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Dammar bee honey as a natural anti-inflammatory agent: evidence from cell culture and biochemical assays

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Natural bioactive compounds with anti-inflammatory properties are increasingly explored as safer alternatives to synthetic drugs. This study evaluated the anti-inflammatory potential of dammar bee honey, a phytochemically rich but underexploited natural product, using a series of *in vitro* biochemical assays. The selected assays targeted key inflammatory pathways, including redox imbalance, enzymatic activity, and protein destabilization. Specifically, the inhibition of nitric oxide and activities of cyclooxygenase (COX), lipoxygenase (LOX), myeloperoxidase (MPO) and protease enzymes, along with protein denaturation, were assessed. Dammar bee honey significantly reduced nitric oxide production in stimulated cultures, indicating immunomodulatory potential. It effectively inhibited COX, LOX, and MPO activities, suggesting strong antioxidant and anti-inflammatory effects. Additionally, it stabilized proteins by reducing both denaturation and protease activity. Dammar bee honey demonstrated substantial inhibitory activity across all tested inflammatory markers, with effects closely approaching those of the standard anti-inflammatory drug diclofenac, particularly in COX inhibition and protein denaturation. These findings demonstrate the multi-targeted anti-inflammatory efficacy of dammar bee honey and support its potential as a natural therapeutic agent for modulating inflammatory responses.

Keywords: Bioactive compounds, Cell line study, Inflammation, Medicinal properties of honey, Protein denaturation, Therapeutic potential, *Trigona iridipennis*

Honey is increasingly valued for its anti-inflammatory properties, largely attributed to its rich profile of bioactive compounds such as flavonoids, phenolic acids, and enzymes. Inflammation is a tightly regulated biological response involving a cascade of enzymatic activities that, when dysregulated, contribute to tissue damage and chronic disease progression. Among the key players in this cascade are cyclooxygenase (COX) and lipoxygenase (LOX), which initiate the synthesis of pro-inflammatory eicosanoids, lipid mediators that enhance vascular permeability, pain sensitivity, and leukocyte recruitment. The inflammatory response is further intensified by myeloperoxidase (MPO), a neutrophil-derived enzyme that generates reactive oxidants, thereby amplifying oxidative stress at inflamed sites[1,2].

This oxidative environment activates inducible nitric oxide synthase (iNOS), which produces high concentrations of nitric oxide (NO). While NO serves key signalling roles, its overproduction contributes to nitrosative stress and sustains inflammatory signalling. In parallel, proteolytic enzymes such as matrix metalloproteinases (MMPs) degrade structural components of the extracellular matrix, facilitating immune cell infiltration but also promoting tissue remodelling and degradation when unregulated. These structural alterations are further compounded by protein denaturation, which not only reflects oxidative damage but also acts as a pro-inflammatory stimulus by altering antigenicity and cell signalling[3,4]. Together, these enzymes form an interconnected network that perpetuates the inflammatory response through biochemical amplification, oxidative damage, and tissue disintegration. Investigating the inhibitory effects of honey on these specific enzymatic targets provides a mechanistic understanding of its potential as a natural anti-inflammatory agent.

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However, the extent and mechanisms of anti-inflammatory activity can vary significantly depending on the floral and entomological origin of the honey. Dammar bee honey is produced by *Tetragonula iridipennis*, a species of stingless bee widely found in South and Southeast Asia. This honey is darker, more viscous, and distinctively rich in bioactive compounds compared to that of *Apis mellifera*. Owing to its higher concentrations of phenolic acids, flavonoids, and organic acids, dammar bee honey exhibits superior antioxidant, anti-inflammatory, and antimicrobial activities. These properties not only enhance its therapeutic potential but also position it as a more efficacious alternative to conventional honey varieties in the management of inflammation and oxidative stress-related conditions[5]. This study investigated the anti-inflammatory potential of dammar bee honey by evaluating its effects on inflammatory markers and enzymes including COX, LOX, MPO, nitric oxide, proteases, and protein denaturation, using cell culture and biochemical assays.

