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Attenuation of carrageenan-induced hind paw edema and plasma TNF-α level by Philippine stingless bee (*Tetragonula biroi* Friese) propolis

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Abstract: Despite decades-long existence of the Philippine stingless bee industry, the biological activity of propolis from this native bee species (*Tetragonula biroi* Friese) remains poorly understood and sparingly investigated. Herein, we examined the potential anti-inflammatory efficacy of Philippine stingless bee propolis using the lambda (*λ*)-carrageenan-induced mice model of hind paw edema. Thirty (30), six-week-old, male ICR mice were randomly assigned into three treatment groups (n=10/group) as follows: distilled water group, diclofenac sodium group (10 mg/kg), and propolis group (100 mg/kg). All treatment were administered an hour prior to the injection of the phlogistic agent. As observed at 3 h post-injection, *λ*-carrageenan remarkably evoked the classical signs of hind paw edema exemplified grossly by swelling and hyperemia. The ameliorative effect of propolis became apparent at the onset of 6 h post-injection with a statistically significant finding evident at the 24-h period. This gross attenuation histologically correlated to a considerable and specific reduction of the dermal edema, which mirrored those of the diclofenac sodium group. Furthermore, both propolis and diclofenac sodium significantly attenuated the *λ*-carrageenan-induced increase in the protein expression levels of the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) depicting more than two-fold decrement relative to the distilled water group. Altogether, these suggest that Philippine stingless bee propolis also exhibited a promising *in vivo* anti-inflammatory property, which can be partly mediated through the inhibition of TNF-α.
Key words: hind paw edema, inflammation, Philippine stingless bees, propolis, tumor necrosis factor-α (TNF-α)

Introduction

Propolis, sometimes referred to as bee glue, is a natural resinous substance that maintains the hive's structure [1–3]. It is commonly used to coat the inner walls, polish the honeycomb cells, protect the hive from foreign substances, and maintain the concentration of bacteria and fungi at a minimum level [4, 5]. At least 300 compounds have already been identified in different propolis samples and this includes various chemical components like esters, polyphenols, terpenoids, essential oils, vitamins, and cinnamic acid derivatives [6–8]. Propolis is also widely known to possess various therapeutic properties such as antibacterial [9, 10], antioxidant [11, 12], antiviral [13], fungicidal [14, 15], anticancer [16], antihepatotoxic [17], and anti-inflammatory [18–22].

A number of studies have shown that propolis can prevent inflammation by interfering with the production of (1) inflammatory cytokines such as IL-1β, IL-2, IL-5, IL-6, and tumor necrosis factor-α (TNF-α) [18, 19, 21–23], (2) granulocyte-macrophage colony stimulating factor (GM-CSF) [18], (3) nitric oxide (NO) and nitric

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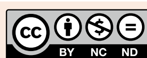
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oxide synthase (iNOS) [18, 24, 25], (4) eicosanoid (i.e. prostaglandin E_2 or PGE_2) [19, 20, 26–28], and (5) cyclooxygenase and lipooxygenase activity [29]. Additionally, It may act by depressing the migration of neutrophils and monocytes towards the inflammatory site and by increasing leukocyte apoptosis through modulation of NF-κB binding and pathway [30].

In the Philippines, experimental investigations demonstrating the therapeutic potential of indigenous stingless bee propolis have been greatly limited to our study on its neuroprotective efficacy against ischemic stroke [31] and those from another group detailing its antimicrobial activity against *Pseudomonas aeruginosa* [32]. More recently, we have illuminated the pronounced specificity in its anticancer activity towards the differentiated subtype of gastric adenocarcinoma but not the diffuse-type [33]. However, no existing evidence has been documented to date concerning its possible regulatory action on the inflammatory process. Hence, in this study, we described the anti-inflammatory potency of Philippine stingless bee propolis in a mouse model of λ-carrageenan-induced hind paw edema.

Materials and Methods

All procedures conducted in mice were approved by the Institutional Animal Care and Use Committee of the University of the Philippines Los Baños (UPLB) (IACUC Approval Number: 069).

Propolis sample preparation

The samples of the Philippine stingless bee propolis were provided by the UPLB Bee Program, Institute of Biological Sciences, UPLB. Propolis was extracted to obtain a 30% ethanolic fraction with a final concentration of 300 mg/ml as indicated in our earlier publication [31]. Upon phytochemical screening, this crude extract has been found to contain more than 500 chemical constituents, which include carbohydrates, steroids, alkaloids, anthraquinones, phenols, and terpenoids, among others [33]. Moreover, a previous report on propolis samples taken from the same colonies of Philippine stingless bees revealed high amounts of flavonoids and polyphenols i.e. pinobanksin-5, 7-dimethyl ether, artepillin C, apigenin, quercetin, luteolin-5-methyl ether, pinobanskin 3-0 butyrate or isobutyrate, and kaempferol [34].

