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Original Article

Abrogation of Acute Inflammation in ICR Mice with Topical Administration of Philippine Stingless Bee (*Tetragonula biroi* Friese) Honey

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ABSTRACT

Background: Honey, a sweet sugar-rich bee-derived product, has been widely recognized in the realm of Ayurveda and Pharmacognosy for the treatment of various inflammatory conditions including inflammatory bowel disease, asthma, arthritis, and gastric ulcer. However, the anti-inflammatory effect of Philippine honey obtained from local stingless bees remains unexplored and poorly investigated. Hence, this study delved on the anti-inflammatory potential of topically applied Philippine stingless bee honey (PSH) using the λ-Carrageenan-induced mouse model of paw edema. **Materials and Methods:** A total of 30, male ICR mice of six weeks of age were randomly divided into the following groups (n=10 per group) as follows: Distilled water-, 1% Diclofenac sodium, and Honey-treated group. All treatments were administered immediately after Carrageenan injection and then every 8 hr during the 24-hr test duration. **Results:** Repeated topical application of PSH significantly attenuated the gross hind paw swelling observed at the onset of the 6 hr post-induction (*pi*) until the 24 hr *pi* with Carrageenan in contrast to the Distilled water-treated group. This corresponded histologically to a marked reduction in dermal thickness, dermal edema, and leukocytic infiltration. Whereas, at the cytokine level, this correlated to significant suppression of the circulating blood levels of pro-inflammatory cytokines, TNF-α, IL-1β, and IL-6. **Conclusion:** Based on these results, honey from the Philippine stingless bees shows convincing anti-inflammatory activity which is partly mediated through the regulation of the expression of inflammation-associated cytokines.

Keywords: Anti-inflammatory, Honey, IL-1β, IL-6, Philippine stingless bees, TNF-α.

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INTRODUCTION

Honey is a natural, pale yellow to dark amber, sweet viscid material produced by bees that have a long history of use in ayurvedic medicine for the treatment of bronchial asthma, diabetes, cough, tuberculosis, worm infestation, leprosy, eczema, throat infections, and ulcers.[1,2] It contains a complex mixture of chemical compounds such as water, carbohydrates, proteins, organic acids, enzymes, vitamins, minerals, aromatic substances, and bioactive molecules like flavonoids and phenolics,[3,4] which varies widely across geographical locations owing to differences in bee species, botanical sources, and climatic condition.[5] These chemical components especially the bioactive constituents are mainly responsible for the multitude of pharmacologic properties of honey including antibacterial,[6] anticancer,[7,8] antioxidant,[9,10] wound healing,[11,12] and anti-inflammatory.[13-15]

Accumulating evidence in the literature has accounted for the potent anti-inflammatory activity of honey obtained from different regions of the world. For instance, Brazilian honey and Malaysian Tualang honey exert significant anti-inflammatory activity by reducing edema formation, inhibiting leukocyte migration, and decreasing the production of reactive oxygen species (ROS) and cyclooxygenase-2 activity (COX-2).[1,16] On the other hand, Malaysian Gelam honey controls inflammation through tight regulation of the nuclear factor kappa B (NF-κB) pathway including its downstream targets like tumor necrosis

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factor-α (TNF-α), prostaglandin E_2 (PGE_2), interleukin 1β (IL-1β), interleukin 6 (IL-6) and nitric oxide (NO).[17-19] Meanwhile, Manuka and jelly bush honey from New Zealand and Australia respectively display potent anti-inflammatory properties by impeding the activation of COX-2 as well as inducible nitric oxide synthase (iNOS), and decreasing the synthesis of prostaglandins and inflammatory cytokines.[2,19-21]

In the Philippines, honey is primarily produced by the indigenous population of stingless bees (*Tetragonula biroi* Friese) which are locally known as "Kiwot". This particular honey has been reported to possess a unique composition of nutrients and chemical constituents including reducing sugars, hydroxymethylfurfural, and polyphenols such as quercetin, luteolin-5-methyl-ether, pinobanksin-5-7-dimethyl ether, methoxy pinobanksin, kaempferide, pinobanksin-3-0 (butyrate or isobutyrate) and 3-(2-2-dimethyl-3,4-dihydro-8-prenyl-1-ben zopyran-6-yl)-2-propenoic acid.[22-25] However, unlike other known honey, the documented biological activity of the PSH has been restricted to a study on its potential neuropreservation property in an ischemic stroke rat model.[26] Therefore, the present study explored the *in vivo* anti-inflammatory activity of topically applied PSH using carrageenan-induced hind paw edema mice model to further enhance the knowledge of its bio functional properties and to provide a basis for its development as a viable anti-inflammatory agent.