While several studies have highlighted the antimicrobial, antioxidant, and wound healing properties of dammar bee honey[6,7], its anti-inflammatory mechanisms remain largely unexplored. Existing literature on honey's anti-inflammatory potential primarily attributes its effects to flavonoids such as luteolin, quercetin, and naringenin. These compounds have been shown to inhibit cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX), thereby reducing the synthesis of prostaglandins and leukotrienes, key pro-inflammatory mediators[8,9]. They also downregulate nitric oxide production via suppression of inducible nitric oxide synthase (iNOS), as demonstrated in RAW 264.7 macrophages[10]. However, this flavonoid centric understanding overlooks the complex phytochemical makeup of honey.

Dammar bee honey, in particular, is known to be rich in phenolic acids, enzymes, organic acids, antimicrobial peptides, and trace minerals[11,12]. The role of these non-flavonoid bioactives in modulating inflammatory processes remains poorly characterized. Additionally, most studies on honey's anti-inflammatory effects are limited to individual mechanisms or single-pathway assessments, rather than capturing the multi-targeted nature of the inflammatory response. There is a lack of systematic *in vitro* studies evaluating the impact of dammar bee honey across multiple inflammatory markers and enzymatic pathways in a unified experimental framework.

This study was designed with the objectives to investigate the anti-inflammatory activity of dammar bee honey through a panel of complementary cell-based and biochemical assays. By assessing its effects on key inflammatory markers and enzymes such as nitric oxide, COX, LOX, MPO, proteases, and protein denaturation the study aims to provide a mechanistic understanding of its multi-targeted immunomodulatory potential. These findings are expected to contribute valuable evidence toward establishing dammar bee honey as a scientifically validated natural anti-inflammatory agent.

Materials and Methods

Dammer honey

Dammar bee honey used in this study was sourced from the Department of Agricultural Entomology, College of Agriculture, Kerala Agricultural University, Thrissur, India. The raw honey was filtered through sterile muslin cloth to remove physical impurities and stored in amber glass bottles at 4±1°C in the dark to protect thermolabile and photosensitive bioactives. To confirm authenticity, the honey sample was screened using standard purity parameters including moisture content, HMF levels, and sugar profile. All analyses were performed within four weeks of collection to ensure bioactivity.

Cell culture

RAW 264.7 murine macrophage cells were purchased from the National Centre for Cell Science (NCCS), Pune, India. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (HiMedia, India) supplemented with 10% foetal bovine serum (HiMedia, India) and incubated at 37°C in a humidified atmosphere with 5% CO_2.

Cell treatment and preparation of cell lysate

RAW 264.7 cells were grown to 60% confluence and stimulated with 1.0 µg/mL lipopolysaccharide (LPS). The LPS activated cells were treated with varying concentrations of the test sample and the standard diclofenac. Six key anti-inflammatory parameters were evaluated: nitric oxide production, and inhibition of cyclooxygenase (COX), lipoxygenase (LOX), myeloperoxidase (MPO), protease activity, and protein denaturation. Dammar bee honey was tested at concentrations ranging from 6.25 to 100 µg/mL, with specific ranges selected

based on the assay sensitivity and dynamic response window of each assay. Broader concentration ranges (20–100 µg/mL) were applied in nitrite quantification, LOX inhibition, MPO activity, and protease inhibition assays, where preliminary observations indicated that lower concentrations did not elicit measurable responses. In contrast, finer two-fold serial dilutions (6.25–100 µg/mL) were employed in COX and protein denaturation assays, where early-phase inhibition was observed at lower doses, necessitating greater resolution to characterize their dose-response behaviour more precisely.

Dammar bee honey was accurately weighed and dissolved in sterile culture medium to prepare a 1000 µg/mL stock solution. Working concentrations were obtained by serial dilution, and all test solutions were freshly prepared and sterile-filtered prior to use. The treated plates were incubated for 24 h. After incubation, the cells were trypsinized, harvested, and centrifuged at 3000 rpm for 2 min. The pellet was resuspended in Tris-HCl buffer (pH 8.0), followed by sequential freeze-thaw cycles at -20 °C and 65 °C to lyse the cells. The lysate was centrifuged at 1200 rpm for 2 minutes, and the supernatant was collected for further assays.

