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Profiling the Non-Targeted Proteins in Sunda Porcupine (*Hystrix javanica*) Quills to Identify the Wound Healing Potential Substance

(Memprofilkan Protein Tidak Bersasar pada Duri Landak Jawa (*Hystrix javanica*) untuk Mengenal Pasti Bahan Potensi Penyembuhan Luka)

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ABSTRACT

Sunda porcupine (*Hystrix javanica*) has quill as the main appendage of its skin and used as a means of self-defence against predators. In some regions of Indonesia, roasted porcupine quills are employed as a traditional remedy for dental pain and wound healing. This treatment is likely to use protein and other compounds in the quills that interact directly with tissues to produce pharmacological effects. The aim of this study was profiling the protein composition in the porcupine quills in order to screening the potential substances which helps improve wound healing. Natural detached porcupine quills were collected and extracted for total protein. The protein composition was identified using SDS-PAGE and LC-MS/MS instrument. The data were analyzed using bioinformatics approach. This study demonstrates that porcupine quills have a soluble protein concentration of 2.11 ± 1.14 mg/mL composed mainly of proteins with molecular weights of 245 kDa and 60 kDa. LC-MS/MS utilisation showed the presence of 19 suspected proteins, including keratin 1, 2, 5, 6a, 8, 10, 14, 17, and 84. Bioinformatic analysis showed that three proteins with more than 1 unique peptide were potentially affecting wound healing, which were Keratin Type II, cytoskeletal 1 (Keratin 1); Keratin Type II, cytoskeletal 6a (Keratin 6a); and Annexin. The results of study provide scientific evidence regarding the potential substance of porcupine quills to help improve wound healing.

Keywords: Annexin; bioinformatic; keratin; proteomic; wound healing

ABSTRAK

Landak Jawa (*Hystrix javanica*) mempunyai duri sebagai pelengkap utama kulitnya dan digunakan sebagai alat pertahanan diri terhadap pemangsa. Di sesetengah kawasan di Indonesia, duri landak panggang digunakan sebagai ubat tradisi untuk sakit gigi dan penyembuhan luka. Rawatan ini berkemungkinan menggunakan protein dan sebatian lain dalam duri yang berinteraksi secara langsung dengan tisu untuk menghasilkan kesan farmakologi. Matlamat kajian ini adalah memprofilkan komposisi protein pada duri landak untuk menyaring bahan berpotensi yang membantu meningkatkan penyembuhan luka. Duri landak gugur secara semula jadi dikumpul dan diekstrak untuk jumlah protein. Komposisi protein dikenal pasti menggunakan instrumen SDS-PAGE dan LC-MS/MS. Data dianalisis menggunakan pendekatan bioinformatik. Kajian ini menunjukkan bahawa duri landak mempunyai kepekatan protein larut sebanyak 2.11 ± 1.14 mg/mL yang terdiri terutamanya daripada protein dengan berat molekul 245 kDa dan 60 kDa. Penggunaan LC-MS/MS menunjukkan kehadiran 19 protein yang disyaki, termasuk keratin 1, 2, 5, 6a, 8, 10, 14, 17 dan 84. Analisis bioinformatik mendedahkan bahawa tiga protein dengan lebih daripada 1 peptida unik berpotensi menjejaskan penyembuhan luka, iaitu Keratin Tipe II, sitoskeletal 1); Keratin Jenis II, sitoskeletal 6a (Keratin 6a) dan Annexin. Hasil kajian memberikan bukti saintifik mengenai potensi bahan daripada duri landak untuk membantu meningkatkan penyembuhan luka.

Kata kunci: Annexin; bioinformatik; keratin; penyembuhan luka; proteomik

INTRODUCTION

The sunda porcupine (*Hystrix javanica*) is one of the endemic animals found in the Indonesian Islands of Java, Bali, and Lombok. The characteristics of the body covering of quill and hair make this animal unique (Prawira et al. 2018; van Weers 1983). Naturally, quills are used as a means of self-defence against predators (Akers & Denbow 2013). In some regions of Indonesia, porcupine quills are employed as a traditional remedy for dental pain (Kakati & Doulo 2002; Krisyanto, Ardian & Anwari 2019). Previous studies showed some anatomical and biochemical characteristics of porcupine skin and quills. Porcupine skin is known to contain up to 22 types of fatty acids that vary from short-chain (C8) to very long-chain (C23) fatty acids (Prawira et al. 2022). The main composition of porcupine quills is protein, followed by lipids and fatty acids. Quills are a modification of the protein structure of keratin and other proteins that grow and develop from the skin. Furthermore, proximate and Kjeldahl analysis methods show that the quills are composed of 93.66% N-total protein (Inayah, Farida & Purwaningsih 2020).

In ethnobiology, the use of quills without an extraction process as a medicine for pain relief or wound healing in several regions of Indonesia is likely to use protein and other compounds in the quills that interact directly with tissues to produce pharmacological effects. Identifying the protein profile of porcupine quills is necessary to determine which proteins are potentially involved in producing healing effects and pain relief. This study was aimed to profiling proteins composition in the porcupine quill. This profiling data was analysed bioinformatically to identify proteins with potential as active ingredients that affect the wound healing process.

MATERIALS AND METHODS

ETHICS STATEMENTS

This study was approved by the Ethics Committee of the National Agency for Research and Innovation under number 002/KE.02/SK/6/2022.

