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Antinociceptive activity of marine derived chitosan coated triphala extract against formalin-induced zebrafish pain model

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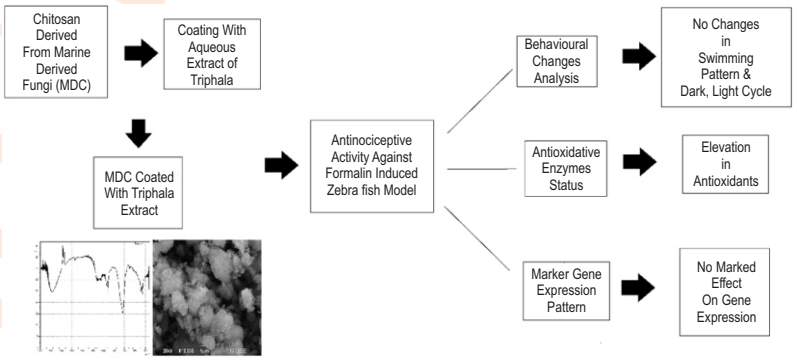
Abstract

Aim: The present study aimed to evaluate the antinociceptive effect of Triphala extract coated with marine derived chitosan against the formalin-induced adult zebrafish (*Danio rerio*) model.

Methodology: Marine derived chitosan was extracted from *Aspergillus niger* and the extracted chitosan was coated witha commercial formulation of Triphala by a simple dispersion method. The prepared formulation was administrated to formalin-induced zebra fish model followed by studying changes in various behavioural parameters like swimming pattern, dark-light cycle pattern, antioxidants which include catalase, superoxide dismutase, lipid peroxidase, glutathione S. transferase, glutathione peroxidase and the expression pattern of inflammatory markers genes like tumour necrosis factor (TNF) and induced nitrous oxide synthase (, iNOs).



Results: Marine derived chitosan-coated extract reveals notable antinociceptive activity by modulation of behavioural changes and inflammatory marker gene expression. The marine derived chitosan treatment group exhibits normal swimming patterns, dark-light cycle patterns, and morphological parameters as in the control group marine derived chitosan-coated Triphala treatment showed notable changes in the antioxidants. MARINE DERIVED CHITOSAN treatment did not induce any inflammatory signals, confirmed by a less expression pattern of inflammatory marker genes.

Interpretation: These findings imply that marine derived chitosan-coated Triphala can be used as an antinociceptive agent through active phyto-principles involved in the molecular mechanism of pain manifestation.

Key words: Antinociceptive, Chitosan, Marker genes, Triphala, Zebrafish

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Introduction

Marine derived chitosan is regarded as a nontoxic and biologically compatible polymer, extensively studied for diverse biomedical, pharmaceutical and applications, including the formulation of small-scale drug delivery systems (Amer *et al.,* 2019). Various *in-vitro* and *in-vivo* studies determine marine derived chitosan' spotential activities as immunomodulatory, bacteriostatic, fungicidal, antioxidative, wound healing, regenerative, and analgesic. Due to its excellent bioavailability, unique functional groups, and biocompatibility, MARINE DERIVED CHITOSAN can be suggested as effective delivery of bioactive compounds (Antunes *et al.,* 2021). The production of chitosan begins after selecting appropriate chitin sources or marine sources. The physico-chemical properties of chitosan are highly dependent on the source selection for chitosan extraction (Kaczmarek *et al.,* 2019; Rastogi *et al.,* 2021). Chitosan production on a commercial scale is carried out by deacetylation of chitin–the major structural component of various marine-based organisms like shrimp and crab shells through the complex treatment process in various forms like solutions, flakes, fine powder, beads, and fibres (Abirami and Nagarajan, 2018).

Chitosan production using marine-based organisms has been reported in various parts of the world, adopting different treatment strategies. A study on chitosan extraction from shrimp and prawn co-products (Islam *et al.,* 2020), shrimp shell waste (Nadia *et al.,* 2019, Abirami *et al.,* 2021), crab shell waste (Tissera *et al.,* 2021), shrimp and crab shell wastes (Premasudha *et al.,* 2017), sea prawn (Sneha Paul, 2014), Mussel shell (Abdulkarim *et al.,* 2013), seafood waste (Shruti *et al.,* 2021) suggest the possible utilisation of marine organism as the major source of chitosan. Though marine organisms are an important source for chitosan production, the extraction process is complex, involving difficulties in the collection, availability (seasonal), and high cost. Chitosan thus obtained by this process is heterogeneous concerning its physio-chemical properties (Islem and Rinaudo, 2015). Hence, it is mandatory to develop eco-friendly, less cost-effective methods for chitosan production with high purity.