Mice

Thirty (30), six-week-old, male ICR mice were obtained from the Research Institute for Tropical Medicine (RITM), Alabang, Muntinlupa City, Philippines. Mice were randomly allocated into three (3) treatment groups (n=10/group) namely: Distilled water (negative control); Diclofenac sodium, a non-steroidal anti-inflammatory drug (NSAID) given at a concentration of 10 mg/kg body weight [35] and served as a positive control; and propolis, which was administered at a concentration of 100 mg/kg body weight [31, 33]. Right before use, ethanolic extract of propolis was diluted to several folds of distilled water thereby allowing the vehicle alcohol to fall to negligible concentration (<1%). All mice were caged individually in standard polycarbonate cages with stainless steel top under 12h light: 12h dark period, 22 ± 4°C, and 30–50% humidity. Commercial pellets and distilled water were provided *ad libitum*. All animals were acclimatized for one week prior to experimentation.

Treatment administration

Mice were given distilled water, diclofenac sodium, or propolis via oral gavage one hour prior to the injection of the phlogistic agent, λ-carrageenan [35, 36]. All treatments were duly coded and independently prepared by a member of the group who was not directly involved in the administration process and scoring of hind paw edema. This was done to ensure that those who were tasked to perform the aforementioned procedures would be blinded to the assigned treatments.

λ-carrageenan-induced hind paw edema in mice and edema paw scoring

The λ-carrageenan was prepared as a 1% suspension in a sterile normal saline and then 0.1 ml was injected subcutaneously into the right dorsal hind paw of each mouse [37]. The extent of inflammation was then assessed by performing edema scoring starting at 0 h (prior to treatment), and 3, 6, and 24 h post-injection. The hind paw scoring system reported by Jeengar *et al.* [38] was adapted, with slight modifications, as follows: 0 = absence of swelling of the paw, 1 = one toe inflamed and swollen, 2 =>1 toe swollen but not the entire paw inflamed and swollen, 3 = entire paw inflamed and swollen, and 4 = very inflamed and swollen paw or ankylosed paw. Gross assessment of the effects of each treatment on the development of hind paw edema was performed by two independent researchers, who were completely oblivious of the treatment assignment.

Euthanasia, collection and histopathology of the paw

Twenty-four hours following induction with λ-carrageenan, 250 μl of blood was collected from the retro orbital plexus of each mouse for subsequent TNF-α cytokine analysis. Afterwards, mice were euthanized by intraperitoneal injection of pentobarbital sodium (Dole-

thal®, Vetoquinol UK Ltd., Buckingham, UK) at a dose of 50 mg/kg body weight. The right hind paw was excised using surgical scissors, fixed in 10% formalin for at least 72 h, decalcified overnight, embedded in paraffin, and sectioned at 5 μm in thickness. Tissue sections were stained with hemotoxylin and eosin (H&E) and examined using a microscope for pathological changes.

Microscope assessment of the hind paw was modified and adapted from the protocol by Jansen and Haveman [39] and Hussein *et al.* [40]. Microscopic thickness of the three sections of the paw dermal region was measured using a binocular research microscope and the mean thickness of these sections was recorded. Extent of lymphocytic infiltration was examined and categorized as: 0 = no infiltration, 1 = mild, 2 = mild to moderate, 3 = moderate, 4 = moderate to severe, and 5 = severe. Edema, on the other hand, was evaluated as: 0 = no edema, 1 = minimal edema, 2 = moderate edema, and 3 = extensive edema [40]. Activation of endothelium was also assessed as: 0 = no vascular changes, and 1 = swollen endothelial cells. The scores in each parameter were averaged and recorded. Analysis and interpretation of the histological data were independently carried out by a veterinary pathologist. All the slides were deliberately coded to make sure that the expert was completely unaware of the treatment assignment.

Cytokine measurement

Five blood samples from each treatment group were randomly selected for the assay. Additional five blood samples were taken from the normal non- λ-carrageenan-treated mice (n=5) to serve as mock (negative) control. Each blood sample was transferred into a properly labeled 1.5 ml microcentrifuge tubes and centrifuged for five minutes at 3,000 rpm. Plasma samples were recovered and utilized for subsequent measurement of TNF-α using mouse TNF-α ELISA kit (Abcam, Cambridge, MA, USA) and microplate reader at 450 nm.