Cellular nitrite assay

The nitrite concentration in the cell lysate was measured using the Griess reagent method[13]. Briefly, 50 µL of culture supernatant was mixed with 150 µL of Griess reagent (Sigma-Aldrich, USA) and incubated at room temperature for 10 minutes in a 96 well plate. The absorbance was recorded at 540 nm using a microplate reader. Sodium nitrite was used as a standard for the experiment.

Cyclooxygenase (COX) inhibition assay

The COX enzyme activity was assayed as per the method of Walker and Gierse[14] with slight modifications. The reaction mixture consisted of Tris-HCl buffer (pH 8.0), cell lysate, glutathione (5 mM), and hemoglobin (20 µg/L). The reaction was initiated by the addition of arachidonic acid (200 mM) and incubated at 37 °C for 20 minutes. The reaction was terminated by the addition of 10% trichloroacetic acid in 1 N hydrochloric acid. After centrifugation, 1% thiobarbituric acid was added, and COX activity was determined by measuring absorbance at 632 nm.

Lipoxygenase (LOX) inhibition assay

The 5-LOX activity was measured according to Axelrod *et al.*[15]. The reaction mixture contained Tris-HCl buffer (pH 7.4), 50 µL of cell lysate, and sodium linoleate (200 µL; 10 mg/mL). The formation of 5-hydroxyeicosatetraenoic acid was monitored by measuring absorbance at 234 nm.

Myeloperoxidase (MPO) activity assay

The MPO activity in the cell lysate was determined based on the method of Bradley *et al.*[16] and Renlund *et al.*[17]. The cell lysate was homogenized in a solution containing 50 mM potassium phosphate buffer and 0.57% hexadecyl trimethyl ammonium bromide (HTAB). After centrifugation at 2000 g for 30 min at 4 °C, the supernatant was collected. The MPO activity was assayed by adding phosphate buffer (pH 6.0) containing 1.67 mg/mL guaiacol and 0.0005% hydrogen peroxide. The change in absorbance at 460 nm was measured, and MPO activity was expressed as units per mL of cell lysate.

Protease inhibition assay

Protease inhibitor activity was determined using the method of Kunitz[18] with modifications. Trypsin (0.5 mg/mL in 0.1 M phosphate buffer, pH 7.0) was pre-incubated with the test sample at 37°C for 15 minutes. Following this, 2 mL of 1% casein (prepared in 0.1 M phosphate buffer) was added and incubated at 37°C for 30 minutes. The reaction was stopped by adding 2.5 mL of 0.44 M trichloroacetic acid (TCA). After centrifugation at 10,000 rpm for 15 minutes, the absorbance of the supernatant was measured at 280 nm. The protease inhibitor activity was calculated based on the difference in absorbance between the control and test sample.

Protein denaturation inhibition assay

The inhibition of protein denaturation was assessed using the method of Mizushima and Kobayashi[19], Sakat *et al.*[20]. The reaction mixture contained 0.4 mL of 3% bovine serum albumin (BSA) and varying volumes of the test sample. The samples were incubated at 37°C for 20 minutes, followed by the addition of 2.5 mL of phosphate-buffered saline (pH 6.3). The mixture was then heated at 80°C for 10 minutes, and absorbance was measured at 660 nm. The percentage inhibition of protein denaturation was determined by comparing the absorbance of the treated and control samples.

Statistical analysis

All experimental data were expressed as mean ± standard deviation of triplicate determinations. One-way analysis of variance (ANOVA) was performed to

determine the significance of differences between treatments, followed by Tukey's post hoc test wherever applicable. The analysis was conducted using RAISINS (Integrating R and AI for Agricultural Data Analysis)[21].