Biotechnology principles through fermentation provide substitute chitosan from non-marine based animal sources that require high or complex parameters for extraction. Through the fungal biotechnology principles, chitosan extraction can be done in an eco-friendly manner with minimal cost. Chitin is the major structural component of fungal cell wall that plays a significant role in the mechanical properties of cells. With this objective, chitin can be extracted from the fungal organism that is cultured under an in-expensive or simple culture medium followed by conversion into chitosan. Chitosan extraction from fungi and its biological activities reported in previous studies reveal fungal-derived chitosan's advantages. Amer and Ibrahim (2019) extracted chitosan from marine fungal strain *Penicillium spinulosum* MH2 cell wall and exhibited potential antimicrobial activity, including antifouling activity. Chitosan with high purity has been extracted from Ugandan mushrooms (Kenneth *et al.,* 2022). This study uses simple green science principles to use chitosan derived from marine fungal strain *Aspergillus niger* cell wall. Marine derived chitosan thus obtained was coated with Triphala – a polyherbal medicinal formulation, and themarine derived chitosan-coated Triphala was evaluated for antinociceptive activity against formalin-induced zebrafish pain model. Triphala, a well-known poly herbal approved ayurvedic formulation comprising equal proportions of dehydrated and ground fruits of *Emblica officinalis* Gaertn, *Terminalia chebula*, and Retz *Terminalia bellirica* (Gaertn.) Roxb. It is known for its anti-inflammatory, antimicrobial, immunomodulatory, and antioxidant properties due to the presence of potentially bioactive compounds (Namasivayam *et al.,* 2021). However, the poor instability, reduced solubility, and limited bioavailability of active phytochemical constituents of medicinal plants, including Triphala, are the major constraints in utilising phyto-based principles as a therapeutic agent. Hence, it is necessary to develop a novel green delivery system that enhances stability, solubility, bioavailability and less non-target toxicity.

Various drug delivery systems based on small-scaled particles system can overcome the constraints in the phyto-principles due to their capacity to control drug delivery and provide effective solutions (Wang *et al.,* 2021). Among the diverse drug systems, chitosan has been utilised as a carrier component of organic drug delivery procedures for encapsulation and controlled release of plant-derived compounds with hydrophobic biomolecules traditionally encased by a chitosan-based shell hydrophilic biomolecules entrapped within the chitosan matrix (Antunes *et al.,* 2021). In this present investigation, marine derived chitosan was selected for Triphala-based delivery system. The proposed marine derived chitosan-coated Triphala was evaluated for antinociceptive activity using zebrafish pain model by measuring changes in various behavioural parameters, antioxidative enzymes status and marker genes expression pattern.

Materials and Methods

Selection of fungal strain and growth condition: Marine derived chitosan was extracted from marine fungal isolate *Aspergillus niger* isolated by culture-dependent serial dilution method. The fungal strain was maintained on sabouraud maltose yeast extract agar with 5% NaCl. For culturing and extraction of chitosan, sabouraud maltose yeast extract broth (SMYB) with 5% NaCl was used.

Chitosan extraction: For chitosan extraction, the known volume of fungal spore suspension (1 ml) derived from SMYA slant was inoculated into 1 l of SMYB with 5% NaCl under shaking condition (250×g) at ambient condition for four days. After the incubation phase, the broth was filtered through a muslin cloth. The collected biomass was used as the source for chitosan extraction. In this method, the collected biomass was dried and homogenised followed by suspending with alkali (1M NaOH). Alkali-treated biomass was sterilised by autoclaving and centrifuged at 12,000 ×g for 15 min. The collected pellet was washed with distilled water at pH 7. The washed pellet was extracted with acetic acid at 95°C

for 8 hr and centrifuged at 12, 000×g. The collected pellet was centrifuged again at 12,000×g, and the precipitated chitosan was washed with respective solvents like distilled water, 95% ethanol, and acetone. After washing with a respective solvent, the extracted chitosan was dried at 60°C.

Characterisation: Determination of functional groups and morphology of extracted chitosan was done by Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscopy (SEM). For FTIR analysis, a potassium bromide pelletised sample was analysed for recording the FTIR spectrum (FT-IR8300). Scanning electron microscopy analysis was used to analyse size and shape or particle morphology. SEM micrograph of processed sample was recorded using carl –Zeiss Supra 55.