MATERIALS AND METHODS

Collection and preparation of PSH

The samples of PSH were harvested from the Meliponary of the UPLB Bee Program using the cut and drip method described previously.[26] Honey pots were chopped, sieved, filtered using a muslin cloth, and pasteurized at 45°C for 15 min before sealing. To ensure the production of standardized samples, honey pots obtained only in the supper but not around the brood area were sourced from the same set of colonies of the Philippine stingless bees which did not receive any sugar, pollen, or supplements of any kind. The authenticity of collected honey samples was confirmed by comparing the results of the physicochemical analysis to the normal range established by the UPLB Bee Program and to that of the Codex or Harmonized Methods for the European Honey Commission.

Animals

A total of 30, male, six-weeks-old, Institute of Cancer Research (ICR) mice, which were purchased from the Department of Health- Research Institute for Tropical Medicine in Alabang Muntinlupa City, Philippines, were assigned in a random fashion into the following groups: Distilled water (Group 1; n=10), 1% Diclofenac sodium (10mg/1ml gel) (Group 2; n=10), and PSH group (100mg/kg BW) (Group 3; n=10). They were kept in individual cages made from standard polycarbonate material and housed at the Department of Basic Veterinary Sciences- Laboratory Animal Room, College of Veterinary Medicine, University of the Philippines-Los Baños. All mice were given commercial rodent diets (Altromin, Germany) and *ad libitum* supply of distilled water, and maintained under standard husbandry conditions as follows: 22 ± 4°C temperature, 30-50% relative humidity, and 12 hr light: dark period. Before the actual start of experimentation, a one-week acclimation period was observed.

All procedures in mice strictly adhered to both international and local regulations on laboratory animal husbandry and care and secured the approval of the UPLB Institutional Animal Care and Use Committee with approval number, UPLB-2021-049.

λ-Carrageenan-induced hind paw edema

Following the method of Winter *et al.* (1962) the phlogistic agent-λ-carrageenan, was used to induce hind paw inflammation.[27] Each mouse per treatment group was injected into the dorsal right hind paw region with 1% λ-carrageenan (0.1 mL). Immediately after administration, 0.1 mL distilled water, 1% diclofenac sodium gel (Voltaren® Emulgel®, Novartis Consumer Health, Inc., Philippines), and 0.1 mL freshly prepared honey were applied topically, and then every 8 hr during the 24-hr test duration. The extent of the paw inflammation was scored at 0 hr (prior to Carrageenan injection), 15 and 30 min, and then at 1, 3, 6, 12, and 24 hr post-injection (*pi*) using Jeengar *et al.*'s (2014) scoring system as follows: 0 for no evident paw swelling, 1 for only one toe evidently swollen, 2 for more than one toe but not the entire paw evidently swollen, 3 for the entire paw evidently swollen, and 4 for the entire paw severely swollen.[28] The mean paw edema score for each of the defined time points was computed using the following formula: sum paw score of mice per treatment group/ total number of mice per treatment group. The gross effects of each intervention was assessed by two independent researchers who were not involved in the actual experiment and completely unaware of the assigned treatment.

Blood Collection

Twenty-four (24) hr *pi* with λ-carrageenan, mice were injected intraperitoneally with pentobarbital sodium (Dolethal®, UK; 30 mg/kg BW) to induce anesthesia. After this, one ml of blood was collected via cardiac puncture using a 1mL syringe with a gauge 25 needle, placed into 1.5 mL microcentrifuge tubes (Eppendorf, USA) and centrifuged at 3,000 rpm for five minutes. The plasma was then recovered and stored at -20°C until further use for cytokine analysis.

Euthanasia and histopathology

Immediately after intracardiac blood collection, mice were humanely sacrificed using the cervical dislocation method. The right hind paw was collected by excision using a surgical scissor and quickly soaked in a container with 10% formaldehyde for 72 hr to allow efficient fixation. This was followed by overnight

decalcification using hydrochloric acid/formic acid solution, and then followed by embedding in paraffin and cutting into thin sections (5 μm) before final staining using hematoxylin and eosin (H&E).

