanoctanoic acid (C17:0) and hyenic acid (C25: 0). Moreover, the fatty acids found only in the male TD region were isooleic (C18:1) and nervonic acid (C24:1). In the female TD region, 18 types

Table 2. Total lipid percentage

Parameter	Male TD	Male LS	Female TD	Female LS
Total lipid (%)	8.23[a]	3.27[b]	1.82[c]	1.91[c]
Total lipid (Sex)	11.50% (Male)[a]		3.73% (Female)[b]	
Total lipid (Region)	10.05% (TD)[a]		5.18% (LS)[b]	

Different superscript letter in a row, show significant difference at $P<0.05$. TD: thoracodorsal, LS: lumbosacral.

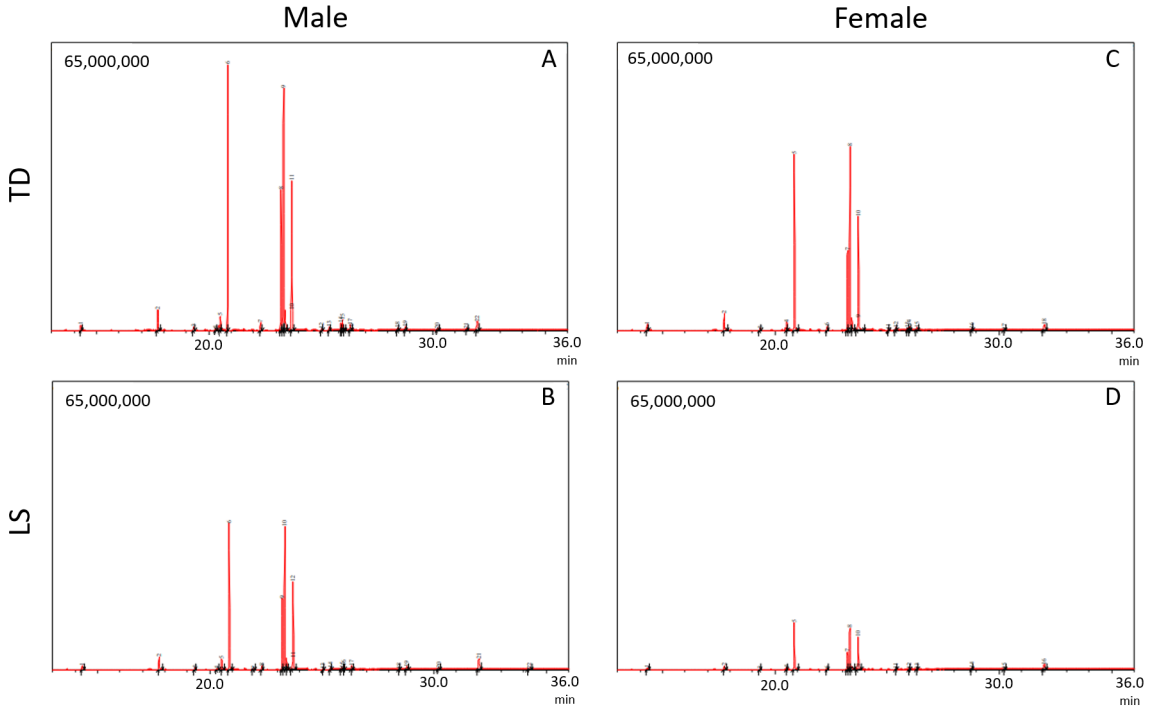

Fig. 1. Gas chromatography mass spectrometry (GCMS) peaks pattern of the fatty acid composition in Sunda Porcupine skin. Male thoracodorsal [TD] (**A**), male lumbosacral [LS] (**B**), female thoracodorsal [TD] (**C**), female lumbosacral [LS] (**D**).