Preparation of marine derived chitosan-coated Triphala extract: A commercial formulation of Triphala was used in this study. The aqueous extract was prepared from the collected powder. For extraction, 5 g of powder was suspended in 100 ml of distilled water, boiled in a water bath (50°C, 1 hr), followed by a filter through Whatman No. 1 filter paper. The collected filtrate was concentrated in the Rotary evaporator. The concentrated extract thus obtained was used for further studies. Followed by extraction, marine derived chitosan coating was done as described below. A known volume of MARINE DERIVED CHITOSAN was suspended in 100 ml of distilled water with 0,1 ml of acetic acid followed by 1 ml of aqueous extract of Triphala, thus prepared, stirred under magnetic stirrer at ambient temperature for 3 hr. After stirring, the suspension was centrifuged, and the collected pellet was lyophilised. FTIR changed the functional groups and morphology, and SEM analysis was done as described earlier.

Antinociceptive activity against formalin-induced zebrafish pain model

Collection and maintenance: Healthy adult zebra fishes (*Danio rerio*) free from malformations or infections were selected for the study. Before the initialization of the experiment, fishes were acclimatized to laboratory conditions according to OECD guidelines (OECD, 203). This work was approved by the Institutional Animal Ethics Committee (IAFC No. VBLT/B/TECH 03/2019).

Treatment groups: Adult zebrafish of both sexes with 0.4-0.5 g were selected in this study. Selected fishes were maintained in a treatment tank separately with different concentrations of plant extracts (5 mg l^{-1}, 10 mg l^{-1}, 15 mg l^{-1}, 20 mg l^{-1}). The control group was maintained in a separate tank without plant extracts. Study group was divided into 14 treatment groups Group I = Control (without formalin), Group 2= formalin, Group 3-6- 5,10, 15 and 20 mg l^{-1} of marine derived chitosan, Group 7-10- 5,10, 15 and 20 mg l^{-1} of Triphala extract, Group 11- 14- 5,10, 15 and 20 mg l^{-1} of marine derived chitosan-coated Triphala. Respective treatment groups were maintained separately for one week.

Formalin test: The formalin test is one of the most popular methods for pain induction. A known volume of formalin (5µl of 0.1 %) was injected into the tail of the selected fishes using an insulin syringe. After 30 min of formalin injection, the respective concentration of MARINE DERIVED CHITOSAN coated Triphala was administrated separately. To check the efficacy, marine derived chitosan and Triphala was administrated separately. Triplicates were maintained for each treatment.

Analysis of behavioural parameters: After 30 min of treatment, the changes in various behavioural parameters like swimming pattern (any notable change in the swimming behaviour in the respective treatment compared to the control and formalin treatment), aggression (analysing the attitude of respective treatment group when contact with other treatment groups-physical and behavioural change such as the erection of dorsal, caudal, pectoral regions, fast-swimming), freezing time (by analysing the maximum time spent by the respective treatment groups without any movement). Active conditions parameters include identifying active, moderate and less active fishes from the respective treatment groups), and dark-light cycle parameters. In this test, half of the tank was covered with a black background whereas the remaining half was exposed to light side (duration of respective treatment on the light and dark side of the tank) were studied. Determination of colour changes also revealed behavioural changes (colour changes in the respective treatment group).

Status of antioxidative enzymes: Determination of antioxidative enzymes in the treatment group reveals antinociceptive activity. The assay was carried out after the tenth day of respective treatment. Tested enzymes include catalase (CAT), superoxide dismutase (SOD), lipid peroxidase (LPO), glutathione S transferase (GST), and glutathione peroxidase (GPO). In brief, tissue homogenate from the treatment group was incubated with a suitable substrate. The chromogenic end products thus formed were read spectrophotometrically at a specific wavelength followed by measuring the enzyme activity. Tissue homogenate derived from the respective treatment group was used as a source for enzyme assays. The tissue section derived from the respective treatment was homogenised in ice-cold homogenising buffer, and the homogenised suspension was centrifuged at 10000×g for 10 min. The collected supernatant was used for enzyme assays (Weydert and Cullen, 2010).

Marker genes expression pattern: The effect of marine derived chitosan-coated Triphala on major inflammatory marker genes viz alpha TNF, and iNOs expression was studied by gradient PCR. Respective gene primers were designed with the NCBI Primer-BLAST tool. β-actin was taken as control for the housekeeping gene. DNA samples were isolated from the respective treatment group adopting standard isolation protocol after 24 hr of post-treatment. Gradient PCR was carried out with the respective primer and isolated DNA under standard conditions (The initial denaturation was done at 95°C for 10 min, trailed by 45 cycles of 3-step amplification consisting of denaturation at 95°C for 15 sec, annealing at 60°C for 15 sec and the extension was done at 72°C for 30 sec. Amplified products were resolved in agarose gel electrophoresis under standard