PREPARATION OF PORCUPINE QUILL POWDER

The porcupine quills were collected from naturally shed quills at the Animalium animal breeding center, KST Soekarno, National Research and Innovation Agency (BRIN). A total of 100 grams of porcupine quills were collected and washed three times with soap and water. The quills were cut into small pieces, and then ground into a fine powder. The powder was stored in a zip-lock plastic bag and kept at 4 °C until use.

PROTEOMIC ANALYSIS

Samples preparation

The sample preparation was performed following procedure by Fatchiyah et al. (2011). A total of 1.5 g of porcupine quills were split into three replicates of 0.5 g each. Prior to pulverization on a chilled container, the quills were washed with phosphate-buffered saline (PBS). The pulverized samples were subsequently extracted using a buffer that contained 1 mM phenylmethanesulfonyl fluoride (PMSF) in dimethyl sulfoxide (DMSO), 50 mM potassium dihydrogen phosphate (KH_2PO_4) at pH 7.4, and distilled water. The samples were homogenized with the buffer and centrifuged at 10,000 RPM at 4 °C for 20 min. The resulting supernatant was taken for further processing. Subsequently, 20 µL was utilized for electrophoresis, and the remaining amount was used for protein concentration measurement via spectrophotometry.

Separation of Protein Fractions by Gel Electrophoresis

A total of 20 µL of supernatant from each replicate of quill and skin extraction was processed for separation of protein fractions based on molecular weight by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method. The gel used consisted of 3% stacking gel and 12.5% separating gel. A total of 10-20 µL of porcupine quill protein extract sample was added to a 1:1 buffer stack and heated at 100 °C for 5 min. After cooling, the sample was loaded into gel wells placed in the electrophoresis chamber along with a running buffer. The electrophoresis process was carried out at a current of 20 mA (100 V) for 40-50 min. The gel was taken out of the chamber and treated with Comassie blue using a solution of methanol, acetic acid, and distilled water (methanol 450 mL, distilled water 450 mL, and glacial acetic acid 100 mL). After 15 min of continuous agitation, the gel was de-stained in a mixture of methanol acetic acid and distilled water until the protein bands were visible. Protein fractions were analyzed further using a liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) instrument.

In-gel digestion

The protein fractions that have been separated are then prepared into peptides. The preparation process utilized the in-gel digestion method outlined in Goodman et al. (2018). The 2×2 cm gel from the SDS-PAGE fraction underwent de-staining, reduction, alkylation, digestion, peptide clean-up, and elution, following the appropriate procedure.

The de-staining step was performed by adding 200 µL of de-staining solution to the gel pieces and incubating for 30 min at 37 °C, then, discarding the solution and repeating the process twice. For the reduction step, 30 µL of reduction buffer was added and incubated for 5 min at 95 °C, allowed to cool, and then alkylation buffer was added and incubated for 1 h at room temperature in the dark. The buffer was discarded and incubated with de-staining solution at 37 °C for 15 min. For gel digestion, 50 µL of acetonitrile (ACN) was added to the sample tube and incubated at room temperature for 15 min. After discarding the ACN, the sample was allowed to dry for 5-10 min and then incubated with activated trypsin as an amount of 10 µL for 15 min at room temperature. Digestion buffer was added as much as

25 µL to the sample and incubated for 16-18 h at 37 °C then transferred to a new tube and concentrated with a vacuum concentrator for 2 h.

The peptide clean-up process followed by mixing the sample with buffer (2% formic acid and 20% ACN) in a ratio of 3:1. This process utilizes a C18 spin column with 50% methanol as an activation solution to wash the resin and an equilibration solution (0.5% formic acid in 5% ACN) to eliminate flow-through (FT). After preparation, the sample was applied to the resin and placed in the receiver tube, then centrifuged at 1500×g for 1 min to collect the FT fraction. The subsequent step involved washing. The column was inserted into the receiver tube and washed with 200 µL of wash solution (0.5% FA in 5% ACN). After centrifuging the column at 1500×g for 1 min to remove the flow-through, elution was performed. The column was placed in a new receiver tube and 20 µL of elution buffer (70% ACN) was added to the top of the resin. The column was centrifuged at 1500×g for 1 min, and the FT fraction was collected. This step was repeated twice, and the FT fractions from each repetition were combined. Then the sample was dried in a vacuum concentrator. After the in-gel digestion process is complete the sample is ready to be analysed on LC-MS/MS.

Identification using Liquid Chromatography Mass Spectrometry/Mass Spectrometer (LC-MS/MS)

The prepared peptides were then analysed using Liquid Chromatography Mass Spectrometry/Mass Spectrometry (LC-MS/MS) instrument. The Liquid Chromatography instrument (type UltiMate 3000 RSLCnano, Thermofisher, USA) tandem with Quadruple Mass spectrometry (Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer, Thermofisher, USA). The column trap was 30 µm in diameter and 5 mm in length (Thermo Scientific tm 164649), and capiller column was PepMap RSLC C18, 75 um×15 cm in size, 3 µm for particle size, 100 pore size (ThermoScientific part number ES800). The flow rate was 300 nL/min with eluent was using aquadest and 0.1% format acid (A) mixed with Acetonitrile and 0.1% formic acid (B) using gradient technique. The mobile phase condition was as follows: 0-3 min (2% B), then gradually increased from 2%-35% (B) for about 3-30 min, and the mobile phase of B was increased from 35%-99% about 30-40 min and held for 40-55 min (99%). After that, the mobile phase was returned to the initial condition (2%) and held for 55-65 min (2%). Mass range detected was in range of 200-2000 m/z.

