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Acute Dermal Irritation Study of Philippine Stingless Bee (*Tetragonula biroi* Friese) Propolis in New Zealand White Rabbits

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Abstract

Background: Propolis, a natural flavonoid-rich substance produced by bees, is recognized and used worldwide for various therapeutic applications. Apitherapy studies on Philippine stingless bee (*Tetragonula biroi* Friese) (PSB) propolis have proven its immunomodulatory, anti-inflammatory, analgesic, and wound-healing properties. However, safety evaluation tests have yet to be conducted. Hence, an acute dermal irritation study of propolis from PSB using New Zealand white (NZW) rabbits was performed. **Methods:** PSB propolis at a concentration of 2,000 mg/kg body weight (BW) was applied on the dorsum and occluded for four hours. Gross assessment and scoring of skin test site for erythema and edema were done at 0- and 1-hour post-removal of control and test materials and then every 24 hours throughout a 14-day observation period. **Results:** There was the absence of gross and microscopic changes on the skin as well as morbidity, and mortality with propolis treatment. **Conclusions:** The results of this study indicate that the propolis from PSB is non-irritating, affirming its safe usage and application in various industries, including the development of biomedical products.

Keywords

acute dermal irritation; edema; erythema; Philippine stingless bee; propolis; New Zealand white rabbits

1. Introduction

Propolis from the Philippine stingless bees (PSB), *Tetragonula biroi* (Friese) is the resinous substance produced from the collected buds, barks, and saps of plants and trees [1]. It is lipophilic, hard but brittle in consistency. Its chemical components and color vary from yellowish-green to dark brown depending on the plant sources of the bees. To date, the PSB propolis contains more than 500 compounds including carbohydrates, steroids, alkaloids, anthraquinones, phenols, terpenoids, and other similar compounds based on GC-MS/MS analysis [2]. Utilizing a variety of plants such as mango, jackfruit, pili, chico, and caimito by the PSB lead to the production of propolis rich in flavonoids and phenolic compounds such as pinobanksin-5,7-dimethyl ether, artepillin C, luteolin-5-methyl ether, pinobanksin-3-0-

(butyrate or isobutyrate), apigenin, kaempferide, and pinocembrin-5-methyl ether [1]. In another study, the PSB that utilize plants such as avocado, jackfruit, mango, pili, and rambutan produce propolis containing flavonoids and phenolic compounds such as p-coumaric cinnamyl ester, artepillin C, and pinobanksin-3-0-hexanoate [3]. Most of these compounds present in propolis have medical application. Artepillin C has antimicrobial, antitumor, antioxidant, and immunomodulation activities [4]. Apigenin has anti-tumor, antioxidant, and anti-inflammatory activities [5]. Kaempferide has antifungal and antimicrobial properties [6-7].

In recent years, *in vitro* and *in vivo* apitherapy studies were conducted on the PSB propolis. Oral administration of ethanolic extract of PSB propolis showed tumor-suppressing activity using *in vitro* models of gastric cancer with specificity towards differentiated-type human gastric cancer cell lines [2]. Treatment with PSB propolis remarkably reduced gastric adenocarcinoma in A4gnt knockout mice [2]. Furthermore, oral treatment of propolis abrogated the neurologic motor deficits and neural damage induced by ischemic stroke in rats [8]. Inflammation of mice hind paw induced by γ-carrageenan injection was also significantly reduced with oral and topical propolis treatment through inhibition of the pro-inflammatory cytokines tumor necrosis factor α (TNF α), interleukin 1ß (IL-1ß), and IL-6 [9-10].

Findings from various wound model studies demonstrated that treatment with PSB propolis dressing enhanced healing of incisional wounds [11-12] and sutured wounds [13-14] in mice and cats. In addition, the application of the PSB propolis dressing hastened the healing of surgical wounds in cats [15]. This was attributed to the capacity of the PSB propolis dressing to reduce erythema, edema, and exudation and prevent pus formation, scab formation, and necrosis in the aforementioned types of wounds. Moreover, PSB propolis was also proven to promote hair growth in mice by stimulating the development of hair follicles (HFs) via activation of Wnt/ß-catenin signaling pathway [16]. These evidences highlight the high potential of PSB propolis as an alternative substance for biomedical product development.