Results

Nitrite assay

To assess the inhibitory effect on lipopolysaccharide (LPS) induced nitric oxide (NO) production, nitrite levels were quantified in cell culture supernatants following treatment with dammar bee honey and diclofenac. Nitrite concentration was estimated using a sodium nitrite standard curve (Supplementary Table 1, Supplementary Fig 1).

In the diclofenac-treated group, the LPS control exhibited a nitrite level of 71.75±1.61 µg, which decreased progressively to 53.13±2.27, 40.66±2.99, and 17.01±1.23 µg at concentrations of 25, 50, and 100 µg/mL, respectively (Supplementary Table 2, Supplementary Fig 2). Similarly, in the dammar bee honey-treated group, nitrite levels declined in a dose-dependent manner from 70.62±1.79 µg in the control to 62.10±2.10, 54.95±1.84, 39.49±2.14, 26.49±2.30, and 21.99±1.47 µg at 20, 40, 60, 80, and 100 µg/mL, respectively (Table 1, Supplementary Fig 3).

Statistical analysis revealed highly significant differences among treatments in both groups ($P < 0.01$), with strong F-statistics (347.85 for diclofenac and 304.27 for honey) and low error variance, indicating robust treatment effects. Tukey's HSD test confirmed distinct groupings, with significantly different means at most concentrations. The coefficient of variation remained below 5%, ensuring data reliability. These results demonstrate a concentration-dependent inhibition of nitric oxide production, with dammar bee honey exhibiting comparable efficacy to diclofenac at higher doses.

Table 1 — Nitrite concentration under different honey treatment

Concentration (µg/mL)	Nitrite (µg)±SD
Control	70.62±1.79[a]
20	62.10±2.10[b]
40	54.95±1.84[c]
60	39.49±2.14[d]
80	26.49±2.30[e]
100	21.99±1.47[e]
F-statistic	304.27**
P-value	0.00
HSD (Tukey)	5.37
MSE	3.84
SE(m)	1.13
SE(d)	1.60
CV (%)	4.26
Cohen's F	11.26

Treatments with same letter grouping are not significantly different

Cyclooxygenase (COX) enzyme activity assay

The cyclooxygenase (COX) assay was employed to quantify the extent of enzymatic inhibition exerted by dammar bee honey in comparison to diclofenac, targeting COX mediated prostaglandin biosynthesis a central pathway in the inflammatory cascade.

Diclofenac treatment resulted in a concentration-dependent increase in COX inhibition, with per cent inhibition of 13.51±1.95, 23.87±2.41, 36.51±2.43, 54.52±2.15, and 68.39±1.91 at 6.25, 12.5, 25, 50, and 100 µg/mL, respectively (Supplementary Table 3, Supplementary Fig 4). The IC_{50} value for diclofenac was calculated to be 52.19 µg/mL. Correspondingly, dammar bee honey exhibited a progressive inhibitory response, fom 9.36±1.96 at 6.25 µg/mL to 54.29±2.58 % at 100 µg/mL (Table 2, Supplementary Fig 5). The IC_{50} value for honey was 90.05 µL.

Analysis of variance indicated highly significant differences across all treatment concentrations ($P < 0.01$), supported by high F-values (313.23 for diclofenac; 229.96 for honey) and low MSE. Post hoc analysis using Tukey's HSD test revealed distinct groupings for most concentrations, reinforcing the dose-responsive nature of inhibition. Coefficient of variation values remained below 7%, demonstrating consistent assay performance. These findings

Table 2 — Percentage inhibition of COX enzyme activity by honey

Concentration (µg/mL)	Percentage of inhibition (Mean±SD)
Control	0±0f
6.25	9.36±1.96[e]
12.5	17.63±2.00[d]
25	31.26±1.94[c]
50	42.91±1.87[b]
100	54.29±2.58[a]
F-statistic	229.96**
P -value	0.00
HSD (Tukey)	5.60
MSE	4.34
SE(m)	1.20
SE(d)	1.70
CV (%)	6.70
Cohen's F	9.59

Treatments with same letter grouping are not significantly different

underscore the capacity of dammar bee honey to inhibit COX activity in a concentration dependent manner, with efficacy approaching that of the standard NSAID at higher doses.