Data acquisition

The raw data from LC-MS/MS were then proceeded to obtained protein identity by matched the protein sequences to the databases using Thermo Proteome ver. 2.2 Software, using the Sequest HT as the search engine. The strict False discovery rate was set on 1%, while the relaxed False discovery rate was set on 5%. The strategy used was percolator. Mass tolerance was 10 ppm. Dynamic modification was using Acetyl/+42.011 Da (N-terminus), oxidation/ +15.995 Da (M), while the static modification was using Carbamidomethyl/ +57.021 Da (C). The acquired data from data base were in the form of protein ID based of UniProt, Coverage (%), Annotated Sequences, Protein Accessions, Peptides, Unique Peptides, Score Sequest HT: Sequest HT, and Molecular Weight (kDa). The proteins with Score Sequest HT: Sequest HT more than 0 were selected and listed. Moreover, the proteins with unique peptides more than 1 were selected to further analysis of Protein Function and Protein-Protein Interaction (PPI) network analysis.

GENE ONTOLOGY (GO) ANALYSIS AND PROTEIN-PROTEIN INTERACTIONS (PPI) NETWORK ANALYSIS

Protein functions were analysed computationally using the Gene Ontology (GO) and UniProt online database (https://www.uniprot.org/). The protein functions were divided into 3 GO terms, which were Cellular Component, Molecular function, and Biological Process. Each term has several annotation and specific function that relate to the protein ID based on publications and experiments.

PPI were analysed using STRING online (https://string-db.org/) and Cytoscape software ver. 3.10 software (Shannon et al. 2003). The selected suspected proteins based on the unique peptide numbers were analysed to construct the PPI and the strength of the node and interaction. The analysis in the STRING were set with parameters, including Physical subnetwork type, medium confidence (0.400), less than 25 interactors, and active interactive source from Text mining, Experiment, and Databases. The networks were then clustered to a specified 'MCL inflation parameter' by 3 inflation parameters. The biological process of each cluster then analysed by GO annotation. The constructed PPI network in STRING then send to Cytoscape software to further analysis, including strength of nodes, edges, and radiality.

RESULTS AND DISCUSSION

PROFILE OF PROTEINS IN THE PORCUPINE QUILLS

The total soluble proteins concentration measured in the porcupine quills in this study was 2.11 ± 1.34 mg/mL (Table 1). The proteins fractions extracted from the quills produced nine bands, consisting of seven thin bands and two thick bands (Figure 1). These two thick bands had molecular weights of about 245 kDa and 60 kDa. Further analysis using LC-MS/MS identified 19 suspected proteins from the thick bands, which were matched with databases from eight species. Among the species, *Mus spicilegus*, *Cavia porcellus*, and *Mus musculus* were the most frequently matched (Table 2). The identification of proteins was based on unique sequences detected in the samples, which were matched to known proteins sequences in databases. The identified keratin profiles included type I, type II, and unclassified keratins, all derived from other

defined species. Currently, no proteins database exists specifically for the genus *Hystrix*, meaning the proteins identified were considered suspected matches based on sequence similarities to proteins in the existing database. This profiling data provided preliminary insights into the protein composition, specifically the keratin, present in porcupine quills.

Among the 19 proteins identified in this study, there were 10 types of keratins, consisting of three type I, four type II, and three unclassified types (Table 2). The proteins with the highest sequence coverage from the porcupine quills sample were keratin type II cytoskeletal 1 (26%), annexin A2 (ANXA2) (21%), ANXA2 (17%), and keratin type I cytoskeletal 17 (16%). Moreover, only five proteins with a minimum of two unique peptides were detected (Table 3), which belonged to various species such as *Homo sapiens, Cavia porcellus, Mus spicilegus*, and *Mus musculus*. These proteins were further analyzed for their functions and protein-protein interaction (PPI) networks. Porcupine quills were a modified part of the integumentary system and were primarily composed of keratin. Keratin was classified into two types, including type I (acidic) and type II (basic) (Lee et al. 2014). Based on their biosynthesis mechanisms, keratin was further divided into soft and hard keratins. The sulfur content in soft and hard keratin was approximately 1% and 5%, respectively. Soft keratin, found in cellular tissues, such as the stratum corneum in the skin, was classified as Ib (acid-soft) and IIb (basic-soft) (Karthikeyan, Balaji & Sehgal 2007; Yu, Yu & Checkla 1993). In contrast, hard keratin, which was the main component of hair, feathers, nails, shells, claws, and horns, was divided into types Ia (acid-hard) and IIa (basic-hard) (Dhuailly et al. 1989; Heid, Moll & Franke 1988).

This study showed that porcupine quills consisted of nearly equal proportions of acidic and basic keratin. However, one type of keratin could not be classified due to a lack of corresponding data in existing databases. Compared to soft keratin, hard keratin was characterized by high cysteine content and low glycine content, providing it with superior toughness and strength (Strand et al. 2011). The study further found that only keratin type II cytoskeleton 6a (P50446) was categorized as hard keratin, while others belonged to keratins without any hard or soft groups. The peptide sequence of keratin type I cytoskeletal 1 (P04264) showed a high content of glycine (Table 3), suggesting it was likely a soft keratin.