Microscopic assessment of the hind paw inflammation was carried out following the modified procedures outlined by Jansen and Havemen (1990),[29] and Hussein *et al.* (2013).[30] Image J/Fiji software was used to accurately determine the thickness of the dermal region of the hind paw.[31] The magnitude of infiltrating lymphocytic cells was scored as 0 for no lymphocytic infiltration, 1 for mild grade, 2 for mild to moderate grade, 3 for moderate grade, 4 for moderate to severe grade, and 5 for severe grade. On the other hand, edema was assessed using the succeeding scoring system: 0 for absence of edema, 1 for minimal grade, 2 for moderate grade, and 3 for extensive grade of edema. Meanwhile, endothelial activation was scored as 0 for absence of vascular changes and 1 for presence of activated endothelial cells. The average scores per treatment group for these aforementioned parameters were obtained. A Veterinary Pathologist who was not involved in the actual experiment and was completely unaware of the assigned treatments independently carried out the microscopic assessment to preclude bias and uphold the integrity of the output.

Measurement of blood plasma TNF-α, IL-1β, and IL-6 cytokine levels

For cytokine assay, five blood plasma samples per treatment group along with an additional five plasma samples from non-Carrageenan-injected mice serving as negative control were obtained to measure the protein levels of TNF-α, IL-1β, and IL-6 cytokines (ABCAM, USA). Briefly, a polyclonal antibody-coated 96-microplate was rinsed for 10 sec with wash buffer, followed by aspiration and repeat rinsing step. After the final wash step, the excess wash buffer was removed by gently tapping the microplate unto a clean, absorbent paper towel. A standard curve was generated using 100μL of mouse TNF-α, IL-1β, and IL-6 at concentrations of 2500, 1250, 625, 312.5, 156.3, 78.1, and 39.1 pg/mL. Blood samples used were diluted with 50μL sample diluents at a 1:1 ratio before adding to the wells. After this, biotin-conjugate was added at 50μL to both standard and sample wells and the plate was sealed using an adhesive film, placed onto a shaker and set aside for 2 hr at room temperature (RT) and then underwent four washing step before addition of 100 μL per well streptavidin horse radish peroxidase. The plate was placed again on a shaker, incubated for 1 hr at RT and subjected to four changes of wash buffer before addition of 100 μL per well TMB substrate solution and RT incubation for 10 min protected from light. To stop the reaction, a 100μL of stop solution was added into each well. The absorbance reading (450 nm) was measured using a microplate reader.

Statistical analysis

One-way Analysis of Variance (ANOVA) followed by Tukey HSD posttest was employed to ascertain the statistical significance ($p<0.05$) among the treatment groups. The statistical software, SPSS v.25 (IBM Corp., Armonk, NY, USA), was used to analyze the generated data.

RESULTS

Effect of PSH on the gross morphology and hind paw edema scores

At the onset of the 15-min *pi* mark, subcutaneous injection λ-carrageenan, strongly induced an acute inflammation in all treated mice as evidenced by the profound swelling of the entire right hind paw (Figure 1A). In Distilled water-treated group, a heightened extent of this gross inflammatory response was invariably observed throughout the temporal course of the study. Contrarily, repeated topical application of both PSH and Diclofenac sodium considerably attenuated the severity of the hind paw swelling produced which became visible starting at one (1) hr *pi* period, although statistically significant reductions were only achieved from six (6) hr up to 24 *pi* period. This was confirmed by the result of the hind paw edema scoring system employed wherein a mean score of 2.70 ± 0.82 and 2.60 ± 0.67 were obtained at 6 hr *pi* whereas a mean score of 2.50 ± 0.85 and 2.50 ± 0.97 was obtained at 24 hr *pi* in the PSH-treated group and Diclofenac sodium-treated group, respectively, as opposed to their Distilled water-treated counterpart (VS. 4.00±0.82 and 3.90±0.32) (Figure 1B).

Effect of PSH on microscopic parameters

Contrary to the Distilled water-treated control, PSH significantly restrained the λ-carrageenan-induced expansion of the dermis showcasing a 47% reduction in the dermal thickness. Moreover, PSH treatment markedly alleviated the formation of dermal edema by 58% (Figure 2B) and impeded the influx of inflammatory leukocytes by about 31% (Figure 2C). Meanwhile, Diclofenac sodium closely mirrored those of the PSH-treated group as semi-quantitative assessment revealed significant improvement in the dermal thickness, dermal edema, and leukocytic infiltration by 39%, 67%, and 52%, respectively, as contrasted to the group treated with Distilled water (Figure 1A-C). No notable difference, however, was detected across all treatment groups with respect to changes in vascular permeability.