Lipoxygenase (LOX) enzyme activity assay

The lipoxygenase (LOX) assay was employed to investigate the inhibitory effect of dammar bee honey on LOX-mediated oxidation of polyunsaturated fatty acids, a key enzymatic step in leukotriene-driven inflammatory responses.

Diclofenac exhibited a clear concentration-dependent inhibition of LOX activity, with per cent inhibition increasing from 8.84±1.97 at 6.25 µg/mL to 72.14±3.50 at 100 µg/mL. Intermediate inhibition levels were observed at 16.01±1.98, 31.70±3.02, and 49.96±2.54 % for 12.5, 25, and 50 µg/mL, respectively (Supplementary Table 4, Supplementary Fig 6). The IC_{50} value for diclofenac was calculated to be 60.22 µg/mL.

Dammar bee honey also demonstrated a dose-dependent inhibition of LOX, with per cent inhibition ranging from 7.92±1.66 at 20 µg/mL to 53.04±1.37 at 100 µg/mL. Inhibition percentages at 40, 60, and 80 µg/mL were 17.43±1.93, 27.78±2.65, and 43.36±2.96, respectively (Table 3, Supplementary Fig 7). The IC_{50} value for honey was determined to be 96.38 µL.

Statistical analysis revealed highly significant differences among treatment concentrations ($P < 0.01$), supported by robust F-statistics (279.95 for diclofenac; 210.97 for honey) and low MSE values. Tukey's HSD post hoc test confirmed significant differences across concentrations, and CV values remained within acceptable limits (<8%). Collectively, the results demonstrate the capacity of dammar bee honey to inhibit LOX activity in a concentration-dependent manner, with considerable potency comparable to diclofenac at higher doses.

Myeloperoxidase (MPO) estimation

The myeloperoxidase (MPO) assay was performed to evaluate the impact of dammar bee honey on MPO enzymatic activity, a critical oxidative enzyme released during neutrophil activation and often associated with chronic inflammatory responses.

In the diclofenac-treated group, MPO inhibition increased progressively with concentration. The per cent inhibition were 16.31±1.86, 30.49±2.35, 92.01 ± 2.64, 94.30±4.25 and 98.10±1.60 at 6.25, 12.5, 25, 50 and 100 µg/mL, respectively (Supplementary Table 5, Supplementary Fig 8). Each concentration showed statistically significant differences from the others, indicating a strong dose-dependent inhibitory effect of diclofenac on MPO activity.

Dammar bee honey also demonstrated a clear concentration-dependent inhibition of MPO activity. The per cent inhibition observed were 8.41±3.38, 19.22±2.76, 47.55±3.23, 63.45±2.83 and 82.52±2.78 at 20, 40, 60, 80 and 100 µg/mL (Table 4, Supplementary Fig 9). All concentrations were significantly different based on Tukey's test, reinforcing the dose-responsive nature of honey's inhibitory activity.

One-way ANOVA revealed highly significant treatment effects for both diclofenac (F = 775.39**) and honey (F = 429.46**), with low mean square

Table 3 — Percentage inhibition of LOX enzyme activity by honey

Concentration (µg/mL)	Percentage of inhibition (Mean±SD)
Control	0±0f
20	7.92±1.66[c]
40	17.43±1.93[d]
60	27.78±2.65[c]
80	43.36±2.96[b]
100	53.04±1.37[a]
F-statistic	210.97**
P -value	0.00
HSD (Tukey)	5.91
MSE	4.83
SE(m)	1.27
SE(d)	1.80
CV (%)	7.35
Cohen's F	9.19