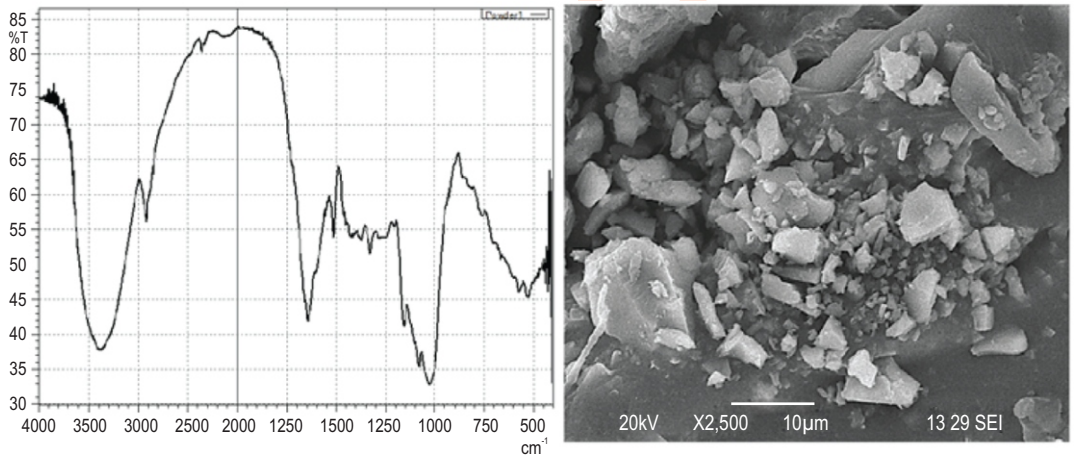
conditions. The amplification pattern of PCR products reveals the changes in the expression pattern.

Statistical analysis: The statistical significance of the results was evaluated by t-test.

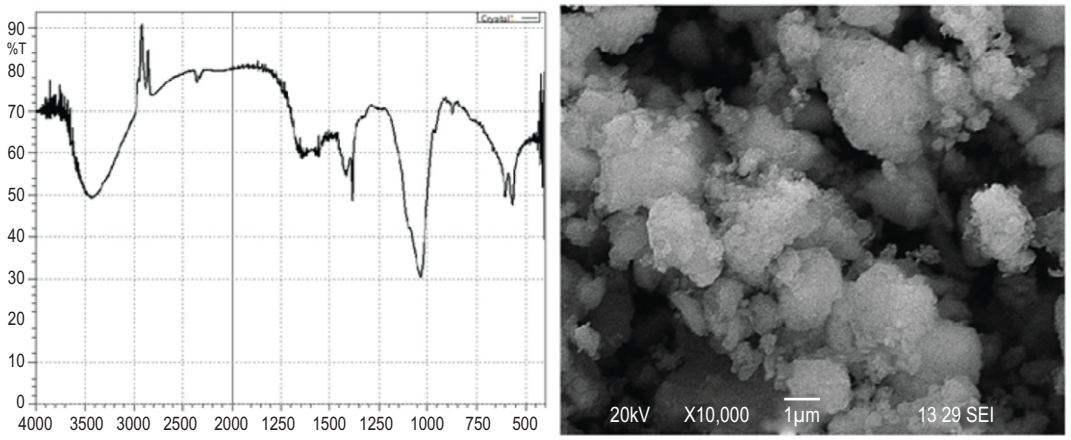
Results and Discussion

A wide range of drugs is available in the global market to manage inflammation and pain, however, utilization of these drugs as anti-inflammatory and analgesic agents is highly affected by several factors which include poor solubility, bioavailability which in turn influences the desired biological activity and undesirable side effects (Hishe *et al.,* 2018). As an alternative to these drugs, different medicinal plants have been traditionally used to treat different types of pain since antiquity. Hence, it is believed that natural products with a potential analgesic effect could be used in amalgamation to reduce some of the unwanted side effects associated with these traditional anti-pain medications as this approach enables decreased dosages of these analgesics without affecting efficacy. Encapsulation of bioactive phytoconstituents with natural and synthetic polymers has now gained more attention in biomedicine as this formulation increases stability, solubility and bioavailability.

Chitosan is an important biopolymer used to formulate important pharmacological products because of its high entrapment or encapsulation efficacy, biodegradability and biocompatibility. Among the various pain-inducing agents in various animal models, formalin is considered to be an important model of nociception as its high rate of reliability with specificity, ease of induction and marked responses are shown by the models exactly resemble clinical pain type (Demsie *et al.,* 2019). In this present work, marine derived chitosan-coated Triphala extract was tested for its enhanced antinociceptive effect against formalin-induced zebra fish nociceptive model. Marine derived



(a)



(b)

Fig. 1: Characterization of MDC and MDC coated *Triphala* (a) FTIR spectra and SEM micrographof extracted MDC; (b) FTIR spectra MDC coated *Triphala* and SEM micrograph of MDC coated *Triphala*.

