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Marine-derived polysaccharides target the canonical nf-κb pathway to attenuate inflammation

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Conventional anti-inflammatory therapies provide symptomatic relief but are often constrained by adverse effects and reduced long-term efficacy. As a result, there is a growing interest towards the development of natural compounds as safer alternatives. Marine-derived polysaccharides are known for the biocompatibility, low toxicity and immunomodulatory properties. Our earlier study evaluated the anti-inflammatory potential of the polysaccharide fractions extracted from the marine bivalves. The fractions were isolated from *Saccostrea cucullata* (SCP), *Perna viridis* (PVP), *Perna indica* (PIP) and *Geloina erosa* (GEP). All of them exhibited potent anti-inflammatory activity in formalin induced paw oedema model in mice. In the present study, the underlying molecular mechanisms of these polysaccharides were investigated using RT-qPCR analysis. The reference drug used was indomethacin at the dose of 10 mg/kg. Formalin induction led to pronounced upregulation of pro-inflammatory mediators. Among the four polysaccharides, GEP markedly downregulated NF-κB along with its downstream mediators TNF-α, IL-6, iNOS, and COX-2. PVP, PIP, and SCP also demonstrated dose-dependent inhibitory effects, although their activities were comparatively less pronounced. Taken together, these findings suggest that the polysaccharide derived from marine bivalves have potent anti-inflammatory efficacy mediated through modulation of multiple molecular targets within the canonical NF-κB signalling pathway.

Keywords: Bivalves, Formalin-induced paw oedema, *Geloina erosa,* NF-κB signalling, *Perna indica*, *Perna viridis, Saccostrea cucullata*

Inflammation is the major defence mechanism of the mammalian immune system. Inflammation acts as an early host response against tissue injury, pathogenic infiltration and various harmful stimuli[1,2]. Acute inflammation plays an important role in homeostasis and tissue regeneration. However, the sustained or uncontrolled inflammation contributes to the onset and advancement of numerous pathological disorders[3,4]. These include cardiovascular diseases, cancer, diabetes, asthma, Alzheimer's disease, gastrointestinal inflammatory disorders and autoimmune syndromes[5–8]. The selective regulation of immune signalling helps to manage the pathological inflammation while preserving the overall immune function[9]. Nuclear factor-kappa B (NF-κB), a transcription factor, is crucial in modulating inflammatory reactions occurring in cells[10]. The NF-κB family proteins are produced throughout the body and act as "molecular on-off switches" which are stimulated by various agents[11]. NF-κB controls the transcription of genes involved in the immune cell activation, which further activates the innate and acquired immunity. Besides activation, NF-κB also coordinates the inflammatory cascade for leukocyte recruitment which helps in the secretion of cytokines *e.g*. tumour necrosis factor-alpha (TNF-α), interleukin – 1 beta (IL-1β), interleukin – 6 (IL-6) and the production of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2)[12,13]. Hence, NF-κB has a dual role in the initiation and regulation of inflammation[14]. However, the dysregulation of these pathways at any stage can lead to pathological inflammation and immune-mediated diseases. The NF-κB system mainly acts through two pathways, the canonical and non-canonical pathways. The canonical NF-κB signalling cascade is the most common and rapidly activated pathway whereas the non-canonical pathway is a slower and tightly regulated one.

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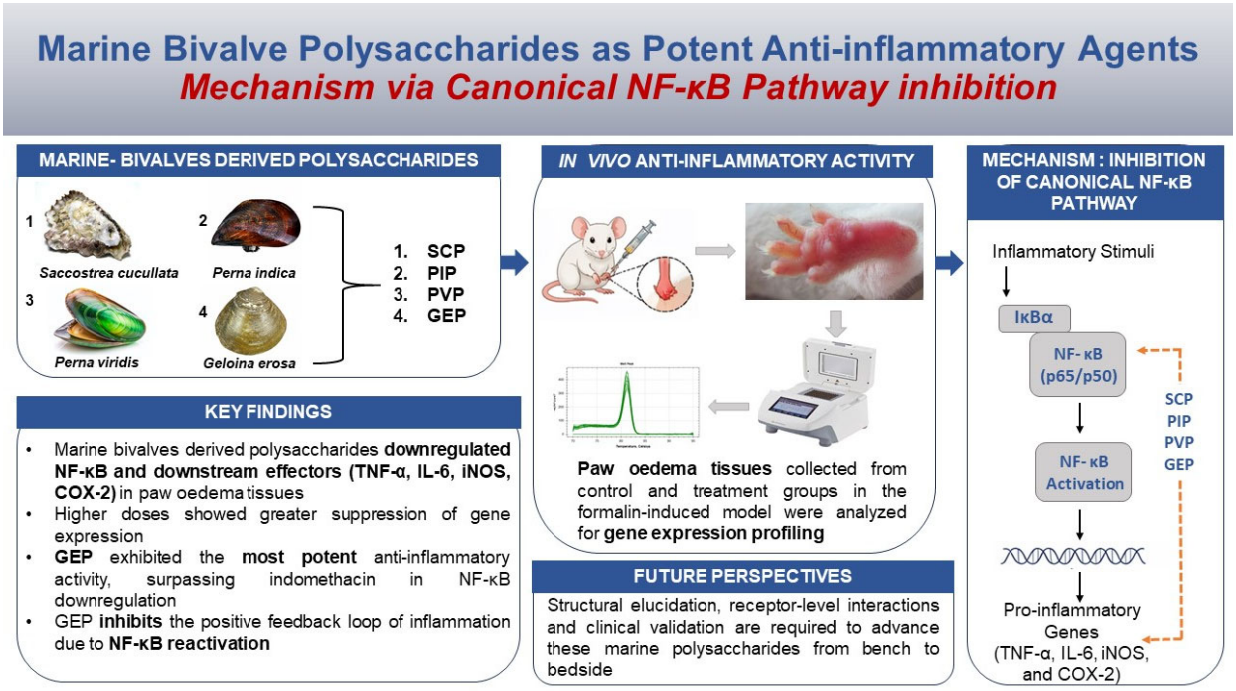
Suppl. data available on respective page of NOPR