Treatments with same letter grouping are not significantly different

Table 4 — Percentage inhibition of MPO activity as influenced by honey

Concentration (µg/mL)	Percentage of inhibition (Mean±SD)
0	0.00±0.00f
20	8.41±3.38e
40	19.22±2.76d
60	47.55±3.23c
80	63.45±2.83b
100	82.52±2.78a
F stat	429.46**
P -value	0.00
HSD (Tukey)	7.52
MSE	7.52
SE(m)	1.58
SE(d)	2.24
CV(%)	7.44
Cohen's F	13.38

Treatments with same letter grouping are not significantly different

errors (MSE = 6.43 for diclofenac; 7.52 for honey) and low coefficients of variation (4.94% and 7.44%, respectively), indicating high precision in the data. The calculated Cohen's f values (17.97 for diclofenac; 13.38 for honey) reflected very strong effect sizes for both treatments. Overall, the results demonstrate that both diclofenac and dammar bee honey significantly inhibited MPO activity in a dose-dependent manner, with statistically distinct responses observed at each concentration tested.

Protease inhibition assay

To assess the modulatory effect of dammar bee honey on proteolytic pathways involved in extracellular matrix degradation and inflammation, a protease inhibition assay was conducted using diclofenac as the reference standard.

Diclofenac treatment resulted in a concentration-dependent inhibition of protease activity, with percent inhibition increasing from 8.15±1.98 at 6.25 µg/mL to 56.41±2.42 at 100 µg/mL. Intermediate values observed were 19.43±3.02, 27.64±1.92, and 38.81±1.94 percent at 12.5, 25, and 50 µg/mL, respectively. The IC_{50} value for diclofenac was calculated to be 86.11 µg/mL (Supplementary Table 6, Supplementary Fig 10).

Dammar bee honey also showed a dose-dependent inhibitory trend, with inhibition percentages of 12.95±2.37, 24.70±1.95, 38.15±1.99, 49.51±2.12, and 56.57±1.47 at 20, 40, 60, 80, and 100 µg/mL, respectively. The corresponding IC_{50} was 87.79 µL (Table 5, Supplementary Fig 11).

One-way ANOVA revealed highly significant differences between treatment concentrations ($P < 0.01$), with F-statistics of 195.11 for diclofenac and 237.37 for honey. Post hoc Tukey's analysis confirmed distinct groupings for most concentrations, supporting the specificity of dose-dependent effects. CV values remained within acceptable experimental limits (7.63% for diclofenac, 5.50% for honey), indicating high repeatability. These results underscore the protease-inhibitory potential of dammar bee honey, with efficacy closely aligning with that of the standard anti-inflammatory agent.

Protein denaturation inhibition assay

Protein denaturation, a hallmark of inflammatory processes and tissue injury, was assessed to evaluate the protective potential of dammar bee honey against thermally induced protein unfolding. Diclofenac served as the reference anti-inflammatory agent.

Diclofenac showed a marked concentration-dependent inhibition of protein denaturation, with per cent inhibition increasing from 9.58±1.98 at 6.25 µg/mL to 86.77±2.59 at 100 µg/mL. Intermediate concentrations (12.5, 25, and 50 µg/mL) have given 19.35±3.48, 33.73±2.93, and 56.10±3.20 per cent inhibition, respectively. The IC_{50} was calculated as 49.68 µg/mL (Supplementary Table 7, Supplementary Fig 12).

Dammar bee honey also demonstrated significant protective activity, with inhibition per cent of 15.04±1.45, 27.61±1.97, 42.91±1.94, 52.69±1.47, and 64.93±1.43 at 6.25, 12.5, 25, 50, and 100 µg/mL, respectively. The IC_{50} for honey was estimated at 75.72 µL (Table 6, Supplementary Fig 13).