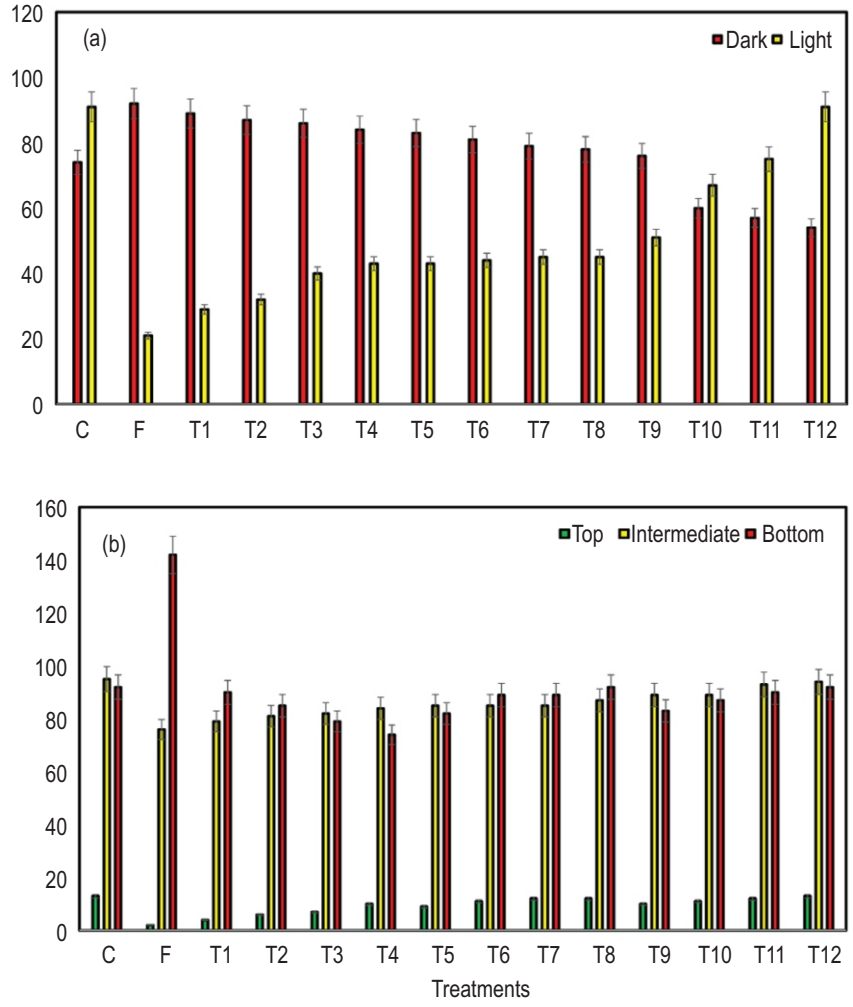


Fig. 2: Antinociceptive activity: (a) Swimming pattern of zebrafish treatment group; (b) Dark-light cycle pattern of zebrafish treatment group.

chitosan used in this study was extracted from marine fungal strain *Aspergillus niger* a marine isolate of *Aspergillus niger* grown in the liquid media was used for chitosan extraction.

Alkali-treated mycelial biomass was extracted with acetic acid followed by a successive purification yield of 1.2% of chitosan. Preliminary characterisation of chitosan was done by FTIR analysis which reveals the characteristic sharp absorption bands corresponding to N-H, O-H, and c= o existed a 3425 to 2880 cm^{-1}, 1646 cm^{-1} and 1650 cm^{-1} compared to pure chitosan standard (Fig. 1a). A scanning electron microscopy study was also carried out to determine the particle morphology. SEM micrograph depicted in Fig. 1b shows aggregated rough crystalline particles with micro-scale dimensions. Marine derived chitosan-coated Triphala extract was done by a simple dispersion method. Characteristic changes in the absorption bands easily conformed to structural modification of marine derived chitosan with Triphala extract. Specific interaction of marine derived chitosan with the phytochemicals of Triphala was known to cause a notable shift in marine derived chitosan absorption peak with the existence of chitosan functional groups (Fig. 1a). SEM analysis also showed the characteristic changes in the morphology (Fig. 2b). In contrast to free marine derived chitosan, Triphala coated marine derived chitosan showed well-agglomerated polymer trapped particles with increased particle size. The average size of the marine derived chitosan was found to be bigger than freemarine derived chitosan, which confirms that Triphala phytochemicals were encapsulated or adsorbed on the membrane surface, which in turn increased the particle size.