Statistical analysis

Data were presented as means ± SD and analyzed using one-way analysis of variance (ANOVA) and Tukey-HSD posttest. All analyzes were performed using SPSS v.23 (IBM Corp., Armonk, NY, USA) and values with P<0.05 were considered statistically significant.

Results

Propolis significantly abrogated the λ-carrageenan-induced gross morphological changes

Subcutaneous injection of λ-carrageenan successfully induced an acute inflammatory reaction in all treatment groups as exemplified by swelling and hyperemia of the mice hind paw at the onset of 3 h post-injection (Figs. 1A and B). Pretreatment with diclofenac sodium and propolis substantially minimized these perceptible signs of gross inflammation, which became more pronounced with each advancing temporal course of observation. In particular, diclofenac sodium pre-treatment exhibited a remarkable improvement in the hind paw scores posting

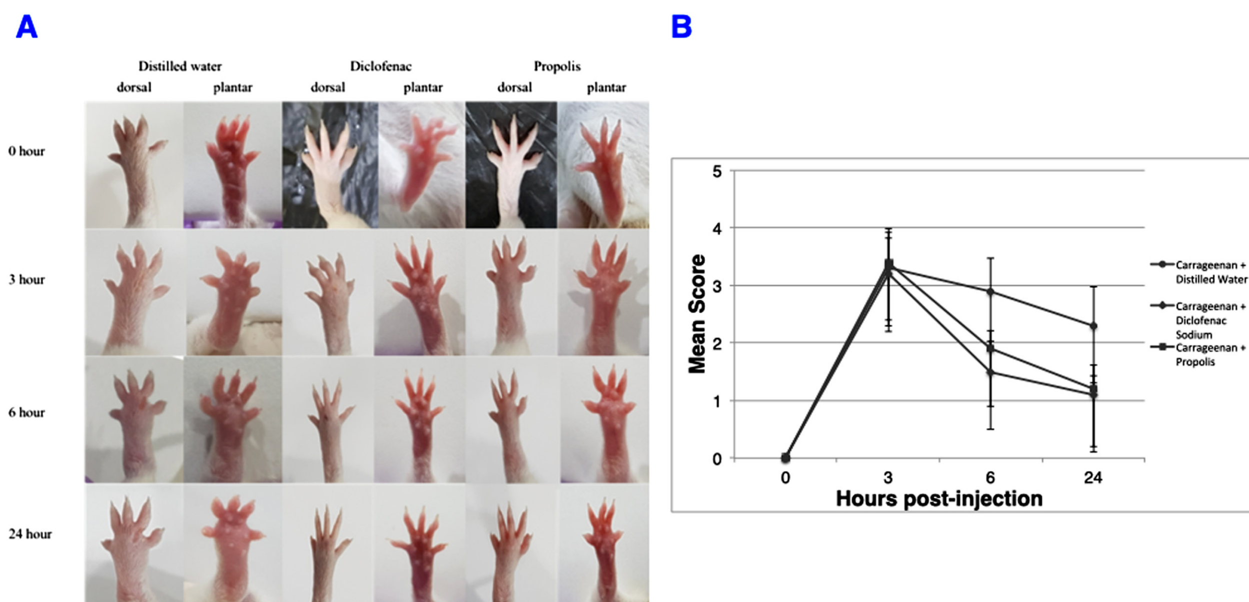

Fig. 1. (A) Gross appearance and (B) mean hind paw scores of mice treated with distilled water, diclofenac sodium, and propolis at 0, 3, 6, and 24 h post-injection of λ-carrageenan. Pronounced erythema and swelling of the hind paw were observed 3 h post administration of λ-carrageenan. A significant attenuation of these gross signs was apparent 6 and 24 h post-injection in the diclofenac sodium-treated group and at 24 h in the propolis-treated group.