Effect of PSH on the protein expression of inflammatory cytokines

Administration of λ-carrageenan substantially altered the inflammatory cytokine expression instigating a 10-fold increment in the circulating blood levels of TNF-α, IL-1β, and IL-6. As shown in Figure 3, topical treatment with PSH effectively negated this pronounced elevation resulting in approximately 37% reduction

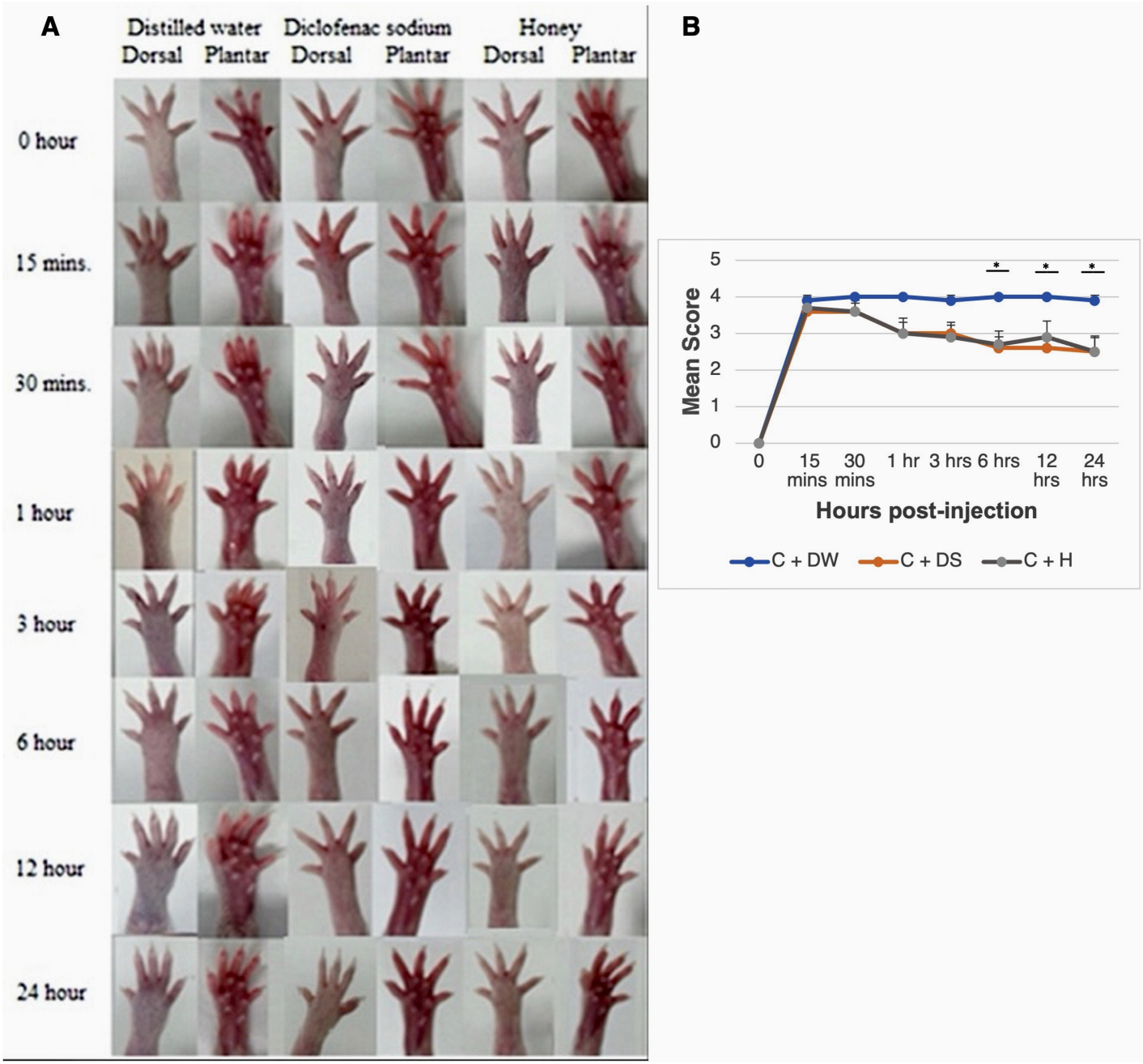

Figure 1: Gross appearance of the right hind paw of representative mice treated with distilled water, diclofenac sodium and honey at 0, 15 min, 30 min, 1, 3, 6, 12, and 24 hr post-injection of λ-carrageenan.