The studies verify the therapeutic properties of PSB propolis. However, safety evaluation tests have yet to be conducted. Hence, an acute dermal irritation test of propolis from the PSB was performed to determine if topical application of propolis causes adverse gross and histopathological changes on the skin of New Zealand white (NZW) rabbits.

2. Materials and Methods

All procedures in rabbits were approved by the University of the Philippines Los Baños (UPLB) Institutional Animal Care and Use Committee with Protocol Approval Number CVM-2018-002.

2.1 Animals

Five (5) 3–4-month-old male NZW rabbits were procured from Tierra Del Menor Farm, Magallanes, Cavite, and individually housed in stainless steel cages measuring 50 cm X 60 cm X 50 cm, which were fitted with feeders and polycarbonate water bottle holders. They were kept under environmental conditions of 22±2°C room temperature, 30-60% relative humidity, 12h:12h light-dark cycle. Commercial maintenance pellets formulated for rabbits (Altromin®, Germany) and distilled drinking water were provided daily, *ad libitum*. Rabbits were acclimated for one week prior to experimentation.

2.2 Preparation of Propolis

Propolis was sourced from a set of colonies at the University Meliponary of the Institute of Biological Sciences, UPLB. A total of 150 g of fresh propolis chips were extracted from cerumen sheets and added to a flask containing 500 mL of 70% ethyl alcohol. The mixture was agitated for five (5) minutes every hour within a 48-hour period, covered with carbon paper, and stored in a dark area. Decantation and filtration were done after one hour, and the filtrate was placed in the rotary evaporator at 40°C to remove the alcohol. The final product was placed in a sterile glass bottle and was kept refrigerated until use. Both the control (distilled water) and propolis were coded prior to experimentation by the UPLB Bee Program Team, Institute of Biological Sciences, College of Arts and Sciences, UPLB, to prevent bias.

2.3 Acute Dermal Irritation Test

The acute dermal toxicity test followed the Organization for Economic Co-operation and Development (OECD) Guidelines on Dermal Irritation/Corrosion (OECD No. 404) [17].

One day prior to experimentation, the left and right dorsal (6 cm x 6 cm) areas of each rabbit were closely shaved using electric clippers, carefully avoiding skin abrasions, scratches, or irritations. Only rabbits with healthy, intact skin were used in the study.

After 24 hours, using a sterile 6 cm x 6 cm plastic guide, 5 mL distilled water was applied uniformly on the shaved area of either the right or left dorsal region of each rabbit. While on the opposite side, PSB propolis at a concentration of 2,000 mg/kg body weight (BW) at a total volume of 5 mL was also uniformly applied. Both distilled water and PSB propolis were applied to the respective test areas using a 5 mL sterile syringe (without needle) (Terumo®, Japan). Test areas in each rabbit were then occluded with sterile gauze (Surgitech®, China) and non-irritating medical tape (3M Micropore™, USA). After four (4) hours, the occlusive dressings were removed, and test areas were cleaned with sterile distilled water and gauze for examination and scoring for erythema and edema based on the system developed by Draize *et al.* [18] as specified in the OECD No. 404. The erythema scores are as follows: 0 = no erythema; 1 = very slight erythema (barely perceptible); 2 = well-defined erythema; 3 = moderate to severe erythema; 4 = severe erythema (beef redness) to eschar formation preventing grading of erythema. The edema scores are as follows: 0 = no edema; 1 = very slight edema (barely perceptible); 2 = slight edema (edges of the area well defined by definite raising); 3 = moderate edema (raised approximately 1 mm); 4 = severe edema (raised more than 1 mm and extending beyond the area of exposure). Erythema and edema scoring were conducted in a blinded manner, meaning the researchers were unaware of which side of the dorsum (left or right) had distilled water applied and which side had propolis. Photo documentation with erythema and edema scoring was done at 0 and 1 hour and then every 24 hours for 14 days post-removal.

2.4 Observation of Physiologic Para meters, Morbidity and Mortality

Feed and water intake were taken daily between 7 – 8 am prior to the provision of fresh feeds and water by measuring leftovers from given pre-weighed pellets (g) and drinking water (mL) using a digital top loading balance (Asuki®, China) and graduated cylinder, respectively.