Graphical abstract

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely accepted anti-inflammatory drugs currently in the market. Since it primarily focuses on the COX pathway, they provide only symptomatic relief. Besides, they are also frequently associated with adverse effects such as gastrointestinal and renal disturbances. This necessitates the need for a safer and sustainable alternative which can collectively regulate inflammatory responses[15]. In this context, natural products have gained attention as promising alternatives[16]. Marine ecosystems have vast biodiversity and greatly untapped potential as sources of pharmacologically active compounds[17]. Their structural diversity and ability to modulate the inflammatory pathways make them ideal candidates for the novel anti-inflammatory drugs. Among marine organisms, molluscs have emerged as valuable source for bioprospecting. Being the second-largest animal phylum, molluscs encompass a wide range of species with significant evolutionary and biochemical diversity[18]. This diversity is reflected in their secondary metabolites which have a major role in their innate immunity, predator attack and microbial defence. Moreover, these compounds have also shown to possess diverse pharmacological activities[17]. Despite the isolation of over 1,100 natural products from molluscan species, only less than 1% of these species have been explored for their pharmacological potential[19].

Within molluscs, bivalves have a major role because of their nutritional value and traditional use as health-promoting foods[20]. However, scientific investigation into their therapeutic potential remains relatively limited. Recent studies have identified that bivalves produce secondary metabolites with significant anti-inflammatory activity. This notion also supports their prospective use as functional food ingredients or natural therapeutic agents[21]. These are likely to have evolved due to their adaptations to the dynamic and pathogen-rich marine environment, where organisms mostly rely on robust biochemical defences.

Polysaccharides are ubiquitously expressed biomacromolecules seen in plants, animals and microorganisms. They are the most naturally abundant class of biological macromolecules[22]. Structurally, they are high molecular weight polymers which are composed of monosaccharide units linked by glycosidic bonds. Their structural complexity and diversity provide them with a remarkable ability to modulate different biological processes[23]. Recent research has provided evidence of the anti-inflammatory potential of naturally derived polysaccharides[24]. They have role in the major inflammatory pathways by acting on the host immune cells and helps in the expression of pro-inflammatory cytokines[25]. Due to their non-toxic nature,

biocompatibility and immunomodulatory effects, bivalve-derived polysaccharides are increasingly being explored as natural agents for managing inflammation-associated disorders[26]. The present study builds upon previously established *in vivo* experimental work involving chronic inflammation models using marine bivalve-derived polysaccharides, a part has been published and the rest is under consideration for publication[27,28]. In the current investigation, we elucidated their molecular mechanisms which play a role in the anti-inflammatory activity with primary focus on the canonical NF-κB pathway.

Materials and Methods

Extraction of crude polysaccharides from marine bivalves and evaluation using the formalin-induced paw oedema model

The extraction of crude polysaccharides from marine bivalves of *Perna viridis* (PVP) and *Perna indica* (PIP) as well as *in vivo* assessment of their anti-inflammatory efficacy were conducted as part of a previously reported studies[27,28]. Meanwhile the extraction and *in vivo* evaluation of polysaccharide fractions from *Saccostrea cucullata* (SCP) and *Geloina erosa* (GEP) is under review. In all the cases, the test substances were administered orally at doses of 22 mg/kg (low) and 110 mg/kg (high) for ten consecutive days, with 6 mice in each group. Indomethacin at the dose of 10 mg/kg served as the standard reference. The methodology of animal grouping, formalin induction, dosing and tissue collection has been described in the aforementioned publications. In the present study, the collected paw tissues were used to quantify the molecular targets of the NF-κB signalling cascade through real-time quantitative polymerase chain reaction (RT-qPCR).

Gene expression studies using RT-qPCR

The gene expression of NF-κB, TNF-α, IL-6, COX-2 and iNOS was analysed using RT-qPCR. The samples include tissues from vehicle control group, the reference drug group and groups treated with low and high doses of polysaccharides. Ribonucleic acid (RNA) was extracted from paw tissues using TRI Reagent (Sigma Aldrich, USA). After isolation, the RNA was quantified using spectrophotometric analysis with a Nanodrop spectrophotometer and the purity of RNA was assessed using the ratio of absorbance of the samples taken at 260 nm, 280 nm and 230 nm. The samples showing A_{260}/A_{280} and A_{260}/A_{230} ratios between 1.8 – 2.0 or above were considered of ideal purity and taken for further study. Further, complementary DNA (cDNA) was synthesized from the isolated RNA with Verso cDNA Synthesis Kit (Thermo Scientific, USA) in accordance with the manufacturer's guidelines.

Primer sequences specific to each target gene were generated using National Center for Biotechnology Information Primer-BLAST tool and their specificity was checked using BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The designed primers were used for PCR amplification of the target genes. The corresponding sequences for each target are provided in (Table 1). β-actin, a housekeeping gene, was used as an internal control and co-amplified alongside the target genes. Gene expression analysis was conducted using GoTaq®qPCR master mix (Cat.no. 4367659, Promega, USA,). The reaction was carried out in six replicates.