Table 3. Percentage abundance of fatty acid in each group

Fatty acid	Carbon	Abundance in regio (%)			
		Male TD	Male LS	Female TD	Female LS
Lauric acid*	C12:0	0.655^{b}	0.875^{a}	1.015^{a}	0.995^{a}
Myristic acid*	C14:0	2.595^{a}	2.655^{a}	2.655^{a}	2.505^{a}
Pentadecanoic acid*	C15:0	0.425^{a}	0.33^{b}	0.3^{b}	0.285^{b}
14-Methylpentadecanoic acid	C16:0	0.205^{a}	0.12^{b}		
Palmitoleic acid*	C16:1	1.835^{b}	2.08^{a}	1.295^{c}	1.2^{a}
Palmitic acid*	C16:0	17.945^{b}	25.945^{a}	26.755^{a}	28.045^{a}
2-hexylcyclopropanoctanoic acid	C17:0		0.165		
14-methylhexadecanoic acid*	C17:0	0.995^{a}	0.765^{b}	0.69^{b}	0.705^{a}
Linoleic acid*	C18:2	17.095^{a}	13.67^{b}	12.775^{c}	11.025^{d}
Oleic acid*	C18:1	30.225^{a}	26.84^{b}	29.93^{a}	25.665^{b}
Isooleic acid*	C18:1	2.835^{a}	2.465^{a,b}	2.375^{b}	2.415^{b}
Elaidic acid	C18:1	1.39			
Stearic acid*	C18:0	18.3^{a}	16.51^{c}	17.95^{b}	19.75^{a}
17-Methyloctadecanoic acid	C19:0	0.16^{a}	0.14^{a}	0.155^{a}	
Arachidonate acid*	C20:4	0.415^{ab}	0.43^{ab}	0.47^{a}	0.355^{c}
Eicosadienoic acid	C20:2	0.97^{a}	0.42^{a}	0.38^{a}	
Eicosenoic acid*	C20:1	0.13^{a}	0.825^{a}	0.71^{a}	0.405^{a}
Arachidic acid*	C20:0	0.73^{a}	0.77^{a}	0.515^{b}	0.485^{b}
Erucic acid	C22:1	0.275^{a}	0.16^{b}		
Behenic acid*	C22:0	0.51^{ab}	0.695^{a}	0.31^{b}	0.695^{a}
Tricosanoic acid*	C23:0	0.235^{a}	0.29^{a}	0.155^{a}	0.28^{a}
Nervonis acid	C24:1	0.145			
Lignoceric acid*	C24:0	1.9^{c}	3.56^{b}	1.58^{c}	5.185^{a}
Hyenic acid	C25:0		0.29		

*Fatty acid in all group. Different superscript letter in a row, show significant difference at $P<0.05$. TD: thoracodorsal, LS: lumbosacral.

Table 4. Actual abundancy of fatty acid based on total area measurement in gas chromatography mass spectrometry

Parameter	Male TD	Male LS	Female TD	Female LS
Total area (× 10^5 unit)	4,056^{a}	2,592.6^{c}	3,243.1^{b}	837.7^{d}
Total area (Sex)	6,684.6^{a}(Male)		4,080.8^{a}(Female)	
Total area (Region)	7,299.1^{a} (TD)		3,430.3^{b} (LS)	

Different superscript letter in a row, show significant difference at $P<0.05$. TD: thoracodorsal, LS: lumbosacral.

(C12-C24) were identified while 16 (C12-C24) were found in the LS. The 2 specific fatty acids identified in the female TD group were 17-Methyloctadecanoic (C19:0) and eicosadienoic acid (C20:2). Lignoceric acid (C24:0) was higher in the LS region both in males and females compared to the TD as shown in Table 3.

The fatty acids in the Sunda porcupine skin were dominated by oleic (C18:1), palmitic (C16:0), stearic (C18:0), isooleic (C18:1), linoleic (C18:2), myristic (C14:0) palmitoleic (C16:1), and lignoceric acid (C24:0). Oleic acid (C18:1), palmitic acid (C16:0), stearic acid (C18:0), linoleic acid (C18:2) were the most abundant with over 10% in all groups as explained in Table 3.