The body weights (kg) of rabbits were measured using a digital top-loading balance (Asuki®, China) before the application of the control and PSB propolis at Day 0, Day 7, and Day 14. Mean bodyweight gain was computed by subtracting the initial weight at Day 0 from the final bodyweight at Day 14 and divided by 14 days.

Morbidity and mortality rates were also recorded. The morbidity rate was calculated as the total number of rabbits that showed signs of toxicity divided by the total number of treated rabbits, multiplied by 100. While the mortality rate was computed by dividing the total number of rabbits that died by the total number of treated rabbits and then multiplying the result by 100.

2.5 Skin Biopsy, Processing, and Examination

At the end of the 14-day observation period, the rabbits were euthanized through intravenous injection via the marginal ear vein of pentobarbital sodium (Dolethal®, UK) at a dose of 100 mg/kg BW. After confirmation of death, an elliptical incisional biopsy method was performed by making a 1-2 cm incision from normal tissue into the test areas through the entire skin thickness of the right and left dorsum of each rabbit. Subcutaneous attachments were severed to remove the skin sample. Skin samples were then fixed in 10% neutral buffered formalin for at least 72 hours, processed using the paraffin technique, sectioned at 5 µm in thickness, stained with hematoxylin and eosin (H&E), and examined under a research microscope (Nikon®, Japan).

Stained skin sections were examined by a veterinary pathologist blinded to the treatments, for histopathologic changes such as dermal edema, hyperemia, hemorrhage, inflammatory cell infiltration, hemorrhage, hyperkeratosis, and acanthosis. Digital photographs of skin sections from each rabbit were obtained using a digital camera attached to a research microscope (Nikon®, Japan).

2.6 Statistical Analysis

All data were presented as means ± standard deviation (SD). Differences in erythema and edema scores between treated groups were analyzed using Kruskal-Wallis Test at $P<0.05$ using IBM SPSS Statistics version 29.0.2.0 (IBM Corp., Armonk, NY, USA).

3. Results

3.1 Gross Findings

Rabbits treated with a single dose topical application of PSB propolis at 2,000 mg/kg BW did not manifest any signs of dermal irritation such as edema or erythema throughout the 14-day test period (Figure 1), thus the mean erythema and edema scores were zero (Table 1).

3.2 Physiologic Parameters, Morbidity and Mortality

Topical application of distilled water and propolis did not negatively influence the mean feed and water intake of rabbits. There was also a consistent increase in the BW of all rabbits every week, which manifested in the positive weekly body weight gain of all rabbits (Table 2).

No rabbit became ill or died throughout the 14-day observation period. Thus, the morbidity and mortality rates for all test animals in control distilled water and PSB propolis-treated rabbits were zero.

3.3 Histopathological Findings

Histopathologic examination of stained skin sections from PSB propolis treated group did not show histopathologic abnormalities and was comparable with the control distilled water group skin sections (Figure 2). The epidermis was lined with 1 to 3 layers of squamous epithelial cells with a very thin layer of keratin observed above the epidermis. The dermis also appeared normal, with the presence of dense collagen fibers, hair follicles, sebaceous glands, and arrector pili muscle, which were evenly distributed.