Separate PCR reactions were set up for each gene. A non-template control (NTC) was included for each gene, along with reverse transcription minus (RT–) controls

Table 1 — Gene expressions primers sequences

Oligonucleotide ID	Annealing temperature	Nucleotide sequence
NF-κB	60ºC	Forward: 5′ CCACTGTCAACAGATGGCCC3′
		Reverse: 5′ AGCGGAATCGAAATCCCCTC3′
COX-2	60ºC	Forward: 5′ TGAGTACCGCAAACGCTTCT3′
		Reverse: 5′CAGCCATTTCCTTCTCTCCTGT3′
TNF-α	60ºC	Forward: 5′ACTGAACTTCGGGGTGATCG3′
		Reverse: 5′CCACTTGGTGGTTTGTGAGTG3′
IL-6	60ºC	Forward: 5′GACAAGCCAGAGTCCTTCAGA3′
		Reverse:5′TGTGACTCCAGCTTATCTCTGG 3′
iNOS	60ºC	Forward:5′GGGACTGAGCTGTTAGAGACAC3′
		Reverse:5′AATCCAACGTTCTCCGTTCTCTTG3′
β -actin	60ºC	Forward: 5′ACTGAACTTCGGGGTGATCG3′
		Reverse: 5′CCACTTGGTGGTTTGTGAGTG3′

Table 2 — Fold change in NF-κB gene expression following treatment with bivalve-derived polysaccharides.

S. No.	Sample	ΔCt	ΔΔCt	Fold change
1	Formalin + SCP @110 mg/kg	5.245 ± 0.089^{bc}	-1.16833	2.247519
2	Formalin + SCP @ 22 mg/kg	4.912 ± 0.227^{ab}	-1.50167	2.831697
3	Formalin + PVP @110 mg/kg	5.87 ± 0.328^{bcde}	-0.54333	1.457336
4	Formalin + PVP @ 22 mg/kg	5.823 ± 0.34^{bcde}	-0.59	1.505247
5	Formalin + PIP @110 mg/kg	5.653 ± 0.2^{bcde}	-0.76	1.693491
6	Formalin + PIP @22 mg/kg	5.367 ± 0.232^{bcd}	-1.04667	2.065751
7	Formalin + GEP @110 mg/kg	6.727 ± 0.194^{e}	0.313333	0.80478
8	Formalin + GEP @ 22 mg/kg	6.363 ± 0.403^{cde}	-0.05	1.035265
9	Formalin + Indomethacin @10 mg/kg	6.605 ± 0.143^{e}	0.191667	0.875594
10	Formalin alone	4.01 ± 0.117^{a}	-2.40333	5.290241
11	Control	6.413 ± 0.165^{de}	0	1

ΔΔCt and fold change values are relative to control. Full qPCR Ct data are provided in (Suppl. Table S1)
The samples were analyzed in six replicates (n =6), and expressed as mean ± standard error.
Means followed by different superscripts (a–e) within the same column indicated significant differences ($P< 0.05$).

for each sample and a negative control containing only nuclease-free water (NFW). The quantitative PCR was performed in a 10 µL reaction mixture which consists of 0.5 µL of cDNA template, 5 µL of qPCR Master Mix (2X), 0.3 µL each of forward and reverse primers (10 pmol/µL), and nuclease-free water (NFW) was added to make up the final volume. The negative control for the reaction was NFW whereas the others consisted of cDNA template. All the reactions were conducted in triplicate. Reaction was initiated with a denaturation step at 94°C for 30 sec. The amplification was carried out for 30 cycles with denaturation at 95°C for 15 sec. Annealing temperatures were 60°C for 30 sec and extension at 72°C for 35 sec. The relative quantification of gene expression was estimated through comparative Ct (ΔΔCt) approach. The expression of target genes was compared with the reference gene to calculate ΔCq and expression of same gene in treatment sample versus control samples were used to calculate **ΔΔ**Cq. The quantification was expressed as fold change relative to the untreated control group. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test in IBM SPSS Statistics The detailed evaluation of various gene expressions is provided as (Suppl. Tables S1-S5).

Results

Effect of polysaccharides of marine bivalves on NF-κB pathway activation in formalin induced paw oedema

The gene expression profile of NF-κB in the samples is presented in (Table 2 and Fig. 1). The formalin alone treated group showed significant upregulation of NF-κB expression in paw oedema tissues. Meanwhile, indomethacin treatment resulted in a marked reduction in NF-κB expression. All

