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Research Article

Keywords: Pinus roxburghii, aqueous extract, zebrafish, crowding stress, oxidative stress.

Posted Date: November 17th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11362758/v1

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Additional Declarations: No competing interests reported.

Effect of *Pinus roxburghii* needle aqueous extract on hepatic antioxidant system of Zebrafish (*Danio rerio*) under crowding stress

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ABSTRACT

Crowding stress is among the major environmental factor in aquaculture that disrupts redox homeostasis in fishes, leading to oxidative damage and impaired physiological function. The present study evaluated the protective role of *Pinus roxburghii* needle aqueous extract on antioxidant mechanisms in zebrafish liver under crowding stress conditions. Adult zebrafish were exposed to two concentrations of the extract (1% and 10%) for 15 and 30 days, either alone or in combination with crowding stress.

Under normal conditions, no significant variations were in catalase, glutathione-S-transferase, reduced glutathione and lipid peroxidation indicating the absence of hepatotoxicity. Crowding stress alone alters the antioxidant enzyme activities and redox balance. Notably, co-treatment with extract mitigated stress-induced oxidative damage by restoring antioxidant enzyme activities, particularly at higher concentration. Histopathological observations also corroborated the biochemical findings demonstrating the potential of *P. roxburghii* potential as a natural phytogenic supplement for improving stress tolerance and liver health in aquaculture systems.

KEYWORDS

Pinus roxburghii; aqueous extract; zebrafish; crowding stress; oxidative stress.

1. INTRODUCTION

Aquaculture is one of the fastest-growing sectors of food production globally; however, the intensification of culture practices has led to increased exposure of fish to various environmental and husbandry-related stressors (Cai et al., 2023). Among these, crowding stress resulting from high stocking densities is a critical challenge, as it is routinely employed to

maximize production efficiency (Song et al., 2026). Prolonged crowding has been shown to adversely affect fish physiology, behavior, and immune competence, ultimately compromising overall fish health and survival (Li et al., 2025).

Crowding stress induces a cascade of physiological responses, primarily mediated through the activation of the hypothalamic-pituitary-inter renal (HPI) axis, leading to elevated cortisol secretion and enhanced metabolic activity (Fernando et al., 2024). This stress response is often accompanied by excessive reactive oxygen species (ROS), resulting in oxidative stress when antioxidant defense mechanisms are overwhelmed. Oxidative stress plays a central role in stress-mediated toxicity, causing lipid peroxidation, protein oxidation, DNA damage, and disruption of cellular integrity (Ma et al., 2025).

The liver is a vital organ involved in metabolism, detoxification, and antioxidant regulation and is therefore particularly susceptible to oxidative damage under stress conditions. Alterations in hepatic antioxidant enzymes such as superoxide dismutase, catalase, glutathione-S-transferase, and reduced glutathione, and along with increased lipid peroxidation, are widely recognized biomarkers of oxidative stress in fish (Hamed et al., 2025). Histopathological changes in liver tissue further serve as sensitive indicators of stress-induced cellular injury and functional impairment.

The zebrafish (*Danio rerio*) has emerged as a powerful vertebrate model organism in ecotoxicology and stress physiology due to its genetic similarity to higher vertebrates, small size, rapid development, and well-characterized antioxidant and stress-response systems (Lundin and Keiter, 2025). Zebrafish exhibit pronounced and histopathological responses to environmental stressors, making them an ideal model for evaluating oxidative stress mechanisms and hepatoprotective interventions (Eachus et al., 2021).

In recent years, there has been growing interest in the use of plant-derived bioactive compounds as natural antioxidants to mitigate stress-induced oxidative damage in aquaculture species. Phytochemicals such as phenolics, flavonoids, terpenoids, and alkaloids possess strong free radial scavenging, metal-chelating, and anti-inflammatory properties, offering a sustainable alternative to synthetic additives (Vasile et al., 2025). The incorporation of plant-based reported to enhance antioxidant enzyme activities, reduce lipid peroxidation, and improve tissue integrity in the fish exposed to environmental stressors.

Pinus roxburghii (Chir pine), a widely distributed conifer species in the Himalayan region has been traditionally used in ethno-medicine and is known to process antioxidants, anti-

inflammatory, antimicrobial, and hepatoprotective properties (Sharma et al., 2025). The needles of *P. roxburghii* are rich in bioactive constituents, including polyphenols, flavonoids, and terpenoids, which contribute to their strong antioxidant potential (Zia-Ul-Haq et al., 2023). Despite these documented pharmacological properties, scientific evidence regarding the efficacy of *P. roxburghii* needle extract in alleviating stress-induced oxidative damage in fish is scarce.

To date, limited studies have explored the role of *P. roxburghii* in aquatic models, particularly under conditions of crowding stress. Understanding its potential as a natural antioxidant supplement is crucial for developing eco-friendly strategies to enhance fish resilience and welfare in intensive aquaculture systems.