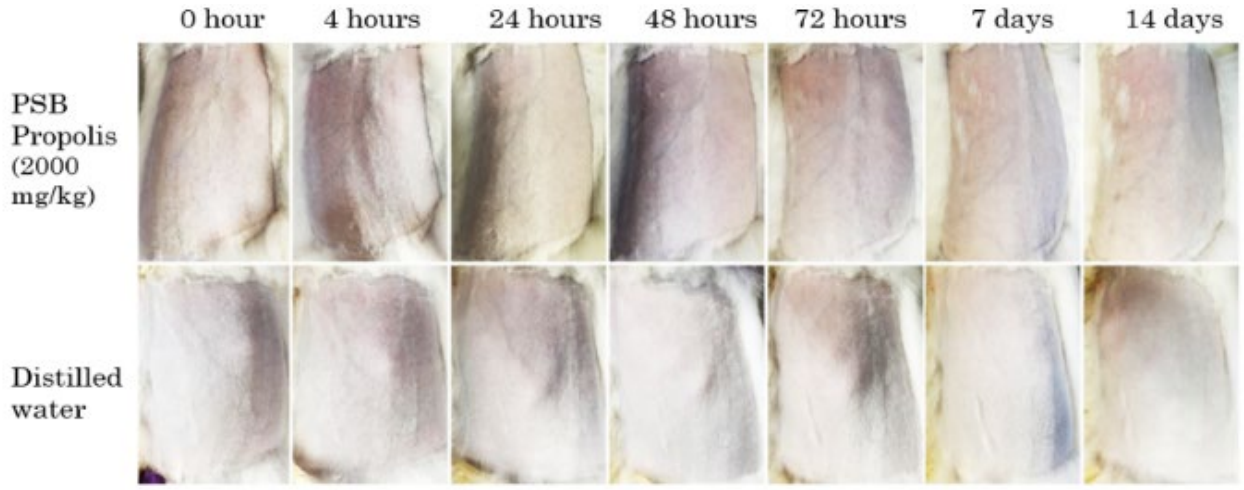

Figure 1. Skin of representative distilled water and PSB propolis-treated rabbits at specific time points showing absence of erythema and edema.

Table 1. Mean daily erythema and edema scores in distilled water and PSB propolis-treated rabbits (n=5).

Treatment	Mean daily erythema score	Mean daily edema score
Distilled water	0.00 ± 0.00[a]	0.00 ± 0.00 [a]
PSB propolis	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]

[a]Means are not significantly different using Kruskal-Wallis Test at P<0.05

4. Discussion

In this study, dermal exposure of NZW rabbits to 2,000 mg/kg BW of PSB propolis did not induce erythema, edema, or eschar formation of the skin. Furthermore, treatment-related pruritus, morbidity, and mortality were absent. The rabbits appeared healthy and maintained a good appetite, with average daily feed intake increasing from 80 g on Day 0 to 92.54 g on Day 14, which was within the average daily feed intake of 33 to 60 g/kg BW [19]. Likewise, the water consumption also increased from Day 0 to Day 14, 73.80 mL to 93.60 mL, respectively, which is well within the typical range of 50 to 150 liters/kg BW for adult rabbits [20]. The rabbits also exhibited normal body weights, falling within the 2 to 5 kg standard for sexually mature NZW rabbits [21]. A weekly average weight gain of 94 to 124 g was observed across all rabbits, indicating that acute dermal application of PSB propolis has no negative effect on rabbit normal physiology.

These findings were similarly observed by Arvouet-Grand *et al.* [22], Ledon *et al.* [23], and Kapare *et al.* [24], in their dermal irritation and

Table 2. Mean (±SD) feed and water intake and mean (±SD) weekly body weight gain of rabbits.

Physiologic Parameters	Mean ± SD
Mean daily feed intake (g)	85.39 ± 4.69
Mean daily water intake (mL)	84.48 ± 8.11
Weekly mean body weight (kg)	2.13 ± 0.11
Mean weekly body weight gain (kg)	0.11 ± 0.03

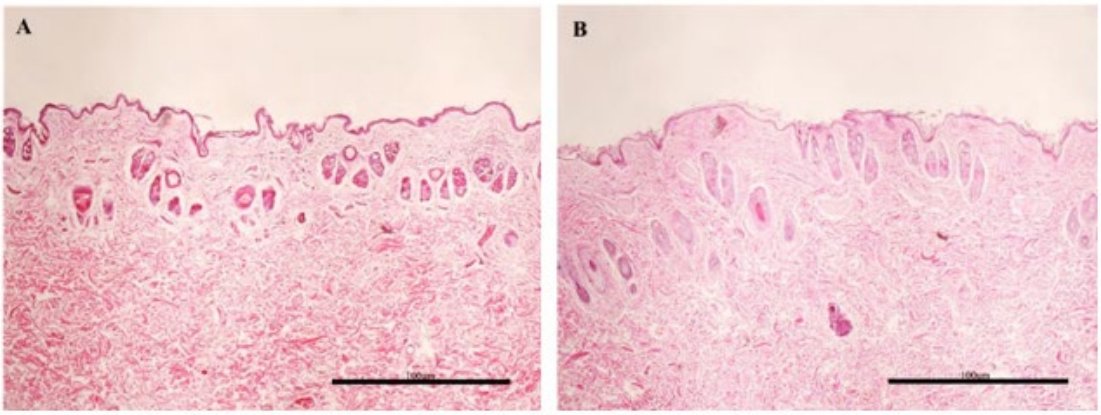

Figure 2. Stained skin sections taken at the end of the 14-day observation period from representative distilled water (A) and PSB propolis (B) treated rabbits. The skin appeared normal microscopically and comparable with the distilled water-treated control. Histopathologic abnormalities of epidermal thickening, dermal edema, hemorrhage, hyperemia, hyperkeratosis, leukocytic infiltration, and acanthosis were absent in skin sections from both control and PSB propolis-treated rabbits (40x, H&E stain).