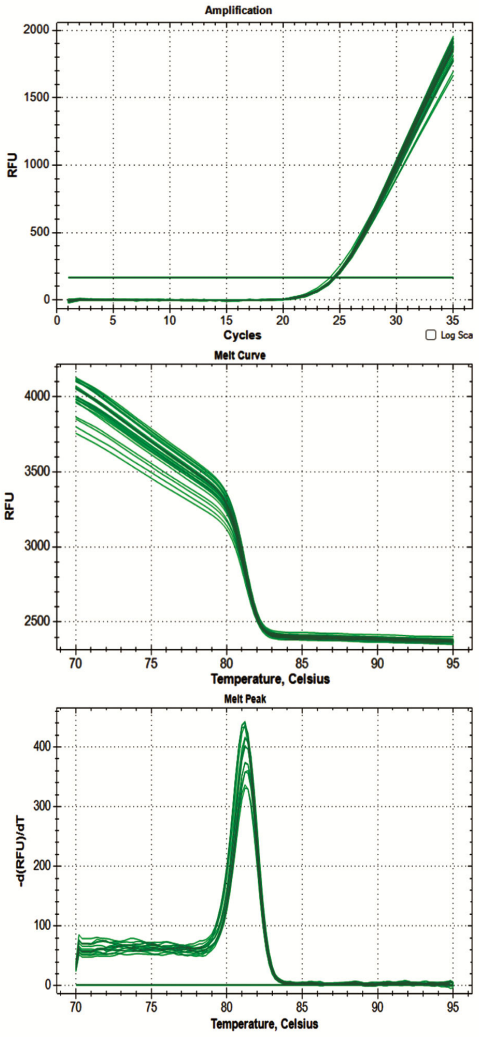


Fig. 1 — Amplification and melt curve of NF-κB gene expression in formalin-induced paw oedema after treatment with marine-derived polysaccharides

Table 3 — Fold change in TNF α gene expression following treatment with bivalve-derived polysaccharides.

S. No.	Sample	ΔCt	ΔΔCt	Fold change
1	Formalin + SCP @110 mg/kg	6.333±0.224^{abc}	-1.08167	2.11648
2	Formalin + SCP @ 22 mg/kg	6.317±0.22^{abc}	-1.09833	2.141072
3	Formalin + PVP @110 mg/kg	7.277±0.248^{bc}	-0.13833	1.100633
4	Formalin + PVP @ 22 mg/kg	6.455±0.378^{abc}	-0.96	1.94531
5	Formalin + PIP @110 mg/kg	6.327±0.269^{abc}	-1.08833	2.126283
6	Formalin + PIP @22 mg/kg	6.347±0.452^{abc}	-1.06833	2.097009
7	Formalin + GEP @110 mg/kg	7.448±0.244^{c}	0.033333	0.97716
8	Formalin + GEP @ 22 mg/kg	7.33±0.184^{bc}	-0.085	1.060688
9	Formalin + Indomethacin @10 mg/kg	5.92±0.38^{ab}	0.805	0.572362
10	Formalin alone	5.118±0.451^{a}	-2.29667	4.913213
11	Control	7.415±0.223^{c}	0	1

ΔΔCt and fold change values are relative to control. Full qPCR Ct data are provided in (Suppl. Table S2)
The samples were analyzed in six replicates (n =6), and expressed as mean ± standard error.
Means followed by different superscripts (a–c) within the same column indicated significant differences ($P< 0.05$).

Table 4 — Fold change in IL-6 gene expression following treatment with bivalve-derived polysaccharides

S. No.	Sample	ΔCt	ΔΔCt	Fold change
1	Formalin + SCP @110 mg/kg	6.77±0.305^{ab}	-1.12	2.17347
2	Formalin + SCP @ 22 mg/kg	6.568±0.193^{ab}	-1.32167	2.499547
3	Formalin + PVP @110 mg/kg	7.833±0.643^{b}	-0.05667	1.04006
4	Formalin + PVP @ 22 mg/kg	7.585±0.528^{b}	-0.305	1.235419
5	Formalin + PIP @110 mg/kg	6.648±0.321^{ab}	-1.24167	2.364716
6	Formalin + PIP @22 mg/kg	6.877±0.379^{b}	-1.01333	2.01857
7	Formalin + GEP @110 mg/kg	7.908±0.343^{b}	0.018333	0.987373
8	Formalin + GEP @ 22 mg/kg	7.737±0.31^{b}	-0.15333	1.112136
9	Formalin + Indomethacin @10 mg/kg	7.953±0.165^{b}	0.063333	0.95705
10	Formalin alone	5.083±0.406^{a}	-2.80667	6.996661
11	Control	7.89±0.202^{b}	0	1

ΔΔCt and fold change values are relative to control. Full qPCR Ct data are provided in (Suppl. Table S3)
The samples were analyzed in six replicates (n = 6), and expressed as mean ± standard error.
Means followed by different superscripts (a–b) within the same column indicated significant differences ($P< 0.05$).

the polysaccharides exhibited dose-dependent suppression of NF-κB expression when compared to formalin alone treated group whereas the high dose of GEP surpassed the level of NF-κB expression than that of indomethacin. For the rest of polysaccharides, the effect observed were in the order of PVP, followed by PIP and SCP.

Effect of polysaccharides of marine bivalves on TNF-α gene expression in formalin induced paw oedema

The study revealed a marked increase in TNF-α expression in the paw tissues of mice treated with formalin alone group. Indomethacin, the standard drug, significantly suppressed the TNF-α expression whereas GEP at higher dose supressed the upregulation of TNF-α. The lower dose of GEP also showed downregulation of TNF-α expression than the formalin alone treated group. All the polysaccharides showed dose dependent anti-inflammatory activity. Meanwhile, the other treatments such as SCP, PIP and PVP showed moderate downregulation when compared to formalin alone treated group. The gene expression data of TNF-α is depicted in (Table 3 and Fig. 2).

Effect of polysaccharides of marine bivalves on IL-6 mRNA expression in formalin-induced paw oedema

Formalin alone-treated group showed a 6.99-fold increase in IL-6 gene expression compared to the control group which indicates a strong inflammatory response. In contrast, indomethacin treatment resulted in a marked downregulation in IL-6 gene expression. GEP at the higher dose showed downregulation of IL-6 expression close to baseline, whereas PIP and SCP treatments showed only mild IL-6 downregulation when compared to formalin-alone treated group. Among the polysaccharides, GEP demonstrated the most potent anti-inflammatory activity in terms of IL-6 expression. The effect of bivalve-derived polysaccharides on IL-6 mRNA expression is given in (Table 4 and Fig. 3).