Therefore, the present study was designed to investigate the ameliorative effects of aqueous *Pinus roxburghii* needle extract against crowding-induced oxidative stress in zebrafish (*Danio rerio*). The study evaluated alterations in hepatic antioxidant defence enzymes, lipid peroxidation levels, liver histopathological levels, and liver histopathological to elucidate the antioxidative and hepatoprotective potential of *P. roxburghii* under crowding stress conditions.

2. MATERIAL AND METHODS

2.1 Plant extract used and procurement of fish

Fresh green needles of *Pinus roxburghii* were collected from Bajhol Village, located within the campus of Shoolini University of Biotechnology and Management Sciences, Solan, India, and authenticated by the department of Botany (Voucher No. SUBMS/BOT-5281). The collected needles were shade-dried at room temperature and pulverized into fine powder. For aqueous extraction, 20 g of the powdered material was soaked in 200 ml of distilled water and subjected to continuous agitation on an orbital shaker at 120 rpm for 48 h. The extract was then subsequently filtered through Whatman No. 1 filter paper, and the filtrate was evaporated at 37°C in a hot air oven to obtain the crude aqueous extract. The dried extract was weighed, transferred into sterile stock vials, and stored at 4°C for further experimental use.

Phytochemical profiling of the *P. roxburghii* aqueous needle extract was performed using liquid chromatography-mass spectrometry (LC-MS; SYNAPT-XS HDMS, UK, Model No. DBA064) at the Sophisticated Analytical Instrument Facility (SAIF), Panjab University, Chandigarh. Chromatographic separation was carried out on a C18 Waters Acquity BEH

column (2.1 × 100 mm, 1.7 µm) with an injection volume of 5 µl, a flow rate of 0.2 ml min^{-1}, and a total run time of 45 min.

Healthy adult zebrafish (*Danio rerio*), measuring 3± 1 cm in length and weighing 0.65 ± 0.13 g, were procured from licensed vendor in Chandigarh, Punjab, for experimental purposes. The fish were transported in oxygen-filled polyethene bags and acclimatized for 15-30 days in glass aquaria (30x30x30 cm) under standard laboratory conditions following ethical approval. A total of 111 fish were randomly into distributed in six experimental groups, each maintained in triplicates. The exposure was conducted under semi-static conditions, with approximately 80% of the water replaced daily. Faecal matter was removed from the bottom of the tanks each day, half an hour after morning feeding, to ensure proper maintenance of water quality.

Water quality parameters were monitored at two-day intervals using a standard test kit. The maintained parameters included nitrate: 10 mg/dm^{-3}, nitrite: 0 mg dm^{-3}, total hardness: 120–180 mg dm^{-3}, pH: 7.2 ± 0.5, and chlorine: 0 mg dm^{-3}. Water temperature was maintained at 24 ± 2 °C, dissolved oxygen at 6.5 ± 0.2 mg L^{-1}, and electrical conductivity at 0.256 ± 0.005 mS cm^{-1}. A 12h light: 12h dark photoperiod was maintained throughout the experimental period. Fish were fed a commercial basal diet at 2% of their body weight per day, divided into two equal feedings (0.006 g per feeding) administered in the morning and evening.

2.2 Experimental setup

The zebrafish were randomly allocated into six experimental groups, each maintained in triplicate. Fish were divided into control and treatment groups and observed for exposure period of 15 and 30 days. Experimental outcomes were compared with those of the corresponding control groups maintained over the same duration. No baseline sampling was conducted at day zero, ethical clearance permitted the use of a limited number of fish, and therefore, comparisons were restricted to treatment and control groups at the respective exposure time periods.

The experimental treatments included control group, two groups exposed to aqueous needle extract of *Pinus roxburghii* at concentrations of 1% and 10%, a crowding stress group (negative control), and two combined treatment groups subjected to both extract exposure and crowding stress. The groups were designated as follows: Group I- Control; Group II – *P. roxburghii* (1 % Conc.); Group III – *P. roxburghii* (10% Conc.); Group IV- Crowding Stress (Negative control); Group V- *P. roxburghii* (1% Conc.) + Crowding Stress; Group VI- *P. roxburghii* (10% Conc.) + Crowding Stress.

In accordance with CPCSEA (2021) guidelines, a stocking density of five adult zebrafish per liter of water was maintained as the standard condition. To induce crowding stress experimentally, the stocking density was increased to ten fish per liter, corresponding to thirty fish maintained in three liters of water. Throughout the exposure period, continuous aeration and optimal water quality parameters were maintained to ensure adequate oxygen availability and to minimize confounding stress factors.