The variation and distribution of keratin among mammals, including those found in porcupine quills, were diverse. Keratin was encoded by several genes in both terrestrial and aquatic mammals, and its variation could depend on factors such as location and protein structure (Ehrlich et al. 20199). Based on the detected peptides, keratin in porcupine quills consisted of keratins 1, 2, 5, 6a, 8, 10, 14, 17, and 84. Keratins 5 and 14 were found in the basal layer, which allowed the proliferation of the epidermis found in all mammals. In contrast, keratins 1 and 10, which formed a major component of the cytoskeleton in the outer (suprabasal) layer of the epidermis in terrestrial mammals, were absent in whales, dolphins (cetaceans), and manatees. While keratins 6 and 17 were only expressed in stress-induced epidermal thickening in terrestrial mammals, dolphins showed consistently high levels of these keratins, indicating their constitutive expression and a compensatory role for the absence of keratins 1 and 10. Keratins 2 and 9 which were expressed in a body-site-restricted manner in the suprabasal epidermis of humans and mice, were absent not only in cetaceans and manatees but also in some terrestrial mammals (Ehrlich et al. 20199). In humans, the diversity of keratin mainly showed the variety of epithelial cell types, including simple epithelium (expressing keratins 8 and 18), oral epithelium (keratins 4 and 13), corneal epithelium (keratins 3 and 12), and different layers of hair follicle epithelial cells (keratins 32 and 82 in the hair cuticle) (Bragula & Homberger 2009). In addition, the keratin found in hair wool from goats (*Capra hircus*), rabbits (*Orcytolagus cuniculus*), and sheep (*Ovis aries*) included keratins 31, 33, 34, and 83 (Thomas et al. 2012).

An uncharacterized protein, E4UQL4, from *Arthroderma gypseum* (*Microsporum gypseum*), with a molecular weight of 249 kDa, were identified. The protein matched the molecular weight of a band detected during proteins fractionation by electrophoresis. The porcupine quills samples were contaminated with the fungus before collection and processing, leading to its detection in the proteomic analysis. *Microsporum gypseum* was a soil-dwelling fungus capable of infecting the keratinized layers of mammals (Chmel & Buchvald 1970; Ginter 2012). Given its ability to colonize the keratin layer of the skin, the fungi also colonized the keratinous layers of the porcupine quills that had fallen to the ground.

GENE ONTOLOGY ANALYSIS

The five selected suspected proteins had several roles according to Gene Ontology (GO) data from the Uniprot online database (Uniprot.org). In the cellular compartment category, there were 10 annotations and 23 functions, with the cytoskeleton (GO:0005856) being the most represented function, comprising four proteins. Similarly, in the molecular function category, there were seven annotations and nine functions, with structural molecular activity (GO:0005198) having four proteins. In the biological process category, there were 16 annotations and 25 functions, with keratinization (GO:0031424), intermediate filament organization (GO:0045109), and fibrinolysis (GO:0042730) showing the highest proteins. Additionally, four proteins were associated with wound healing and related processes, including cell differentiation, signaling, extracellular matrix organization, inflammatory responses, and immune system processes (Table 4).

TABLE 1. Protein concentration

Sample replication	Protein concentration (mg/mL)
I	3.63
II	1.08
III	1.62
Average	2.11 ± 1.34

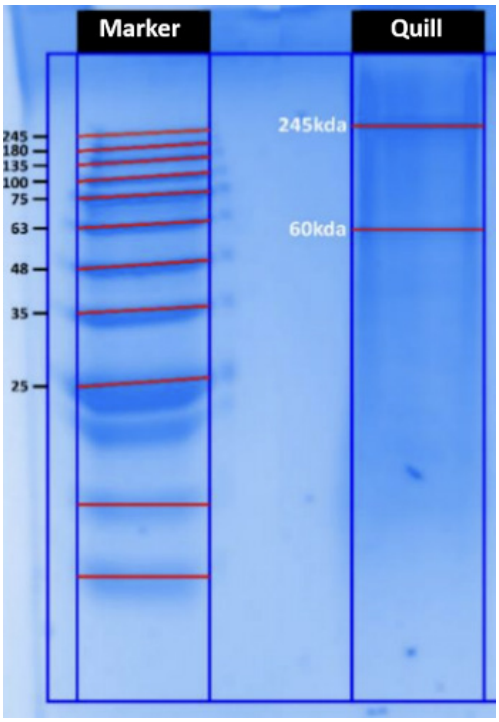

FIGURE 1. Protein fractions separation using SDS-PAGE showed 2 thick bands

Further analysis showed that proteins P04264 (KRT1 gene), H0V7P8 (ANXA2 gene), P50446 (Krt6a gene), and A0A286XKP4 (LOC100732091) mainly contributed to cytoskeleton formation, molecular functions, and anatomical structure development. Proteins P04264 (KRT1 gene), H0V7P8 (ANXA2 gene), and P50446 (Krt6a gene) also played significant roles in wound healing, inflammatory responses, and immune system processes. The specific biological processes associated with these proteins included antimicrobial humoral immune response mediated by antimicrobial peptides (GO:0061844), complement activation through the lectin pathway (GO:0001867), defense response to Gram-positive bacteria (GO:0050830), negative regulation of inflammatory response (GO:0050728), fibrinolysis (GO:0042730), collagen fibril organization (GO: 0030199), angiogenesis (GO: GO:0001525), regulation of angiogenesis (GO:0045765), cell-cell adhesion (GO:0098609), cell-matrix adhesion (GO:0007160), and wound healing (GO:0042060) (Table 5).