Marine derived chitosan-coated Trehala extract formulation thus prepared was checked for antinociceptive activity against the zebrafish model. Zebrafish is an excellent vertebrate model and is increasingly used for diverse pharmacological screening owing to various salient features like short life span, ease of cultivation, transparent embryos and

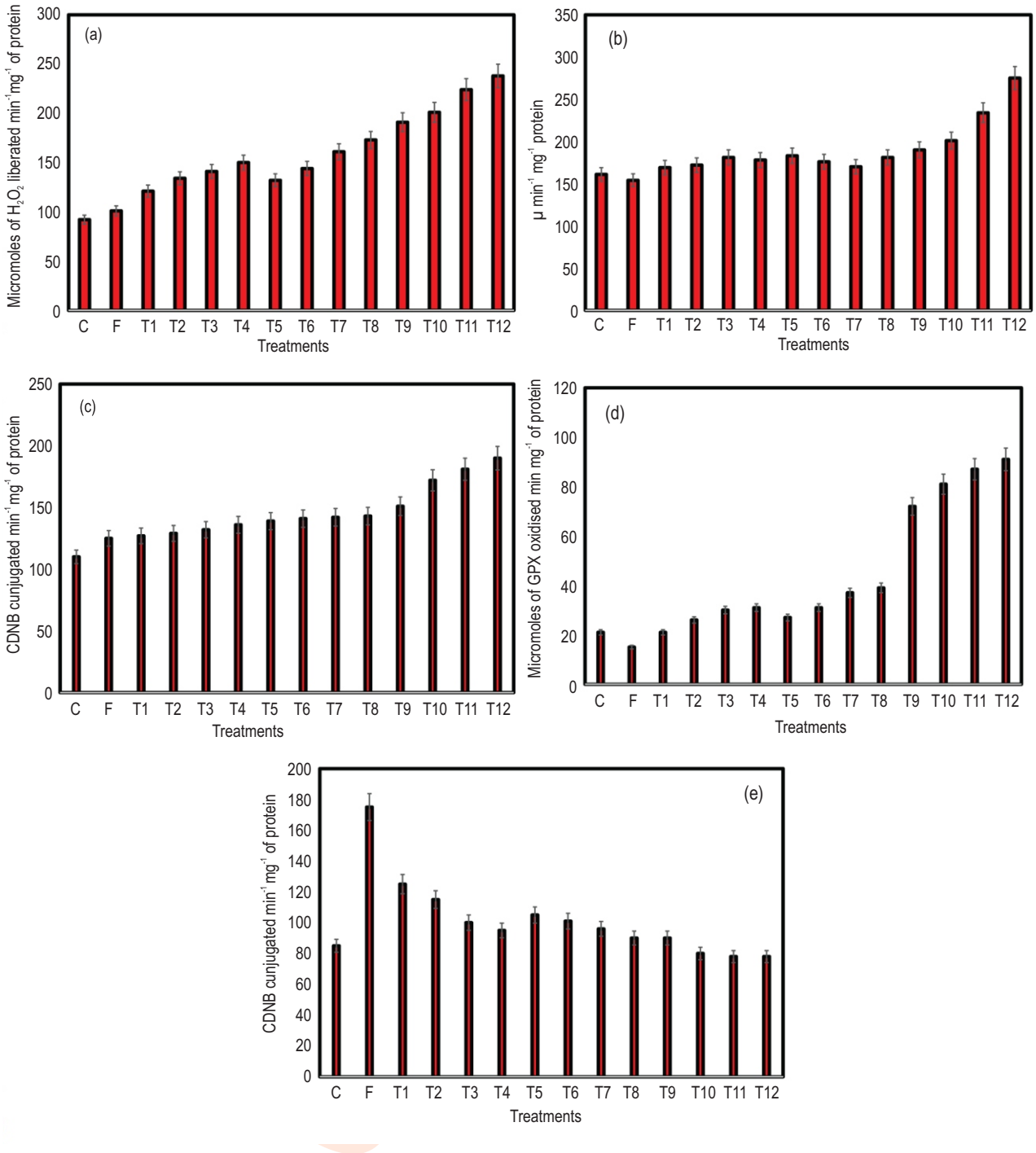


Fig. 3: Antioxidative enzyme activity (a) Catalase enzyme activity of zebrafish treatment group; (b) SOD enzyme activity of zebrafish treatment group; (c) GST enzyme activity of zebrafish treatment group; (d) GPO enzyme activity of zebrafish treatment group and (e) LPO enzyme activity of zebrafish treatment group.

considerable homology of genetic, and biochemical characteristics, which all provide a unique platform for using zebrafish as a versatile tool for efficacy screening and estimating the toxicity of chemicals (Sharma *et al.,* 2018). The zebrafish model extensively utilizes the antinociceptive activity of various drugs because of its well-developed physiological status similar to mammals and its response to toxic compounds. (Altenhofen *et al.,* 2017). This study initially carried out a behavioural analysis of