Mean hindpaw edema scores of various treatment groups at different time points.* Statistically different at $p < 0.05$ using One-way ANOVA and Tukey HSD *post hoc* test. C + DW – Carrageenan + Distilled water, C + DS – Carrageenan + Diclofenanc sodium, C + H – Carrageenan + Honey.

in the plasma concentration of TNF-α, 49% for IL-1β, and 57% for IL-6 cytokine relative to the control group. Interestingly, a statistically comparable result with the PSH-treated group was elicited following topical treatment with Diclofenac sodium which promoted a corresponding decrease in the expression levels of these aforementioned cytokines by around 39%, 57%, and 60%.

DISCUSSION

Though steroids and non-steroidal anti-inflammatory drugs (NSAIDs) are still the mainstays of managing inflammatory diseases and disorders, their collateral adverse effects along with the rising medication costs have driven global efforts to seek new alternatives, hence, invigorating the traditional medicine and

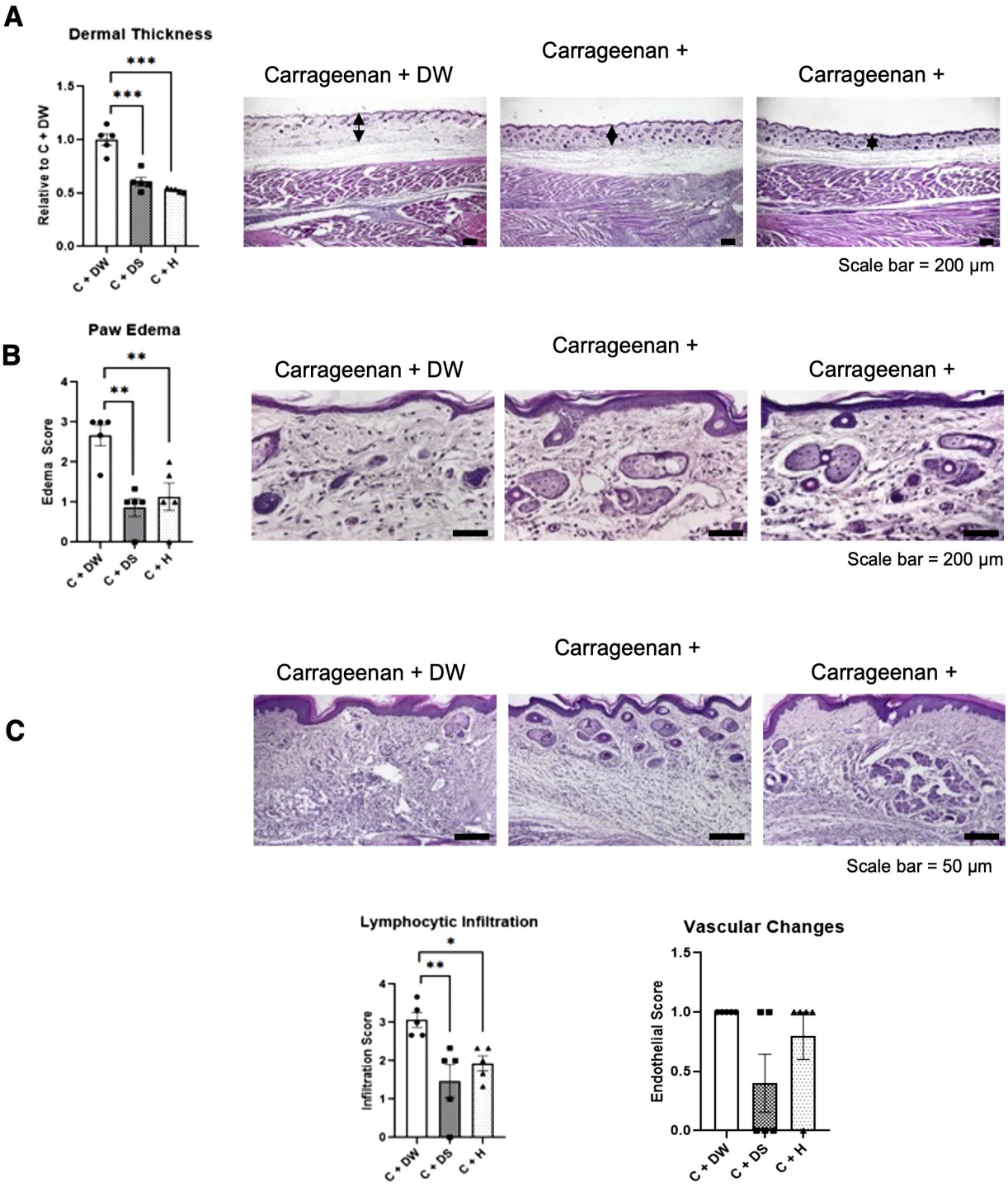

Figure 2: (A). Mean scores of different treatment groups with respect to various microscopic parameters such as (A) dermal thickness, (B) dermal edema, (C) lymphocytic infiltration and vascular changes. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ using One-way ANOVA and Tukey HSD *post hoc* test. C + DW - Carrageenan + Distilled water, C + DS – Carrageenan + Diclofenac sodium, C + H – Carrageenan + Honey.

natural product research.[32] Honey is a natural product derived from bees that has been extensively investigated for its promising anti-inflammatory activity. For decades, numerous studies have documented the anti-inflammatory efficacy of honey obtained from various geographical origins against a wide spectrum of inflammatory conditions, for instance, inflammatory bowel disease (IBD),[13,15,33] corneal injury,[34] airway inflammation,[35,36] gastric ulcer,[37] sepsis,[17] and arthritis.[38] However, no single study in the literature has been published to date which accounts for this biological property of honey taken from the stingless bee population in the Philippines. Therefore, we herein investigated the effect of the topically-applied PSH on the λ-carrageenan-induced