The ethnobiological use of porcupine quills in wound healing could be supported by the results of this study. Keratin, the primary component of the quills structure, has been shown to promote collagen deposition, fibroblast adhesion, and keratinocyte migration, which were crucial for wound healing (Yan et al. 2022). Several investigations have also shown that keratin improved hemostasis by adhering to and activating platelets as well as enhancing fibrin polymerization under both physiological and pathological conditions (Burnett et al. 2013; Sun et al. 2018; Yang et al. 2020). The multiple roles of keratin in wound healing process were in according with the results of the Gene Ontology (GO) analysis. Moreover, keratin extracts from the porcupine quills could be formulated into liposomes to effectively deliver antibiotic substances (Majeed et al. 2023). Peptides derived from the porcupine

TABLE 2. Suspected proteins identified using LC-MS/MS analysis

Protein ID	Protein name	Gen	Species	Coverage [%]	Unique peptides	MW (kDa)
P04264*	Keratin, type II cytoskeletal 1	KRT1	*Homo sapiens* (Human)	26	14	66
H0V7P8	Annexin	ANXA2	*Cavia porcellus* (Guinea pig)	21	3	38.6
A0A8C6HY62	Annexin	-	*Mus spicilegus* (Steppe mouse)	17	1	46.4
A0A8C6G6Q2*	keratin, type I cytoskeletal 17	-	*Mus spicilegus* (Steppe mouse)	16	4	48.1
A0A286XYN3	Galectin	LGALS1	*Cavia porcellus* (Guinea pig)	12	1	14.8
A0A286XKP4*	keratin, type I cytoskeletal 10	-	*Cavia porcellus* (Guinea pig)	11	4	58.7
A0A6P5QMK4*	keratin, type I cytoskeletal 10 isoform X1	Krt10	*Mus caroli* (Ryukyu mouse)	9	1	61.1
A0A286XM26*	Keratin 14	LOC100713547	*Cavia porcellus* (Guinea pig)	9	1	51.4
P50446*	Keratin, type II cytoskeletal 6A	Krt6a	*Mus musculus* (Mouse)	4	3	59.3
A0A286XV95	IF rod domain-containing protein	Krt6a	*Cavia porcellus* (Guinea pig)	4	1	59.9
H0VI24	SERPIN domain-containing protein	-	*Cavia porcellus* (Guinea pig)	3	1	42.4
F8WID5	Tropomyosin alpha-1 chain	Tpm1	*Mus musculus* (Mouse)	3	1	37.4
A0A8C6GV73*	Keratin, type II cytoskeletal 8	-	*Mus spicilegus* (Steppe mouse)	3	1	54.5
Q9QZ83	Gamma actin-like protein	Actg1	*Mus musculus* (Mouse)	3	1	43.6
B2RTP7*	Krt2 protein	Krt2	*Mus musculus* (Mouse)	2	1	70.9
A0A8C6GZQ4	Transglutaminase 3, E polypeptide	-	*Mus spicilegus* (Steppe mouse)	2	1	78.7
A0A8C6GVD8*	Keratin 84	-	*Mus spicilegus* (Steppe mouse)	2	1	65.4
A0A6P5R3S8*	keratin, type II cytoskeletal 5 isoform X1	LOC110310445	*Mus spicilegus* (Steppe mouse)	2	1	62.1
E4UQL4	Uncharacterized protein	MGYG_02256	*Arthroderma gypseum*	1	1	249.9

Asterisk symbol (*) is suspected keratins in the porcupine quill

quills with a molecular weight below 1 kDa have also been shown to induce apoptosis in mammary cancer cells (Wang et al. 2024).

PROTEIN-PROTEIN INTERACTION (PPI) NETWORK

Protein-protein interactions network of the suspected protein using STRING and Cytoscape show the biological process between proteins. Four of five suspected proteins were found to have interactions based on database of STRING, which were P04264, H0V7P8, P50446, and A0A286XKP4, while protein A0A8C6G6Q2 have no protein networks Protein P04264 and H0V7P8 play a central hub in several process of the networks.

PPI network of P04264 (KRT1) was clustered into 4 clusters with 3 main biological processes cluster according to interaction strength, which were Keratin Organization and epithelium development process, Blood Coagulation and Fibrinolysis Regulation, as well as Blood coagulation and cell adhesion process (Figure 2). In addition, protein P50446 (Krt6a) was clustered into 3 clusters with 2 main biological processes cluster, which were Keratin and Keratinocyte Organization, and Blood Coagulation process (Figure 3). Keratin proteins KRT1 and Krt6a played an essential role in wound healing process. KRT1 was included in cornification and fibrinolysis, while Krt6a participated in keratinization and epithelial cell differentiation. Suprabasal cells in the epidermis produced KRT1 along with KRT10 to support post-mitotic differentiation of stratified epithelium and cornification (Bragulla & Homberger 2009). KRT1 was not only found in the upper epidermal layer but also on the surface of endothelial cells. It regulated kinase activities, such as proteins kinase C (PKC) and SRC, which participated in fibrinolysis and contributed to complement