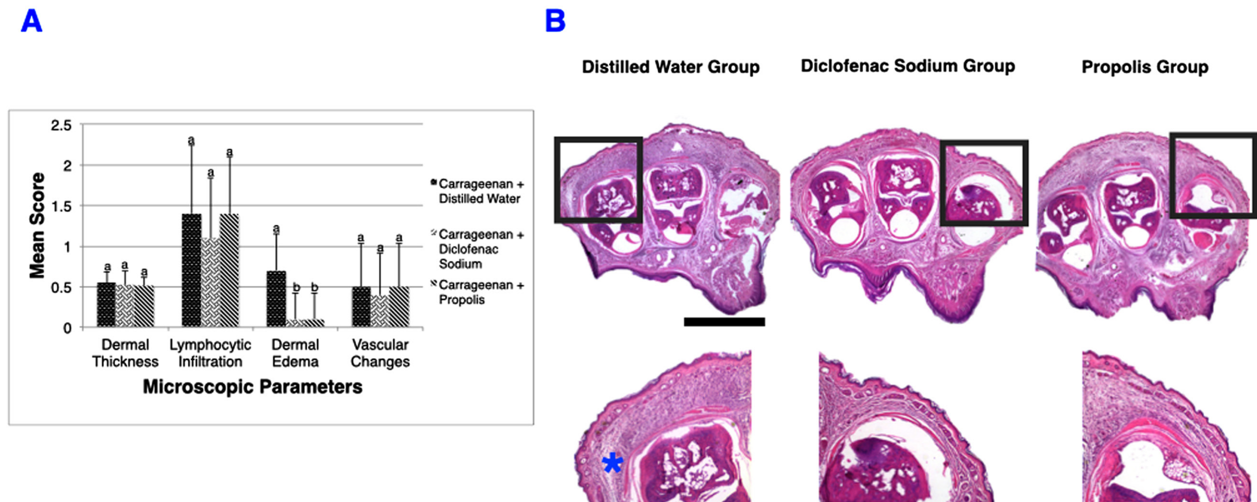

Fig. 2. (A) Mean scores of different treatment groups in relation to various microscopic parameters such as dermal thickness, lymphocytic infiltration, dermal edema, and vascular changes. (B) Representative histological sections of the hind paw of distilled water, diclofenac sodium, and propolis treated groups (Above). Scale bar: 1,000 μm. The distilled water group had a moderate edema (asterisk) whereas both diclofenac sodium- and propolis-treated groups showed a very minimal to almost absent edematous lesion (Inset, Below).

a mean value of 1.50 ± 0.53 at 6 h and 1.10 ± 0.32 at 24 h in converse to those attained by the distilled water control group (vs. 2.90 ± 0.57 and 2.30 ± 0.68, respectively). For the propolis-treated group, although a tendency for a slightly lower mean score was observed at 6 h post-induction, a statistically significant result was only achieved at the 24-h time period (1.20 ± 0.42 vs. 2.30 ± 0.68) (Fig. 1B).

Propolis distinctively attenuated the histological lesion of dermal edema

Microscopically, the inflammatory changes evaluated after carrageenan administration include increased thickness of the dermis, dermal edema, leukocytic infiltration, and dermal vascular alterations like dilation, thickening of dermal blood vessel walls, and perivascular lymphocytic infiltration (Figs. 2A and B). Of these parameters, dermal edema was distinctively reduced by propolis pretreatment reflecting a seven-fold diminution (0.10 ± 0.32) as opposed to those of the distilled water-treated counterpart (0.70 ± 0.95). Whereas, in addition to dermal edema (0.10 ± 0.32), the magnitude of lymphocytic infiltration was also significantly decreased by diclofenac sodium (1.10 ± 0.74 vs. 1.40 ± 0.84). No striking difference was noted, however, when considering the mean dermal thickness and alterations of the dermal vascular epithelium irrespective of the treatment group (Fig. 2B).

Propolis substantially inhibited the profound elevation of plasma TNF-α levels

The mean plasma concentration of TNF-α was subsequently examined to determine the tendency of each treatment to modulate the expression of this classical mediator of inflammation. In relation to the normal mock-treated group, injection of λ-carrageenan promoted a mean TNF-α concentration of around 576 mg/ml, which steeply deviated from the baseline level of about 56 mg/ml (Fig. 3). Interestingly, mice belonging to the diclofenac sodium- and propolis-pretreated groups both effectively alleviated this profound escalation thereby restricting the cytokine expression level to a value of only ~209 mg/ml (Fig. 3).