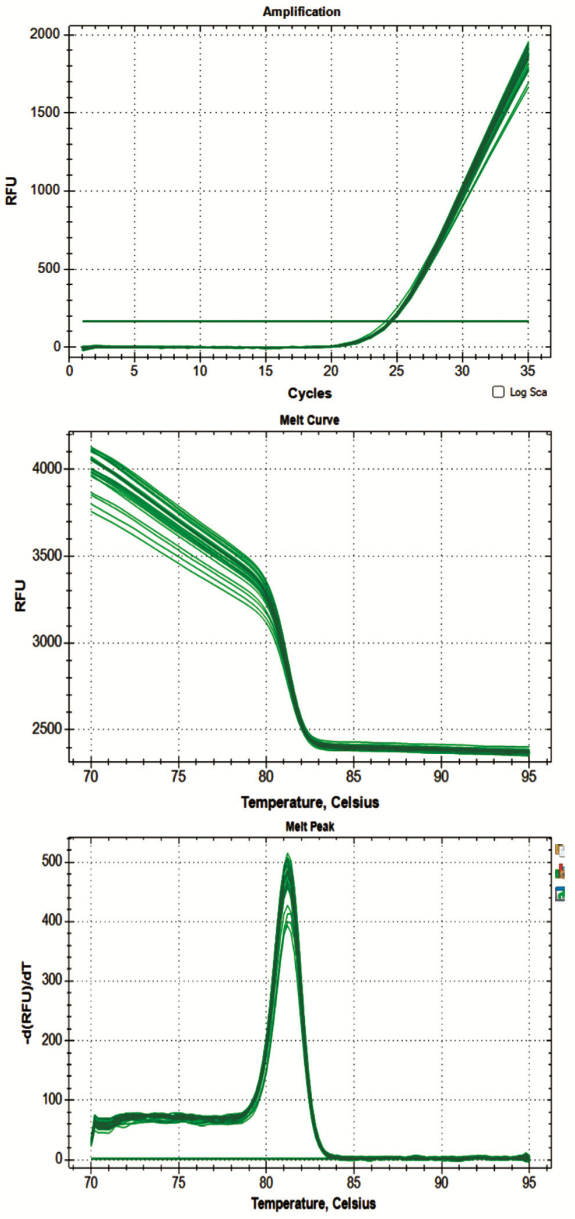


Fig. 2 — Amplification and melt curve of TNF-α gene expression in formalin-induced paw oedema after treatment with marine-derived polysaccharides

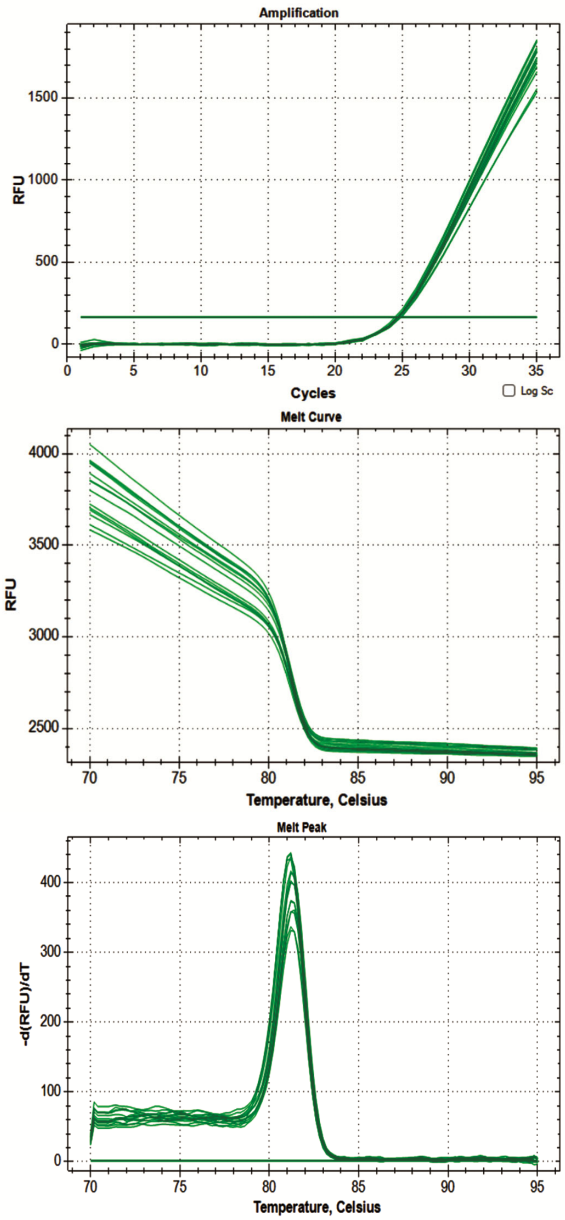


Fig. 3 — Amplification and melt curve of IL-6 gene expression in formalin-induced paw oedema after treatment with marine-derived polysaccharides

Differential regulation of COX-2 mRNA levels following treatment with polysaccharides of marine bivalves

The gene expression analysis of COX-2 revealed differential modulation across treatment groups which is given in (Table 5 and Fig. 4). Indomethacin significantly downregulated COX-2 expression to 0.75-fold, confirming its expected inhibitory effect. Among the test compounds, GEP at high dose demonstrated a strong anti-inflammatory response, closely approaching the suppressive effect of indomethacin. GEP at lower dose regulated expression levels near control, suggesting a moderate anti-inflammatory activity on COX-2 expression. While others treatments as SCP, PIP and PVP showed dose-dependent anti-inflammatory activity when compared to the formalin alone-treated group.

iNOS gene expression across treatment groups in formalin induced inflammation

The expression levels of iNOS were significantly altered following various treatments, indicating diverse inflammatory responses. The formalin group exhibited the highest upregulation of iNOS. In contrast, both

Table 5 — Fold change in COX-2 gene expression following treatment with bivalve-derived polysaccharides.