TABLE 3. Selected protein and the peptides sequences

Protein ID	Protein name	Unique peptides	Peptide sequences	
P04264	Keratin, type II cytoskeletal 1	14	o GGGGGGYGSGGSSYGSGGGSYGSGGGGGGGR*^	o TNAENEFVTIK*
			o GGGGGGYGSGGSSYGSGGGSYGSGGGGGGGR*^	o SKAEAESLYQSK*
			o TNAENEFVTIKK*	o SLNNQFASFIDK*
			o QISNLQQSISDAEQR*^	o SGGGFSSGSAGIINYQR*
			o LNDLEDALQQAK*	o FLEQQNQVLQTK*
			o QISNLQQSISDAEQR*^	o AQYEDIAQK*
			o WELLQQVDTSTR*	o YEELQITAGR*
			o NKLNDLEDALQQAK*	o NmQDmVEDYR*
H0V7P8	Annexin	3	o TDLEKDIISDTSGDFR	o DALNIETAIK*
			o AYTNFDAERDALNIETAIK*	o AYTNFDAER*
			o TPAQYDASELK	o GVDEVTIVNILTNR
			o TNQELQEINR	
A0A8C6G6Q2	Keratin 17	4	o EVATNSELVQSGK*	o ALEQANTELEVK*
			o TRLEQEIATYR	o ASLEGSLAETENR*
			o TKFETEQALR*	o VLDELTLAR
A0A286XKP4	keratin, type I cytoskeletal 10	4	o SQYEQLAEQNRK*	o QSLEASLAETEGR
			o SQYEQLAEQNR*	o LENEIQTYR
			o IRLENEIQTYR*	o VTMQNLNDR
			o QSVEADINGLR	o DAEAWFNEK*
P50446	Keratin, type II cytoskeletal 6A	3	o TAAENEFVTLKK*	
			o TAAENEFVTLK*	
			o YEELQVTAGR*	

Asterisk symbol (*) is a unique peptide identified as similar to the peptide sequence in the protein ID database.
Symbol (^) is identified as repeated peptide sequences

activation through the lectin pathway (Han et al. 2013). Fibrinolysis referred to the breakdown (lysis) of blood clots resulting from activation of the hemostatic pathway, whether in response to vascular trauma or pathological thrombosis (Wilson & Tait 2014). Krt6a interacted with Krt16 and Krt17 to facilitate the process of keratinization. The expression of Krt6a, Krt16, and Krt17 continued until wound closed and the skin returned to normal, serving as a defense mechanism. Krt6a was believed to regulate keratinocyte migration by modulating the cell matrix and intercellular adhesion through activation of the keratinocyte follicle (Wang et al. 2018). It was also expressed constitutively in stratified epithelial tissues and was induced by inflammatory cytokines during tissue repair and injury (Dyrlund et al. 2012). Antimicrobial peptides (AMPs/KAMPs), short peptide derivatives (13-26 amino acids) located in the carboxyl-terminal region of Krt6a, were produced by epithelial cells. KAMPs had potent antibacterial and cytoprotective effects, effectively combating various pathogenic bacteria through electrostatic interactions that disrupted bacterial membranes (Chan et al. 2018).

Protein H0V7P8 (ANXA2) was clustered into 3 clusters with 2 main biological processes cluster, including Cytoskeleton and cellular structural as well as Blood coagulation and Fibrinolysis regulation (Figure 4). On the other hand, the PPI network of A0A286XKP4 (LOC100732091) was only clustered into 1 cluster which involved in Cellular membrane transport activity. Annexin proteins (ANXA2) played a crucial role in wound healing. ANXA2 represented a class of proteins that bind to calcium, phospholipids, and membranes, characterized by unique Ca^{2+} binding sites. This molecular structure allowed ANXA2 to connect phospholipids with different membranes or link membranes to other cellular components (Grill et al. 2018). In particular, ANXA2 was important in fibrinolysis and cellular differentiation during the proliferation phase of wound healing. The

TABLE 4. Gene Ontology (GO) Annotation of suspected proteins

GO terms	Annotation	Selected protein
Cellular component	Cytoplasmic vesicle	P04264
	cytoskeleton	P04264, P50446, A0A286XKP4, A0A8C6G6Q2
	cytosol	P04264, H0V7P8
	endosome	H0V7P8
	extracellular matrix	P04264, H0V7P8
	extracellular region	P04264, H0V7P8
	extracellular space	P04264
	nucleus	P04264, H0V7P8
	plasma membrane	P04264, H0V7P8
	Organelle	P04264
	other cellular component	P04264, H0V7P8
Molecular function	cytoskeletal protein binding	H0V7P8
	Lipid binding	H0V7P8
	hydrolase activity	H0V7P8
	molecular function regulator activity	H0V7P8
	Molecular transduser activity	P04264
	Structural molecular activity	P04264, P50446, A0A286XKP4, A0A8C6G6Q2
	other molecular function	P04264, H0V7P8
Biological process	Anatomical structure development	P04264, P50446, H0V7P8
	Cell differentiation	P04264, P50446, H0V7P8*
	Cytoskeleton organization	P04264, P50446
	Defense respones to other organism	P04264, P50446*
	DNA-templated transcription,	H0V7P8
	MRNA metabolic process	H0V7P8
	extracellular matrix organization	H0V7P8*
	Immune system process	P04264, P50446*
	Inflammatory responses	P04264*
	protein catabolic process	H0V7P8
	protein-containing complex assembly	P04264
	Regulation of DNA-templated transcription	H0V7P8
	signaling	P50446, H0V7P8*
	vesicle-mediated transport	H0V7P8
	Wound healing	P04264, P50446, H0V7P8*
	other biological process	P04264, H0V7P8