Table 5 — Percentage inhibition of protease activity by honey

Concentration (µg/mL)	Percentage of inhibition (Mean±SD)
Control	0±0f
20	12.95±2.37e
40	24.70±1.95d
60	38.15±1.99c
80	49.51±2.12b
100	56.57±1.47a
F stat	237.37**
P -value	0.00
HSD (Tukey)	5.38
MSE	4.01
SE(m)	1.16
SE(d)	1.63
CV(%)	5.50
Cohen's F	9.74

Treatments with same letter grouping are not significantly different

Table 6 — Percentage inhibition of protein denaturation by honey

Concentration (µg/mL)	Percentage of inhibition (Mean±SD)
Control	0±0f
6.25	15.04±1.45e
12.5	27.61±1.97d
25	42.91±1.94c
50	52.69±1.47b
100	64.93±1.43a
F stat	421.08**
P -value	0.00
HSD (Tukey)	4.49
MSE	2.79
SE(m)	0.96
SE(d)	1.36
CV(%)	4.11
Cohen's F	12.98

Treatments with same letter grouping are not significantly different

ANOVA confirmed significant differences among treatment groups ($P < 0.01$), with strong F-statistics (346.18 for diclofenac; 421.08 for honey) and low mean square errors. Tukey's test showed clear stratification across concentrations, while CV values (7.01% for diclofenac, 4.11% for honey) ensured data reliability. These findings confirm the anti-denaturation potential of dammar bee honey, supporting its role as a natural inhibitor of inflammatory protein damage.

Discussion

The findings of this study highlight the significant anti-inflammatory potential of honey, as demonstrated through multiple biochemical assays. Notably, this is the first study to comprehensively evaluate dammar bee honey, a darker, polyphenol rich variety, using a multi biochemical assay framework. Previous research has predominantly focused on commercial honeys such as Apis, manuka or tualang honey, while the therapeutic potential of indigenous honeys with darker hues and richer phytochemical profiles remains under-investigated. The inhibition of nitric oxide production suggests honey's ability to modulate inflammatory signaling pathways, potentially by inter fering with inducible nitric oxide synthase (iNOS) activity[22]. This aligns with previous studies indicating that polyphenol-rich natural products can suppress nitric oxide synthesis, thereby reducing oxidative stress and inflammation[23-25]. In the present study, dammar bee honey demonstrated nitric oxide inhibition that was comparable to diclofenac at higher concentrations, indicating its potential efficacy in modulating immune activation. Given that NSAIDs such as diclofenac are associated with gastrointestinal and renal side effects[26], honey may offer a safer alternative for chronic inflammatory conditions.

The suppression of cyclooxygenase (COX) and lipoxygenase (LOX) enzyme activities further underscores honey's potential in regulating inflammatory pathways. Since these enzymes are key mediators in the biosynthesis of prostaglandins and leukotrienes, their inhibition suggests that honey may help alleviate inflammatory responses associated with chronic conditions[27,28]. In this study, dammar bee honey exhibited concentration dependent inhibition of both COX and LOX enzymes. The COX inhibitory effect was notably close to that of diclofenac, suggesting comparable efficacy in modulating the prostaglandin arm of the arachidonic acid pathway. While diclofenac predominantly targets COX isoforms, dammar bee honey showed dual inhibitory activity across both COX and LOX pathways. Such a dual mechanism, although uncommon among conventional NSAIDs, may offer a broader anti-inflammatory profile with reduced risk of arachidonic acid shunting toward pro-leukotriene pathways. This broader targeting is significant, as inhibition of COX alone may lead to substrate shunting toward the LOX pathway, there by exacerbating leukotriene-mediated inflammation[29]. By attenuating both pathways simultaneously, dammar bee honey may mitigate this compensatory mechanism, positioning it as a potential multi-target anti-inflammatory agent[30,31]. Similar findings have been reported in previous research investigating honey's ability to modulate eicosanoid pathways[32-34].

The reduction in myeloperoxidase (MPO) activity indicates honey's ability to limit neutrophil-driven oxidative damage. MPO is a critical enzyme in the inflammatory response, contributing to oxidative stress through the production of reactive oxygen species[35,36]. The observed decline in MPO activity suggests that honey can intervene in cellular-level inflammatory cascades, supporting its antioxidant and immunomodulatory profile[37,38]. Although diclofenac also reduces MPO activity, it primarily does so by suppressing neutrophil recruitment and inflammatory signalling, rather than by directly inhibiting the enzyme. In contrast, the MPO suppressive effect of dammar bee honey may result from its polyphenol content acting through direct antioxidant or enzyme modulating mechanisms, suggesting a distinct and complementary mode of action.