S. No.	Sample	ΔCt	ΔΔCt	Fold change
1	Formalin + SCP @110 mg/kg	5.388±0.257^{ab}	-0.86	1.815038
2	Formalin + SCP @ 22 mg/kg	5.427±1.643 ^{ab}	-0.82167	1.767447
3	Formalin + PVP @110 mg/kg	6.227±0.244 ^{ab}	-0.02167	1.015132
4	Formalin + PVP @ 22 mg/kg	6.178±0.249 ^{ab}	-0.07	1.049717
5	Formalin + PIP @110 mg/kg	6.162±0.329 ^{ab}	-0.08667	1.061914
6	Formalin + PIP @22 mg/kg	5.687±0.302 ^{ab}	-0.56167	1.475973
7	Formalin + GEP @110 mg/kg	6.458±0.142 ^{ab}	0.21	0.864537
8	Formalin + GEP @ 22 mg/kg	6.182±0.402 ^{ab}	-0.06667	1.047294
9	Formalin + Indomethacin @10 mg/kg	6.668±0.368 ^{b}	0.42	0.747425
10	Formalin alone	3.858±0.196 ^{a}	-2.39	5.241574
11	Control	6.248±0.3 ^{ab}	0	1

ΔΔCt and fold change values are relative to control. Full qPCR Ct data are provided in (Suppl. Table S4)
The samples were analyzed in six replicates (n = 6), and expressed as mean ± standard error.
Means followed by different superscripts (a–b) within the same column indicated significant differences ($P< 0.05$)

Table 6 — Fold change in iNOS gene expression following treatment with bivalve-derived polysaccharides.

S. No.	Sample	ΔCt	ΔΔCt	Fold change
1	Formalin + SCP @110 mg/kg	3.743±0.08^{bc}	-1.43167	2.697582
2	Formalin + SCP @ 22 mg/kg	3.788±0.125^{bc}	-1.38667	2.614738
3	Formalin + PVP @110 mg/kg	3.953±0.239^{bc}	-1.22167	2.33216
4	Formalin + PVP @ 22 mg/kg	4.373±0.497^{cd}	-0.80167	1.743114
5	Formalin + PIP @110 mg/kg	-1.488±0.303^{a}	-1.93284	3.818069
6	Formalin + PIP @22 mg/kg	3.783±0.187^{bc}	-1.39167	2.623816
7	Formalin + GEP @110 mg/kg	5.177±0.163^{d}	0.001667	0.998845
8	Formalin + GEP @ 22 mg/kg	5.467±0.167^{d}	0.291667	0.816958
9	Formalin + Indomethacin @10 mg/kg	5.27±0.231^{d}	0.095	0.936272
10	Formalin alone	2.988±0.38^{b}	-2.18667	4.552524
11	Control	5.175±0.081^{d}	0	1

ΔΔCt and fold change values are relative to control. Full qPCR Ct data are provided in (Suppl. Table S5)
The samples were analyzed in six replicates (n =6), and expressed as mean ± standard error.
Means followed by different superscripts (a–d) within the same column indicated significant differences ($P< 0.05$).

doses of GEP suppressed iNOS expression demonstrating strong anti-inflammatory potential. Indomethacin also showed suppression comparable to GEP treatments, further validating its anti-inflammatory property. Among the other polysaccharide fractions, all the high dose treatments demonstrated notable reductions in fold change compared to the formalin alone-treated group, suggesting effective anti-inflammatory activity. Table 6 and Figure 5 depict the iNOS gene expression across treatment groups in formalin induced inflammation.

Discussion

The aim of this research was to elucidate the molecular mechanisms underlying the anti-inflammatory activity of marine bivalve-derived polysaccharides. An intraplantar injection of formalin induced paw oedema with significant ($P< 0.05$) increase in NF-κB, TNF-α, IL-6, iNOS, and COX-2 in tissues. The oral administration of the all test doses of polysaccharides greatly reduced the paw oedema at both gross and histopathological level as well as lowered the level of signalling molecules. The higher dose of GEP showed the most potent anti-inflammatory activity compared to other treatments

NF-κB signalling system operates through two distinct pathways in the development of inflammation[29,30]. The canonical pathway mediates the inflammatory responses, whereas the non-canonical pathway regulates the immune cell development and the formation of secondary lymphoid tissues[31,32]. The canonical pathway signalling is activated by stimuli such as pathogens or cytokines. Further, the downstream kinase complexes are activated which leads to the induction of pro-