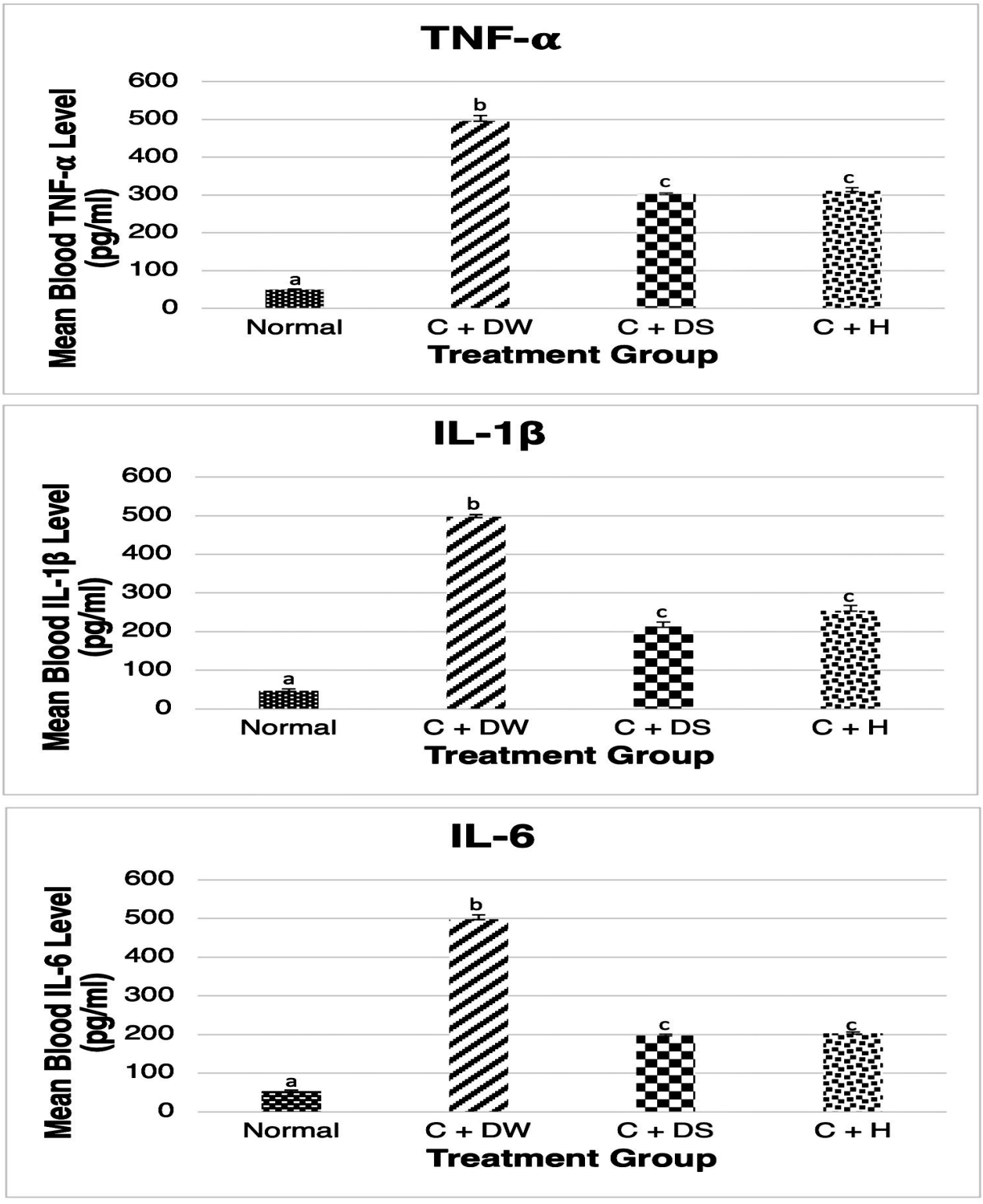

Figure 3: Mean blood TNF-α, IL-1β, and IL-6 levels of mice belonging to different treatment groups at 24 hr following injection of λ-carrageenan. Means with different letters are significantly different at $p < 0.05$ using One-way ANOVA and Tukey HSD *post hoc* test.

mouse model of hind paw edema as a first attempt to describe its potential anti-inflammatory efficacy.

Carrageenan-induced paw edema is a highly reproducible experimental model that has been routinely used to test new drugs, compounds, or substances with potential anti-inflammatory activity.[27,39] Injection of λ-carrageenan invokes an accentuated release of inflammatory mediators which eventually leads to the observable development of the classical hallmarks of acute inflammation represented by edema, hyperalgesia, and erythema.

[40,41] In the present study, these gross signs of acute inflammatory response were successfully induced in all experimental mice as early as 15 min *pi* period. After the 1-hr *pi* period had lapsed, the hind paw swelling in both Diclofenac sodium-treated group and the PSH-treated group was noticeably relieved although statistically significant alleviation was only accounted for from 6 hr *pi* up until the 24 *pi* period compared to the Distilled water-treated group. Consistent with our findings, Sahara honey, Gelam honey, Indian honey, and Yemeni Sidr honey have been previously reported to cause significant attenuation of the inflamed paw tissues of mice following carrageenan injection although with an earlier onset of around 2-4 hr *pi*.