Protease inhibition is another crucial mechanism observed in this study. Proteolytic enzymes play a central role in inflammatory processes, particularly in tissue degradation and immune cell migration[39]. Honey's ability to inhibit protease activity suggests that it may contribute to preserving extracellular matrix integrity and preventing excessive tissue damage in inflammatory conditions[40-42].

The protein denaturation inhibition assay further reinforces honey's protective effects. Protein denaturation is a hallmark of inflammation-related tissue damage, often observed in conditions like rheumatoid arthritis[43,44]. The observed inhibition suggests honey's potential role in stabilizing proteins and preventing structural degradation, which may contribute to its traditional use in wound healing and inflammatory disorders[45,46].

A key strength of this study lies in its comparative design: all six assays were conducted in parallel with the NSAID diclofenac, enabling a direct assessment of honey's relative efficacy. While diclofenac remains the benchmark for anti-inflammatory drugs, its long-term use is limited by gastrointestinal and renal side effects[47,48]. In contrast, dammar bee honey demonstrated dose-dependent inhibition across all six assays, with moderate IC_{50} values and no observed cytotoxicity, suggesting it may serve as a safer, natural alternative in chronic inflammation management[31,49,50].

Additionally, the presence of naturally occurring bioactive compounds in honey may influence inflammatory gene expression at the molecular level. Recent transcriptomic studies suggest that honey-derived flavonoids can modulate the expression of genes associated with the NF-κB and MAPK pathways, which are key regulators of inflammatory cytokines like TNF-α, IL-1β, and IL-6[51,52]. This transcriptional modulation suggests that honey not only blocks downstream inflammatory mediators but may also prevent their synthesis at the gene level.

From a nutritional and functional food perspective, the inclusion of honey as a dietary supplement may offer synergistic health benefits when combined with other polyphenol-rich foods, especially in inflammation-prone populations. Furthermore, the anti-inflammatory effects of honey may vary with its floral origin, mineral content, and antioxidant index. Among the different varieties, dammar bee honey demonstrated particularly strong activity, likely due to its higher total phenolic and flavonoid content. Its deep amber hue reflects a rich phytochemical profile, which contributes to enhanced radical scavenging ability and greater inhibitory action against inflammatory enzymes like COX and LOX[53,54]. This study provides strong preliminary evidence that dammar bee honey holds multi-targeted anti-inflammatory potential. By bridging the existing research gap and offering a comparative biochemical evaluation, these findings lay the groundwork for future *in vivo* and clinical studies exploring dammar bee honey as a novel functional agent in inflammation management.

Conclusion

To substantiate the anti-inflammatory effects of dammar bee honey, we employed a comprehensive *in vitro* strategy targeting key inflammatory mediators and pathways, including nitrite production, eicosanoid (COX and LOX) biosynthesis, oxidative stress (MPO), protease activity, and protein denaturation. The honey exhibited significant, dose dependent inhibition in all assays, with notable suppression of nitric oxide, COX, LOX, and MPO activities, as well as effective inhibition of protease function and protein denaturation. In several assays, particularly COX inhibition and protein denaturation, the inhibitory effect of dammar bee honey closely approached that of diclofenac, a standard anti-inflammatory drug. Importantly, unlike NSAIDs, which are associated with gastrointestinal, renal, and cardiovascular side effects during long-term use, dammar bee honey may offer a safer alternative for chronic inflammation management, owing to its natural origin and antioxidant-rich phytochemical composition. These observations confirm its multi-targeted mode of action. Thus, we conclude that dammar bee honey holds strong promise as a natural anti-inflammatory agent, warranting further investigation through *in vivo* and clinical models.

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Ethical statement

This study did not involve any human participants or animal subjects and therefore did not require ethical approval.

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Conflict of interests

The authors have no relevant financial or non-financial interests to disclose.

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