Asterisk symbol (*) indicate the process that involved in wound healing

TABLE 5. GO annotation of biological process function of selected proteins

Protein ID	Protein name	GO function
P04264	Keratin, type II cytoskeletal 1	Regulation of angiogenesis (GO:0045765), fibrinolysis (GO:0042730) complement activation, lectin pathway (GO:0001867) negative regulation of inflammatory response (GO:0050728)
H0V7P8	Annexin	angiogenesis (GO: GO:0001525) collagen fibril organization (GO: 0030199), cell matrix adhesion (GO:0007160) cell-cell adhesion (GO:0098609),
P50446	Keratin, type II cytoskeletal 6A	antimicrobial humoral immune response mediated by antimicrobial peptide (GO:0061844), defense response to Gram-positive bacterium (GO:0050830), wound healing (GO:0042060)

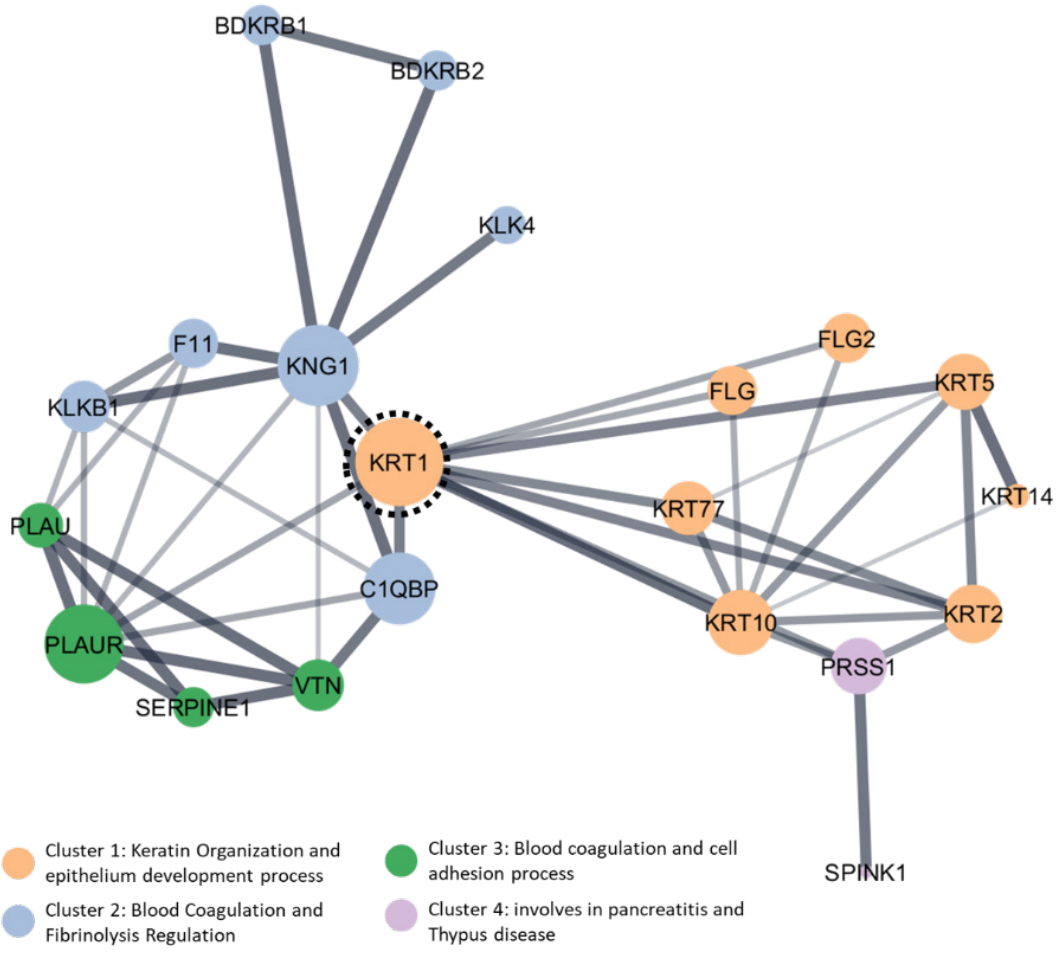

FIGURE 2. Protein-protein interactions of P04264 (Keratin, type II cytoskeletal 1). Keratin, type II cytoskeletal 1 (KRT1) play as the hub among the clusters as it related to all clusters. The size of the node and the thickness of the line between nodes, represent the strength of the nodes and interactions. The diagram and analysis were constructed in Cytoscape