[30,42-44] This may be attributed to the relatively higher dosage of honey (500 mg-2000 mg/kg BW vs 100 mg/kg BW) and the longer duration of treatment utilized in these studies as opposed to the present work. Nonetheless, our results indicate that PSH similarly possesses a promising anti-inflammatory activity.

Microscopically, λ-carrageenan mediates a series of local inflammatory events which is characterized by the substantial accumulation of dermal edema, leukocytic infiltrates, and vascular perturbations resulting in an increased thickness of the dermal layer.[45-47] In concordance with our gross observation, repeated topical application of PSH significantly suppressed the ensuing inflammatory reaction as evidenced by the remarkable inhibition of dermal edema formation and leukocyte leakage into the site of inflammation. Consequently, the dermal thickness in the PSH-treated group was commensurately reduced as compared to the distilled water-treated group. However, PSH treatment did not appear to influence the dermal blood vessels since no prominent changes in terms of vascular dilation, vessel wall thickness, and perivascular leukocyte influx were noted. Interestingly, topical treatment of mice with the standard NSAID drug, Diclofenac sodium, significantly recapitulated the anti-inflammatory action of PSH as depicted by the marked reduction of dermal thickness, dermal edema, and leukocyte infiltration, and the absence of vascular changes. Collectively, these lines of evidence reinforce the anti-inflammatory potential of PSH.