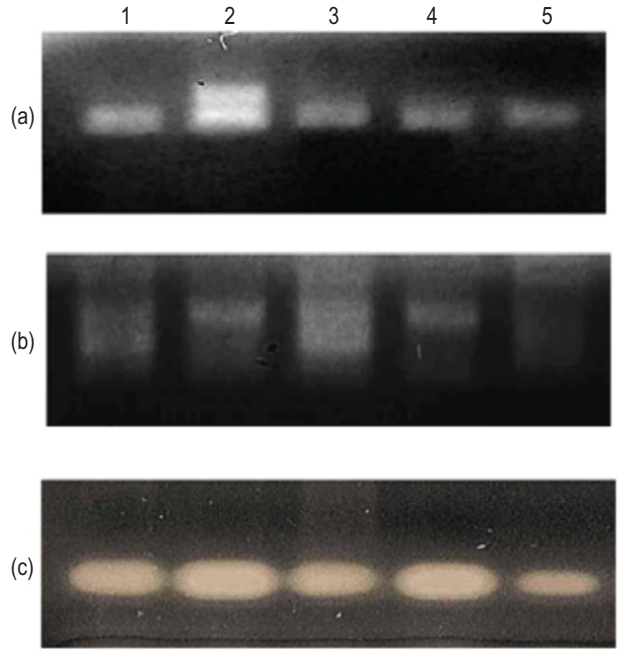


Fig. 4: Gene expression pattern of Zebrafish treatment group marker genes (a) iNOs; (b)TNF- alpha; (c) actin Lane 1- Control, Lane 2- Formalin group, Lane 3- Triphala (high dosage) group, Lane 4- marine-derived chitosan treatment group (high dosage), Lane 5- marine derived chitosan-coated Triphala group (high dosage).

respective treatment groups based on swimming patterns. The data were articulated as Mean and SD analysed by two-way analysis of variance using Graph Pad prism Ver 8.0 software. The values were highly significant (P-value <0.0001) at all the dosages of marine derived chitosan-coated Triphala extract compared to other treatment groups (Fig. 2a). The swimming pattern was observed at the top, intermediate and bottom levels for 5 min. Formalin-injected groups were settled at the bottom for a long duration whereas marine derived chitosan-coated Triphala groups were found to stay at the top level. Staying time at the bottom level significantly decreased when the concentration of marine derived chitosan-coated Triphala extract increased. All the concentrations of marine derived chitosan-coated Triphala (20 µg ml^{-1}) showed similar swimming behaviour as a control group.

The top entry duration of the induced group was very low compared with the control group and treated groups. Administration of toxic compounds to zebrafish may cause anxiety and reduce locomotor activity. It is well known that the reduction in swimming distance and speed shows marked evidence of a stimulus-induced change in behaviour (Ahmad *et al.,* 2018). Formalin-induced anxiety may reduce locomotor activity in the induced treatment group which can be easily inferred from the swimming data. Ethanol extract of neem fruit exhibited note worthy antinociceptive activity in the formalin-induced adult zebra fish model. Their results indicate that the administrated extract significantly reduced nociceptive behaviour. Abubakar *et al.* (2020) tested methanol extract of *Chlorophytum alismifolium* tubers against acetic acid-induced mice model and their findings revealed that the administrated extract significantly enhanced anti-nociceptive activity. Marked reduction of nociception in acetic acid-induced zebrafish model treated with methanol extract of *Bacopa monnieri* was reported recently by Sharma *et al.* (2022). A dark and light test was carried out to study the antinociceptive activity. Recent studies have elicited noxious stimuli (exposure to chemicals) and behavioural response in zebrafish characterised by reduced locomotor activity. In addition to the nociceptors already mentioned, excitatory and inhibitory neurotransmitter systems have been identified in zebrafish, such as dopaminergic, serotoninergic, cholinergic, purinergic and histaminergic, nitrergic, glutamatergic, glycinergic, and GABAergic systems (Hishe *et al.,* 2018). In this study, the dark-light test was done followed by measuring the swimming parameters to determine antinociceptive activity.

The dark-light test is commonly used to study the behavioural changes in zebrafish (Maximino *et al.* 2010). The light and dark duration were observed between control, induced and treated groups for 5 min. The values were highly significant (P-value <0.0001) in marine derived chitosan-coated Triphala treatment which showed that the rate of exposure to light zone was higher than remaining treatment groups (Fig 2b). Marine derived chitosan- coated Triphala extract treatment group spent more time in the light zone, and the maximum exposure time to light zone was recorded at 20 µg ml^{-1}. In contrast, the formalin-induced group spent little time in the light zone. The sustained or controlled release of bioactive phytochemical constituents of Triphalafrom chitosan coating may modulate the anxiety and locomotor activity of formalin-induced zebra fish treatment groups which in turn maintain normal homeostatic mechanism.