The percentage of some fatty acids between groups varied significantly but the actual abundance based on area measured in GCMS showed that the fatty acids in the female LS region were significantly lower compared to the other regions (Table 4). Furthermore, the total abundance in the TD region for both males and females was significantly higher than LS. The male TD and LS showed higher total abundance compared to the female but the difference was not significant (Table 3), these results correlated with the total lipid percentage.

Generally, the dominant fatty acids were those with medium-chain (C12-C14), followed by long-chain fatty acids (LCFA) with 15–20 carbon atoms, while the rest were very long-chain fatty acids (VLCFA) with over 22 carbon atoms. There were 14 types of saturated fatty acids, 3 of them were classified as branched. Moreover, 1 cyclic fatty acid was identified in the male LS group, while 7 monounsaturated and 3 polyunsaturated types were also found.

DISCUSSION

This study identified fatty acids which originated from sebaceous glands, epidermis, and part of the dermis. Fatty acids contained in the skin are generally produced from the sweat, sebum, and epidermal cells containing antimicrobial lipids [13, 33]. These components are presumably important parts of the innate defense mechanisms against bacterial infections on the skin surface. Most of the lipids are composed of fatty acids released from sebocyte triglyceride by the activity of normal flora lipase [9, 23], sebocyte and keratinocytes, as well as in milk [35–37].

Based on the literature search, there are currently no studies on the fatty acid composition in the Sunda porcupine skin. Meanwhile, several investigations have been conducted on the fatty acid content in quill of porcupine. For example, Roze *et al.* [30] in a study on the antibiotic properties in the quill of North American porcupine as well as Inayah *et al.* [12] on the nutritional content in the quill of Sunda porcupine. According to Inayah *et al.* [12], the quill of Sunda porcupine consists of 0.44% crude fat and 18.6% is mainly fatty acids. However, the quills used in the study were not specifically classified [12].

Most of the lipid on the epidermis surface is produced by the sebaceous glands, while those synthesized by the epidermis represent a small fraction of the total lipid, especially in parts of the body that have numerous sebaceous glands [10]. The TD region is known to have a higher sebaceous glandular density than the LS, but the LS region has larger sebaceous glands [25]. This is because the glands density is determined by the number of quills that grow on the skin. Several quills grow in the follicle and form a cluster, the small and flexible types in the TD region culminate in higher growth (1.08 ± 0.29 quill cluster/cm^2) compared to the LS region where the quills are bigger and harder (0.32 ± 0.07 quill cluster/cm^2) [25]. This factor might affect the lipid composition in the region based on the gland density of quill follicle. Additionally, this might be a factor which contributed to the lower bacteria population in the TD ($2.37 \pm 1.91 \times 10^5$ cfu/cm^2) region due to a higher amount and composition of fatty acid on the skin compared to the LS ($2.76 \pm 2.2 \times 10^5$ cfu/cm^2) [25], given that one of the roles of fatty acid is antibacterial at certain conditions [2, 5, 14–16, 21]. The alpha tocopherol in the TD region was also higher compared to the LS with 48.75 and <0.21 nmol/g tissue, respectively [26].

The skin of the male and TD region have more types of fatty acid compared to the female and LS region. Akamatsu *et al.* [1] revealed that fatty acid compositions can also be affected by sex hormones such as androgens and estrogens, especially in the sebaceous glands depending on the location of the gland and the lipid type in sebum. This indicates that estrogen contributes significantly to the inhibition of sebaceous gland activity [31]. Furthermore, the estrus and early diestrus phase of the female porcupine is predicted to cause the lower types of fatty acid in females compared to males. A previous study on bacterial population found more colonies in female with $3.52 \pm 2.25 \times 10^5$ cfu/cm^2 than in male $1.6 \pm 1.14 \times 10^5$ cfu/cm^2 [25].

One of the site-depending physiology of the Sunda porcupine skin is observed by immunoreactivity of UCP-1 protein in sebaceous glands. The TD region showed higher intensity of immunopositive reaction compared to the LS [28]. Additionally, the wound healing in dorsal skin also exhibited the site-depending factor, where the TD region healed faster than the LS [29].