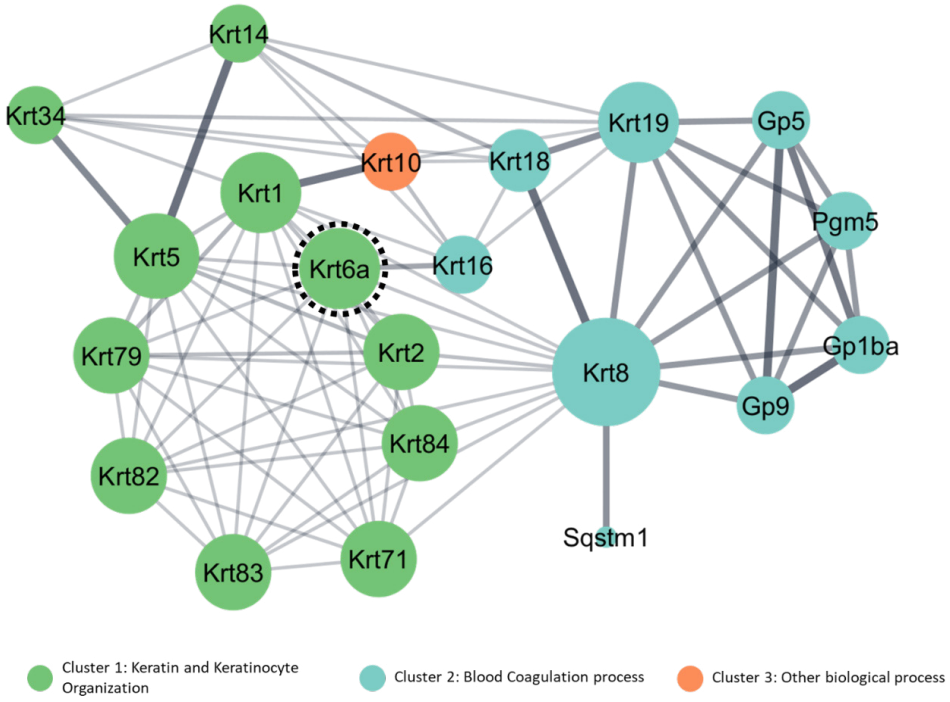

FIGURE 3. Protein-protein interactions of P50446 (Keratin, type II cytoskeletal 6A). Keratin, type II cytoskeletal 6A (Krt6a) was clustered in the Keratin and Keratinocyte organization cluster. The size of the node and the thickness of the line between nodes, represent the strength of the nodes and interactions. The diagram and analysis were constructed in Cytoscape

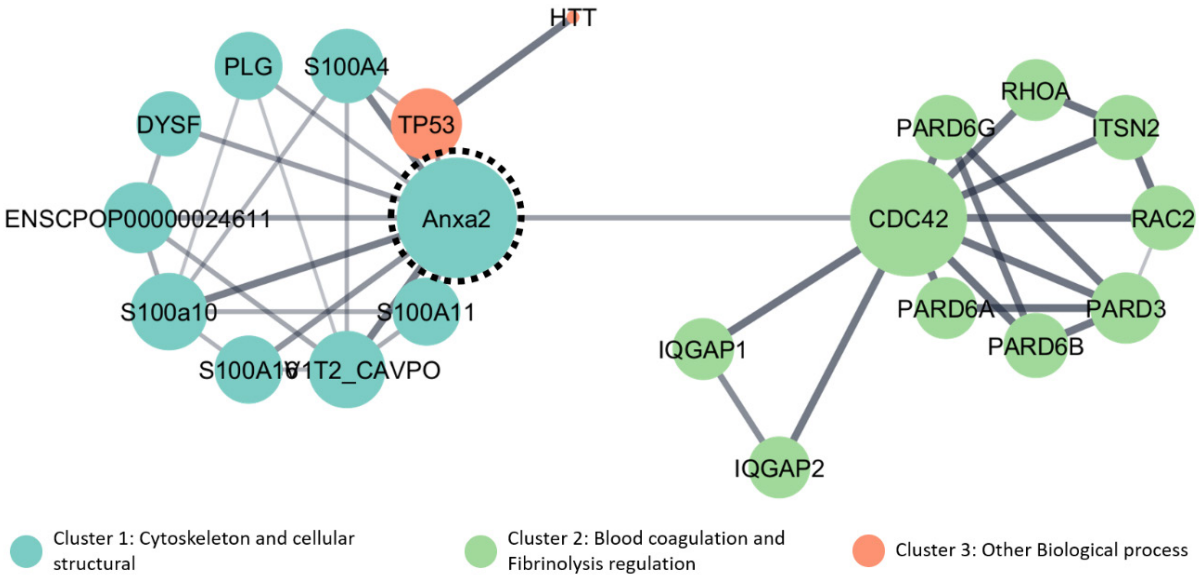

FIGURE 4. Protein-protein interactions of H0V7P8 (Annexin 2). Annexin 2 (Anxa2) clustered in the cluster 1 (Cytoskeleton and cellular structure) and related to the cluster 2 (Blood coagulation and fibrinolysis regulation). The size of the node and the thickness of the line between nodes, represent the strength of the nodes and interactions. The diagram and analysis were constructed in Cytoscape

ANXA2 heterotetramer complex bound plasminogen and tissue plasminogen activator (tPA) on the endothelial cell surface, facilitating the activation of plasmin, and the major fibrinolytic protease. The activated plasmin not only cleared fibrin but also promoted extracellular matrix proteolysis (Lim & Hajjar 2012). Furthermore, ANXA2 contributed to cell differentiation during wound healing process. This was in line with multiple reports indicating that ANXA2 expression correlated with cell proliferation, differentiation, or transformation (Gerke & Moss 2002).

CONCLUSIONS

In conclusion, the analysis results showed that the porcupine quills contained chemicals capable of promoting faster wound healing. Proteins identified in quill included keratin, annexin, and galectin. Specifically, several proteins such as keratin type II cytoskeletal 1, keratin type II cytoskeletal 6a, and ANXA2 showed a potential candidate of substances that related to improving wound healing. The results of this study supported previous observation and provided evidence for the potential substances in porcupine quill in improving wound healing.

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