Changes in the respective treatment group's colour also reveal the behavioural changes Formalin treatment group showed changes in the tail colour to light brown. In contrast, no changes in colour were recorded in the plant extract treatment group. The colour change is also a crucial behavioural parameter that responds to undesirable stimuli that may intercept normal physiology molecular mechanisms. In this study, formalin induction may trigger some inflammatory signals followed by the accumulation of neutrophils in the tail, which causes change in colour in the tail. Sireeratawong (2012) evaluated the antinociceptive activity of Triphala recipein an ethyl phenylpropiolate-induced rat model. Their studies suggest that the antinociceptive action of Triphala recipe was due to inhibition of synthesis and release of inflammatory pain mediators. The present investigation showed the in flammatory cells' accumulation in the formalin-induced groups' tail region, which may be the reason for colour change. The absence of colour change in the marine derived chitosan-coated Triphala treatment group reveals the potential bioactive compounds in the extract may protect the animals from formalin toxicity and maintain normal homeostatic conditions. Antinociceptive activity of various

plant extracts using various animal models has been reported. Rao *et al.* (2017) studied the antinociceptive activity of ethanolic extract of *Fumaria indica* whole plant and methanolic extract of *Clinacanthus nutans* Lindau leaves in a mice model. However, no reports on the polymer encapsulated or coated plant extracts based on antinociceptive activity against zebrafish model. Chitosan is an important biopolymer as cellulose consists of glucosamine and N-acetylglucosamine with unique biocompatibility, high charge density, non-toxicity and mucoadhesion (Pujari and Pandharipande, 2016).

The effect of marine derived chitosan-coated Triphala on the antioxidative enzymes, including lipid peroxidase, was also investigated. Determination of various antioxidative enzymes' status is an indicator of oxidative stress. Oxidative stress is due to the imbalance of reactive oxygen species. (ROS is a critical factor that mediates the transformation of normal cells into cancerous cells or development of various diseases (Patwardhan *et al.,* 2004). Cells have a unique mechanism by which they protect from oxidative stress-related damages. Such a mechanism includes mainly antioxidative enzymes like catalases, glutathione s transferases, and superoxide dismutase (Dastmalchi *et al.,* 2007). Antioxidative enzymes, mainly CAT, SOD and GST are more appropriate markers for oxidants exposure (Davis *et al.,* 2004). In this study, the antioxidant content of the enzyme treatment group showed more variation than the control group (Fig. 3). The total content of CAT, SOD, GPO and GST increased in the marine derived chitosan-coated Triphala treatment group. In the case of LPO, no marked effect was recorded in marine derived chitosan-coated Triphala treatment, whereas enhanced LPO in the formalin treatment group showed a drastic increase in oxidative stress. As in the control group, the marine derived chitosan-coated Triphala treatment showed moderate LPO activity, indicating that lipid peroxidation was not triggered due to the suppression of toxic radicals by the marine derived chitosan-coated Triphala. Followed by determining behavioural changes, the effect of plant extract on the gene expression pattern of iNOs, alpha TNF was studied qualitatively. Respective gene primers were designed with the NCBI Primer-BLAST tool. PCR amplified products were resolved in agarose gel electrophoresis. Amplified DNA bands resolved in agarose gel showed the expression pattern (Fig. 4).

In the case of alpha TNF and iNOs, the formal in treatment group showed high expression. High-level expression of TNF and iNOs is known to cause oxidative stress, which further promotes undesirable disease progression (Zhang *et al.,* 2019). A notable reduction pattern of inflammatory genes after MARINE DERIVED CHITOSAN coated Triphala treatment inhibits disease progression. Based on the results of the present study, marine chitosan-coated Triphala significantly reduced the nociception effects in formalin-induced zebrafish model. Chitosan is an important biopolymer used as an effective drug carrier system due to its unique functional groups and its biodegradability, and biocompatibility properties (Rastogi *et al.,* 2021). It can be seen that the various phytochemical constituents in Triphala were released in a sustained manner from the chitosan that coated the Triphala extract. Released phytochemical constituents in the extract may act on the formalixcitatory and inhibitory neurotransmitter systems, which enhanced the normal behavioural responses.

In conclusion, the formulation proposed in this present investigation has this distinct antinociceptive effect against the formalin-induced zebrafish pain model. The mechanism of antinociceptive action is mainly due to the presence of potential bioactive metabolites in Triphala that are released from the membrane chemistry principles. Isolation, identification and interaction with nociceptive mediators will be useful to suggest the possible utilization of marine derived chitosan for clinical use.

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