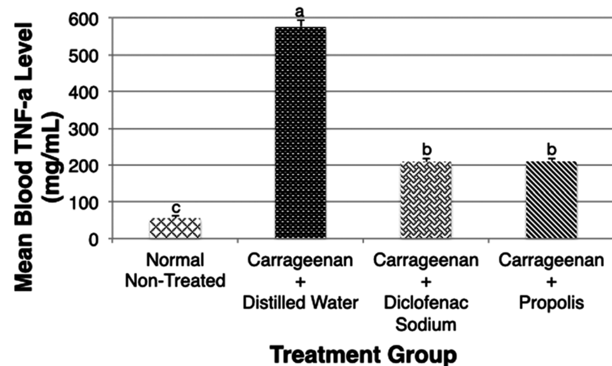

Fig. 3. Mean blood TNF-α levels of mice belonging to different groups at 24 h following administration of λ-carrageenan. Both diclofenac sodium and propolis group exhibited profound inhibition of TNF-α expression, which steeply deviated from those of the distilled water control group.

Discussion

Acute inflammation involves a well-programmed homeostatic response that is usually triggered whenever

the host protective barriers are disrupted by physical, chemical, or biological noxae, and whose duration of action must be short-lived to preclude the occurrence of any unwanted outcomes like organ dysfunction and diseases such as arthritis, diabetes, and cancer [41, 42]. In acute paw edema induced by λ-carrageenan, a complex interaction is typically observed among several factors including immune cells, non-immune mediators, and vascular elements. During the early phase, the ensuing inflammation is mainly invoked through the concomitant release of various endothelial-, platelet-, and mast cell-derived components such as histamine, 5-hydroxytryptamine, and bradykinin, thereby prompting the increase in vascular permeability leading to the enhanced leakage of plasma proteins, fluids, and polymorphonuclear leukocytes [43–45]. These infiltrating immune cells, predominated by neutrophils, in turn liberate a number of inflammatory cytokines i.e. IL-1β, IL-6, and TNF-α, which together with endothelial and sensory nerve cells stimulate the generation of nitric oxide (NO) [46–48]. A further augmentation in vascular permeability is also provoked by an intensified production of prostaglandin species particularly PGI_2 and PGE_2, and this is commonly accompanied by a commensurate increase in the levels of bradykinin, cytokines, and NO [49, 50]. On the other hand, the late phase of λ-carrageenan-induced paw edema is characterized by an excessive degree of inflammation in which the preponderant immunoreactive cell populations are the mononuclear macrophages [51]. In conjunction with neutrophils, these cells instigate the biosynthesis and release of various inflammatory mediators as well as toxic radicals (oxygen and nitrogen species) and their by-products (peroxynitrite). Of these, PGE_2 and NO have been largely thought to be responsible for modulating this delayed phase of inflammatory process owing to the maximal expression of COX-2 and inducible nitric oxide synthase (iNOS) enzymes [50, 52, 53]; unlike in the early stage where the increment in their respective levels are primarily dependent on the activation of COX-1 and constitutive NOS (cNOS) [45, 47, 48]. Also, the enhanced production of these mediators correlated to a profound activation of the transcription factor NF-κB, which directly regulates the downstream transcriptional expression of multifarious inflammatory molecules including chemokines, cytokines, and adhesion markers [54–56]. These result, therefore, in an exuberant amplification of the inflammatory response.

In the present study, subcutaneous injection of the phlogistic agent λ-carrageenan considerably recapitulated these hallmarks of acute inflammatory reaction therefore serving as a useful *in vivo* model for screening compounds with potential anti-inflammatory activity [57, 58]. Here, we demonstrated that pretreatment with Philippine stingless bee propolis caused a significant reduction of the hind paw edema showing a two-fold decrement at 24 h post-induction in relation to those obtained by the distilled water control group. Intriguingly, the data generated at this particular time point almost paralleled those of the positive control group, diclofenac sodium, albeit mice belonging to this latter group had an early onset of marked improvement starting at 6 h post-induction. This result suggests that a promising anti-inflammatory activity is also part of the biofunctional repertory of the Philippine stingless bee propolis.

The histopathological finding of the significant amelioration of dermal edema by propolis and diclofenac sodium on the 24th hour period consistently supported the profound gross attenuation of the hind paw swelling and hyperemia. In fact, there was a complete removal or absence of dermal edema in 9 out of 10 carrageenan-injected mice in both pre-treated groups relative to those of the distilled water-treated counterpart. The same observation was reported by Yasukawa *et al.* [59], Bolfa *et al.* [60], and Jastrzebska-Stojko *et al.* [61] using Brazilian, Romanian, and Polish propolis, respectively. However, the extent of dermal edema formation especially in the distilled water-treated control group, which yielded an output that was only indicative of a mild grade, was rather unexpected. This may be explained in part by the biphasic nature of dermal edema that is being elicited following λ-carrageenan injection [51, 62]. As described elsewhere, administration of 1% λ-carrageenan in mice, as in the present study, promoted a first wave of low intensity reaction at the first 6 h while the second wave of an exaggerated inflammatory reaction could only be perceived after 24 h with peak detected at 72 h [48, 51]. Moreover, this underwhelming response may also be ascribed to the genetic strain of the experimental animals used. For example, mice on a C57BL/6J background have been documented to stimulate a relatively weaker degree of biphasic edema in opposition to BALB/c [46] and CD1 [48] mice. Succeeding experiments, which carefully integrate these crucial factors, are already in place to comprehensively analyze the impact of Philippine stingless bee propolis on the formation of dermal edema.