toxicity tests of European, Cuban red and powdered South Asian propolis, respectively. Likewise, El Kersh *et al*. [25] determined that Indian propolis ethanolic extract was non-toxic at a dose of 2000 mg/kg BW. In contrast to the results obtained from the present study, in the experiment of Balderas-Cordero *et al*. [26], mice treated with 50% w/v Mexican propolis showed signs of mild irritation evidenced by erythema and edema four days post-treatment and excoriation and ulceration eight days post-treatment. These findings suggest variation in the irritating properties of propolis depending on the country of origin and that PSB propolis remarkably show non-irritating properties.

Histopathologic assessment of PSB propolis-treated skin in the present study resembled those of their control counterpart and exhibited normal epidermal and dermal appearance. There were no documented skin microscopic abnormalities like inflammatory cell infiltration, thickness irregularities, edema, hyperemia, hemorrhages, hyperkeratosis, or acanthosis. Microscopic examination of the skin revealed the absence of cellular and structural damage with PSB propolis treatment, further attesting to its non-irritating nature. In contrast to this study's histopathology findings, Basista-Sołtys *et al.* [27] reported neutrophil and monocyte infiltration in the epidermis and numerous mast cells in the dermis after 24 and 72 hours of occlusion with 5% Polish propolis patch, which indicates inflammatory reaction. Balderas-Cordero *et al*. [26] found abundant inflammatory infiltrate, decreased skin thickness, and loss of the stratum corneum in the histological analysis of their samples.

Propolis samples from India, USA, Saudi Arabia, Turkey, and Egypt, although there were no dermal irritation or dermal toxicity test results reported, were found to be effective in treating excisional and burn wounds in dogs and mice [28-31]. Mexican propolis demonstrated anti-inflammatory and wound-healing activity [26]. On the other hand, PSB propolis is non-irritating, non-toxic, and efficacious when applied as a wound dressing in treating incisional, sutured, and surgical wounds in mice and cats [11-15]. From the gathered data, most sources of propolis reassure its efficacy on different types of wounds. However, taking into consideration the variety of compounds present in propolis, together with the aforementioned studies, these imply that dermal irritation and toxicity results vary based on the geographical source of the propolis. This may be attributed to the fact that the precise composition of propolis is influenced by factors such as the plant source, season of harvesting, geographical location, local bee flora, climate variations, and the bee species at the collection site [32].

Moreover, results from this study found that PSB propolis is non-irritating, thereby reinforcing its dermal safety as a biomedical product.

5. Conclusion

In conclusion, PSB propolis is dermally non-irritating at ≤2000 mg/kg BW. Together with previously conducted apitherapy findings PSB propolis is an excellent alternative treatment option and can be utilized for the development of biomedical products. Conduct of subchronic and chronic dermal irritation and skin sensitization tests are recommended.

Availability of Data and Materials

Readers can access all datasets that support conclusions.

Author Contributions

Conceptualization, M.A.C.E.; Methodology and Investigation, M.A.C.E., T.M.A.C., T.V.M., M.M.R.R., R.P.G., J.V.B., C.R.C., M.J.M.D., and C.P.F.C; Writing – Original Draft, M.M.R.R., T.V.M., M.A.C.E., T.M.A.C.; Writing – Review and Editing, M.A.C.E., T.V.M. T., M.A.C., and M.M.R.R.; Funding Acquisition, none; Resources, M.A.C.E., T.M.A.C.; Supervision, M.A.C.E., and T.M.A.C.

Ethics Approval and Consent to Participate

All the pertinent international, national, and/or institutional guidelines for the care and use of animals were adhered to during the conduct of this study. All animal experimental procedures were

approved by the Institutional Animal Care and Use Committee of the University of the Philippines Los Baños.

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Conflict of Interest

The authors declare no conflict of interest.

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