Based on the results on this study and previous studies on alpha tocopherol [26] and bacterial population on skin surface [25], there was a positive correlation between the total lipid, fatty acid abundance, and alpha tocopherol. Meanwhile, the bacterial population had a negative correlation with the total lipid, fatty acid contents, and alpha tocopherol (Supplementary Table 1). The higher amount of total lipid percentage and fatty acid abundance in the TD region contributed to the lower bacterial population compared to the LS. These factors might affect the physiology of wound healing in Sunda porcupine.

Unique fatty acids with odd or branched carbon chains are commonly found in the skin and sebum of mammals and rodents. They also exist as metabolites produced by normal skin flora [22]. Some of the fatty acids with odd and branched carbon chains found in the skin of Sunda porcupine include pentadecanoic (C15:0), tricosanoic (C23:0), 14-methyl pentadecanoic (C16:0), 14-methylhexadecanoic (C17:0, branched), and 17-Methyloctadecanoic acid (C19:0 branched). One other unique fatty acid is the cyclic type, 2-hexylcyclopropanoctanoic acid. They are produced by the normal flora on the skin surface, specific feed, or metabolic products from the fermentation process in the gastrointestinal tract [19].

The composition of sebum is specific to each species of animal [18, 30], in the sebaceous glandular part, GCMS was used to identify C12 to C25 chains with oleic (C18:1), palmitic (C16:0), stearic (C18:0), linoleic (C18:1), 14-methylhexadecanoic (C17:0), lignoceric (C24:0), and lauric acid (C12:0) being the most abundant with greater relative content than other fatty acids. This composition is specific compared to other rodents such as mice, rats, and guinea pigs [34]. The fatty acid in the sebum of common rodents consisted mostly of n-hexadecanoic acid (C16) in Guinea pig, rabbit, and rat with 15.8%, 24%, and 17.4%, while mono-unsaturated nonadecanoic acid (C19) in mouse is up to 10% [34].

Several saturated fatty acids with C6-C18 are known to have antibacterial activities at certain temperatures, concentrations, and time [2, 5, 14–16, 21]. Palmitic (C16:0), 14-methylpentadecanoic (iso C16:0) and oleic acid (18:1) are the major fatty acid in the quil of North American porcupine, *Erethizon dorsatum*, with confirmed antibacterial activity against some Gram-positive bacteria, such as *S. aureus* and *Bacillus cereus* [30]. These types of fatty acids were also found in the skin of Sunda porcupine. Palmitoleic (C16:0), Oleic (C18:1), palmitic (C16:0), stearic (C18:0), linoleic (C18:2), myristic (C14:0) and palmitoleic acid (C16:1) are the most abundant component of fatty acids in the dorsal region, in both male and female.

Long-chain fatty acids with C20-C25 have several activities in the skin, eicosanoid acid (C20) is a mediator formed from unsaturated fatty acids and is present in the cell membrane. Eicosanoid is a local hormone that regulates numerous important physiological and pathological processes such as pain, fever, wound closure, and birth [6, 7]. Other fatty acids with more than 20 carbon found in the skin on Sunda porcupine are closely related to the ceramide component of the cell membrane in the epidermis and do not have antimicrobial activity [4]. However, the lignoceric acid which was more abundant in the LS region might be associated with the origin of the acid. Behenic (C22: 0), nervonic (C24: 1) and lignoceric acid (C24: 0) are known to play a role in the function of nerve cells in

the form of sphingolipids in cell membranes of the myelin sheath [19]. According to previous studies, several nerve fibers are found in the quill follicle, especially in the lumbar dorsal and LS region [24, 25].

Based on the results, the fatty acid composition in the dorsal region consists of at least 16 types ranging from C12-C25. The region in the dorsal trunk and sex contribute to the variation in the composition where the thoracodorsal and male had the highest amount. These results can be used as basic information for further investigations on Sunda porcupine skin physiology and pathology.

POTENTIAL CONFLICTS OF INTEREST. The authors declare that there is no conflict of interest.

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