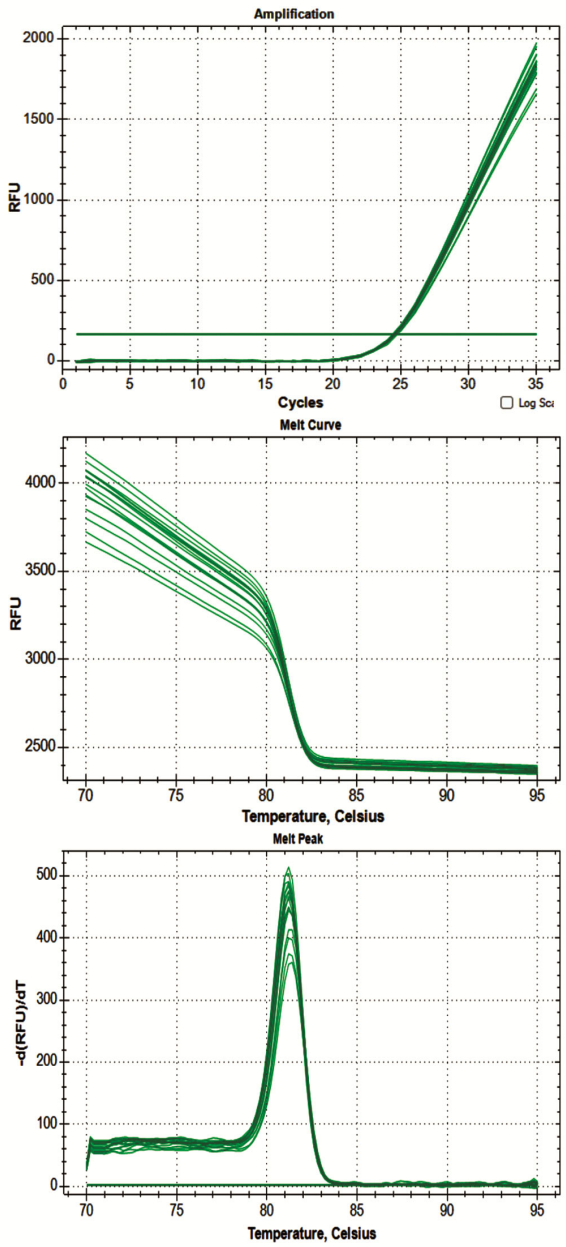


Fig. 4 — Amplification and melt curve of COX-2 gene expression in formalin-induced paw oedema after treatment with marine-derived polysaccharides

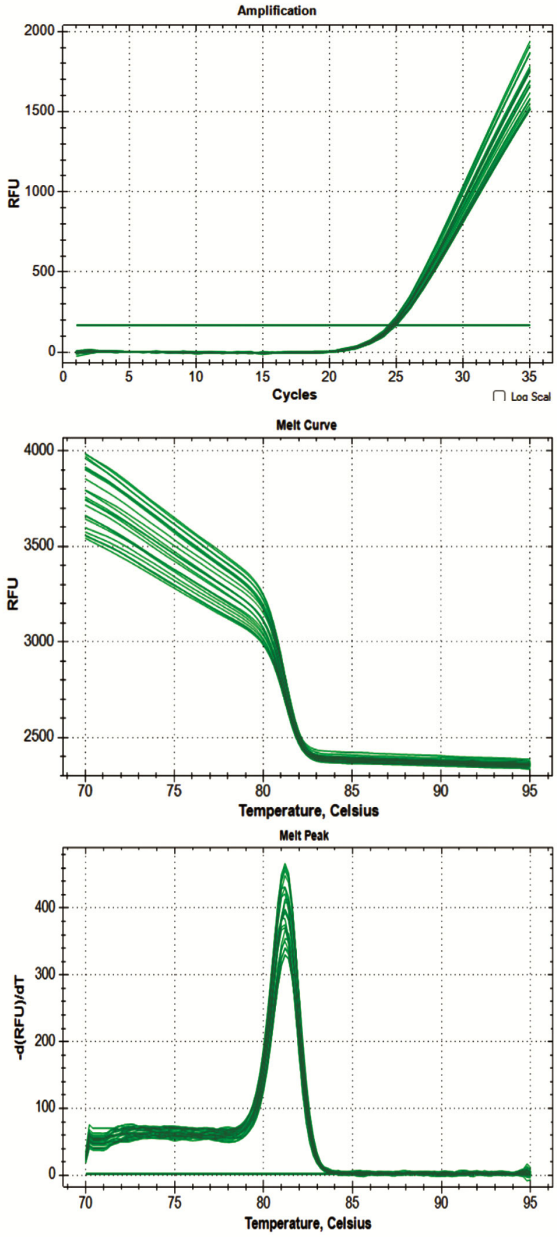


Fig. 5 — Amplification and melt curve of iNOS gene expression in formalin-induced paw oedema after treatment with marine-derived polysaccharides

inflammatory mediators (TNF-α, IL-6, COX-2, and iNOS). These mediators help in production of cytokines, prostaglandins, release of nitric oxide and progress to inflammation[33,34].

In the present study, formalin induction led to an upregulation of NF-κB and its downstream effectors (TNF-α, IL-6, iNOS, and COX-2). This have been previously reported by Huang *et al*. and Nwikwe *et al*. who showed the upregulation of NF-κB and TNF-α in formalin induced paw oedema[35]. Formalin induction led to local tissue injury and immune activation which stimulates the release of TNF-α from macrophages and other immune cells[36,37]. TNF-α plays a pivotal role in the onset and advancement of inflammation in the formalin-induced paw oedema model[38]. This cytokine, in turn, activates NF-κB, initiating the canonical signalling pathway which drives the progression of the inflammatory response[4,39].