λ-carrageenan promotes a biphasic edematogenic response. The first or early phase occurring during the first 6 hr stimulates a low-intensity inflammation due to the release of various inflammatory mediators such as bradykinin, histamine, serotonin, and 5-hydroxytryptamine. On the other hand, the second or delayed phase occurring at the 12-24 hr elicits a more exaggerated inflammatory reaction due to the release of enzymes like COX-2, and iNOS as well as inflammation-associated molecules such as prostaglandins and leukotrienes.[48-51] Moreover, the enhanced degree of inflammation in this second phase is sustained through the synthesis of several pro-inflammatory type of cytokines- the most common of which are TNF-α, IL-1β, and IL-6.[52,53]

TNF-α is a pleiotropic cytokine that plays a critical function in inflammation, proliferation, differentiation, and apoptosis. In Carrageenan paw edema, it principally acts by stimulating leukocyte migration, inducing prostaglandin and NO biosynthesis, and activating the NF-κB signaling pathway that in turn amplifies the transcription of other cytokines including IL-1β, and IL-6.[54-57] IL-1β is a key pro-inflammatory cytokine that is typically liberated in response to injurious, infectious, toxic, or antigenic insults. Like TNF-α, it exacerbates carrageenan-mediated acute injury by increasing leukocyte adhesion and stimulating the production of PGIs.[58-60] Meanwhile, IL-6 is a multifaceted cytokine that is involved in T and B cell proliferation and is responsible for initiating the acute phase proteins following Carrageenan administration.[61,62] Herein, the circulating blood levels of these cytokines were sharply increased by Carrageenan evoking an approximately 10-fold increment with respect to the normal control values which essentially conforms with the data generated earlier by Lopes *et al.* (2020).[60] Noteworthily, topical application of PSH strongly hampered this profound elevation allowing a more or less half-maximal reduction in the measured protein expression levels of these cytokines. In agreement with our findings, Hussein *et al.* (2013) showed that Malaysian monofloral Gelam honey significantly abrogated the plasma expression levels of both TNF-α and IL-6 cytokines in Carrageenan-injected Sprague-Dawley rats.[30] Additionally, the repressive ability of PSH towards these inflammation-associated cytokines may be extrapolated from several *in vitro* and *in vivo* studies using acute inflammatory models. For instance, Manuka honey significantly inhibited the LPS-triggered increase in the plasma cytokine levels of TNF-α, 1β, and IL-6 in treated RAW 264.7 macrophages[63] and exerted a pronounced gastroprotective activity against an ethanol-induced gastric ulcer in Albino rats by decreasing the circulating levels of all these three cytokines.[37] Taken together, these data suggest that the potent anti-inflammatory action of PSH in Carrageenan-induced paw edema is partly mediated through negative regulation of the proinflammatory cytokine production and closely imitates the action of the commercial drug, Diclofenac sodium.

CONCLUSION

The present work unveiled the first report of the potent anti-inflammatory activity of topical PSH, and one of the putative mechanisms involved in expending this biological activity is the specific inhibitory control of the expression of inflammation-associated cytokines. It would be interesting to elucidate in the future whether Philippine PSH can also affect other known mediators of inflammation.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

COX: Cyclooxygenase; **iNOS:** Inducible nitric oxide synthase; **IBD:** Inflammatory bowel disease; **ICR:** Institute of Cancer Research; **IL-1β:** Interleukin-1 beta; **IL-6:** Interleukin-6; **NO:** Nitric oxide; **NF-κB:** Nuclear Factor kappa B; **NSAID:** Non-steroidal anti-inflammatory drugs; **PGE_2:** Prostaglandin E_2; **PGI:** Prostaglandin; **I_2:** Post-injection; **ROS:** Reactive oxygen species; **TNF-α:** Tumor necrosis factor-alpha.

SUMMARY

The present work explored whether repeated topical application of Philippine honey obtained from the indigenous population of stingless bee exemplifies a satisfactory anti-inflammatory efficacy using a mouse model of Carrageenan-induced hind paw edema. The paw samples were subjected to morphological examination to measure the effect on gross and microscopic parameters, as well as cytokine assay to measure the protein expression levels of inflammatory cytokines. Treatment with Philippine stingless bee honey evidently abrogated the gross manifestation of paw edema especially during the 24 hr post-induction period. This was reflected at the histological level by the significant reduction of dermal edema and leukocytic infiltrates and at the cytokine level by the marked inhibition of TNF-α, IL-1β, and IL-6 expression. These results prove that Philippine stingless bee honey also possesses a promising anti-inflammatory activity.

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