TNF-α is a pleotropic cytokine that assumes a pivotal role not only in inflammation but also in the intricate process of apoptosis, survival, proliferation, and differentiation [37, 63, 64]. It has been shown to promote a collateral cytotoxicity in carrageenan-induced mouse paw edema by acting as a stimulator of prostaglandin synthesis [65], an inducer of NO formation (46) and neutrophil migration (66), and an activator of the NF-κB

signal transduction pathway [55]. In addition, it has been intrinsically linked with both phases of the edematogenic response. This was confirmed by the data on TNF-α p55 receptor deficient mice and TNF-α antibody- or Thalidomide-pretreated mice unveiling a superior reduction of edema formation with the early phase exemplifying a higher degree of inhibition (60–90%) than the late phase (40–65%) [66, 67]. In the present work, oral pretreatment with Philippine stingless bee propolis strongly evoked a suppressive action on TNF-α cytokine expression approximating around 63% deduction relative to those obtained by the corresponding control group. This restraining effect appears to be mediated through the modulatory influence exerted by propolis upon mononuclear macrophages most likely via the engagement of TNF-R1 receptor [66, 68]. Congruent with this proposition, propolis treatment has been noted by few independent investigations to considerably obviate the profound upregulation of TNF-α levels in several human and murine monocyte/macrophage cells such as THP-1 [69], J774A.1 [70], and peritoneal macrophages [71]. However, the detailed molecular underpinnings by which propolis regulate the TNF-α signaling pathway remains to be elucidated. Nevertheless, our findings, together with the result of the former studies, underline the key contributory role of TNF-α in the regulation of acute inflammation in the context of this animal model; and more importantly, validates the glaring potential of propolis in addressing various inflammatory-type of human diseases like asthma [72], gout [73], inflammatory bowel disease (IBD) [74, 75], Alzheimer's disease [76], arthritis [77], and diabetes [78]. Indeed, oral intake of propolis capsule by type 2 diabetes mellitus patients, who were recently enrolled in a randomized double-blind clinical trial, substantially improved their lipid profile, post-prandial blood glucose level, and insulin resistance; and effectively abrogated the unconstrained expression of inflammatory biomarkers especially TNF-α cytokine level [79]. Subsequent studies will be directed on identifying the activity of Philippine stingless bees propolis on other established mediators of inflammation.

Propolis is enriched with biologically active constituent compounds such as flavonoids and phenolic acids, which are demonstrated to affect specific mediators of inflammation including cytokines [6–8]. For instance, artepillin C has been previously reported to restrain TNF-α [79] whereas quercetin has been proposed to act by decreasing IL-6, TNF-α, IL-12, and IL-17 [80, 81]. Meanwhile, few studies have recounted that kaempferol, apigenin, and luteolin significantly hampered the expression levels of IL-1, IL-6, and TNF-α [82–85]. Interestingly, all these above-mentioned chemical compounds have been documented to be present at higher concentrations in the crude extracts of the Philippine stingless bee propolis [34]. Moreover, in our most recent publication, we have identified the existence of several components of this crude propolis extract, which may serve as candidate chemical markers with promising anticancer and anti-inflammatory activity. Based on GC-MS/MS analysis, some of these compounds might possibly constitute a new report at least for the propolis samples [33]. Further studies are being undertaken to verify these speculations. Taken together, it seems reasonable to suggest that the anti-inflammatory activity of propolis from the Philippine stingless bees may be due to the collective action of these bioactive compounds, which affect various cytokines involved in inflammation.

In conclusion, we herein reported that propolis from the indigenous population of Philippine stingless bees also exhibited a promising *in vivo* anti-phlogistic/anti-inflammatory activity, which could be partly achieved by reversing the λ-carrageenan-mediated increase in the expression of the pro-inflammatory cytokine, TNF-α.

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