The enhanced gene expression of IL-6, iNOS and COX-2 in formalin induced oedema has also been

previously studied. Abdelhady *et al.* reported the upregulation of IL-6, iNOS and COX-2 in formalin induced rat paw tissue while Farrukh *et al*., reported the upregulation of IL-6, COX-2, TNF-α and NF-κB in formalin induced arthritis model[40,41]. IL-6, iNOS and COX-2 are downstream targets while TNF-α is both activator and downstream effector of the canonical pathway. Formalin induced upregulation in the COX-2 expression leads to increased synthesis of prostaglandins. Likewise, the iNOS-derived NO acts either directly or through enhancing the production of pro-inflammatory prostaglandins. Prostaglandins causes vasodilation, increased vascular permeability and the development of inflammatory pathologies such as paw oedema[42].

In the earlier part of the current study, we have elucidated that the bivalve-derived polysaccharides attenuated the formalin-induced paw oedema at both gross and histopathological levels. The most potent suppression of paw oedema was observed in the GEP-treated groups when compared with other treatments. These findings have already been reported in our earlier publication. The present study explored the underlying molecular mechanisms of the anti-inflammatory activity of these polysaccharides. The results revealed that the bivalve-derived polysaccharides downregulated all the molecular markers in the canonical pathway of inflammation. GEP markedly suppressed the expression of NF-κB and its downstream effectors (TNF-α, IL-6, COX-2, and iNOS) while compared to other groups. Hence the superior anti-inflammatory activity of GEP at cellular level can be attributed to its stronger inhibition of the markers in the canonical NF-κB pathway. Canonical NF-κB pathway is the master regulator of inflammation which controls the gene expression, immune cell activation and resolution of inflammation[11,43].

Canonical NF-κB pathway is a critical therapeutic target[42] and its modulation helps to attenuate the inflammation. In the present study, GEP inhibited both the initiators (*e.g*., TNF-α) as well as effectors (*e.g*., IL-6, COX-2, iNOS) of canonical pathway. Hence, GEP prevents NF-κB activation at an early stage of inflammation[43]. Furthermore, GEP also suppressed–COX-2 and iNOS expression which are involved in prostaglandin and nitric oxide production respectively. These findings are in agreement with the previous work of Zhu *et al.* on marine algae[44]. Polysaccharide-coated selenium nanoparticles from *Ulva lactuca* significantly attenuated the DSS-induced colitis in mice. *Ulva lactuca* prevented the NF-κB translocation to the nucleus, inhibited the macrophage activation[45] and supressed the expression of TNF-α, IL-6, COX-2, and iNOS.

GEP showed superior downregulation activity over indomethacin in NF-κB suppression. The anti-inflammatory activity of NSAIDs is through the suppression of COX-2 enzyme rather than targeting the upstream pathway. Meanwhile GEP strongly suppressed the entire canonical pathway from the stage of NF-κB activation and downregulated all further molecular markers in the pathway. This suggests that GEP can strongly inhibit the amplification phase of inflammatory signalling when compared to the conventional anti-inflammatory drugs.

The polysaccharide PIP, PVP and SCP did not suppress inflammation as pronounced as GEP. However,–they significantly ($P< 0.05$) prevented the extreme upregulation observed in the formalin alone -treated group. Even though these polysaccharide cannot completely block the pathway, they modulated it sufficiently to attenuate the intensity of inflammation. Besides, their effects also appeared to be dose-dependent. Higher doses of the polysaccharide fractions showed better suppression than lower doses, especially in the case of PVP.

Despite the findings of modulation of the canonical pathway by marine-derived polysaccharides at the molecular level, the protein expression of the markers was not assessed, which would provide additional confirmation of the observed gene expression changes. Furthermore, future investigations should aim to undertake comprehensive structural and biochemical characterization of the polysaccharide compound, alongside validation of its anti-inflammatory efficacy across additional animal models to further substantiate its therapeutic potential and translational applicability.

Conclusion

The marine environment is a relatively unexplored source of bioactive compounds with significant potential as therapeutic agents. Within the marine organisms, bivalves have a major role because of their nutritional value and traditional use as health-promoting foods. However, scientific investigation into their therapeutic potential remains relatively limited. Our earlier studies have shown that

polysaccharides derived from *Perna viridis*, *Saccostrea cucullata*, *Geloina erosa*, and *Perna indica* possessed anti-inflammatory activity in formalin induced paw oedema model in mice. In the present study, we investigated the mechanism of action of these polysaccharides in modulating inflammation. These polysaccharides primarily targeted on the canonical NF-κB pathway of inflammation. They exhibited dose dependent effect in downregulation of the molecular markers of inflammation in the canonical pathway. The higher doses of polysaccharide fractions showed better suppression of gene expression than the lower doses, particularly in the case of PVP.

The polysaccharide fraction isolated from GEP showed the most potent anti-inflammatory activity at both cellular and molecular levels. GEP markedly downregulated the expression of NF-κB and its downstream effectors (TNF-α, IL-6, iNOS and COX-2) in paw oedema tissues. GEP also surpassed the activity of indomethacin in NF-κB downregulation. This suggests that GEP has a stronger action in both receptor signalling events as well as in the in the downstream effector molecules. Hence, GEP inhibits the positive feedback loop of inflammation due to NF-κB reactivation. Collectively, these findings indicate that bivalve-derived polysaccharides, particularly GEP, hold promise as a potential anti-inflammatory agent. Future research should aim to undertake validation of anti-inflammatory activity in additional animal models to substantiate their therapeutic potential and translational applicability.

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Conflict of interest

All authors declare no conflict of interest.

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