2.3 Feed preparation

Zebrafish were fed a commercial aquarium diet formulated to meet their nutritional requirements for growth and health. The control diet consisted of a commercially available feed, *Optimum*, containing 28% protein, 3% fat, 4% fibre, and 12% moisture. This was followed by supplementation with fish flakes (*Bugs-In*), were used as a dietary supplement and contained 42% protein, 12% fat, 5% fibre, 9% ash, 10% moisture, 1.50% calcium, 0.80% phosphorus, and 1.10% methionine. Experimental diets were prepared by supplementing the basal feed with *P. roxburghii* needle aqueous extract at concentrations of 0% (control), 1% (low concentration), and 10% (high concentration) per kg^{-1} of feed. The required amount of extract was first dissolved in distilled water and then thoroughly mixed with the feed ingredients to ensure uniform distribution. The feed was subsequently cooled at -20°C, pelleted using a mechanical grinder, and sieved through a 1.2mm mesh to obtain pellet uniform size. The prepared feed was air-dried at room temperature and stored at 4°C until future use.

2.4 Ethical clearance

All experimental procedures involving zebrafish were conducted in accordance with the ethical standards of the Institutional Animal Ethical Committee (IAEC) and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Shoolini University, Solan. Ethical clearance for the study was obtained under reference number **IAEC/SU/22/14**. All fish were cared for, handled, and euthanized following the CPCSEA and guidelines to ensure animal welfare and minimize stress or suffering throughout the experimental period.

2.5 Euthanasia

During the experimental period, *Danio rerio* were humanly euthanized using eugenol, the active component of clove oil, in accordance with CPCESA-recommended practices. As eugenol is insoluble in water, it was appropriately prepared prior to use to ensure effective

administrative. Eugenol (clove oil) was selected as a euthanizing agent due to its rapid induction, cost-effectiveness, making it suitable for the use in fish research.

2.6 Liver histological analysis

For histopathological evaluation, freshly dissected liver tissues were fixed immediately in a suitable fixative to preserve tissue architecture. After fixation, tissues were dehydrated through a graded series of ethanol concentrations, cleared in xylene, and embedded in paraffin wax using standard histological procedures. Paraffin blocks were sectioned at a thickness of approximately 4-5 µm using a rotary microtome.

The tissue sections were mounted on glass slides, deparaffinized, rehydrated, and stained with haematoxylin and eosin (H&E) for general histological assessment. Haematoxylin was used to visualize nuclear morphology, while eosin provided contrast for cytoplasmic and extracellular components (Baker, 1945; Pearse, 1968). Stained sections were examined under a light microscope to evaluate structural integrity and cellular organization of hepatic tissue.

Histopathological alterations such as hepatocyte degradation, cytoplasmic vacuolization, nuclear abnormalities, sinusoidal dilation, and disruption of hepatic cords were assessed qualitatively and compared across experimental groups. The extent of tissue damage was correlated with biochemical findings to better understand the protective role of *Pinus roxburghii* needle extract against crowding-induced hepatic oxidative stress.

2.7 Measurement of antioxidant parameters

Liver tissues were excised immediately after euthanasia, rinsed in ice-cold physiological saline to remove excess blood, blotted dry, and weighed. The tissues were homogenized (10% w/v) in ice-cold phosphate buffer (pH 7.4) using a chilled homogenizer to prevent enzymatic degradation. The homogenates were centrifuged at 10,000 × g for 15 minutes at 4 °C, and the resulting supernatants were collected for biochemical estimations.

The activities of key antioxidant enzymes were assessed to evaluate the oxidative stress status of hepatic tissue. Superoxide dismutase (SOD) activity was measured based on its ability to inhibit the autoxidation of reactive oxygen species, reflecting the first line of enzymatic antioxidant defence (Kono, 1978). Superoxide anions are produced when hydroxylamine HCL auto-oxidizes. The reduction of NBT to blue formazan is caused by these anions. SOD prevents hydroxylamine HCL-induced NBT decrease. Catalase (CAT) activity was determined by monitoring the decomposition of hydrogen peroxide, indicating the tissue's capacity to

neutralize hydrogen peroxide generated during oxidative metabolism (Lück, 1965). The reaction was initiated by adding H_2O_2 solution, and the absorbance decrease was measured at 240 nm. Glutathione-S-transferase (GST) activity was estimated to assess the detoxification potential of liver tissue through conjugation reactions involving reduced glutathione (Habig et al., 1974). To measure GST activity towards CDNB (1-chloro-2,4-dinitrobenzene) spectrophotometrically at 340 nm.

Reduced glutathione (GSH) levels were quantified as a measure of non-enzymatic antioxidant capacity and intracellular redox balance (Beutler et al., 1963). Using spectrophotometry, the level of GSH was determined at 420 nm of wavelength. Lipid peroxidation (LPO) was estimated by measuring malondialdehyde (MDA) formation, which serves as an indicator of oxidative damage to membrane lipids (Beuge, 1978). All biochemical assays were carried out using standard spectrophotometric methods under controlled conditions and were performed in triplicate to ensure analytical reliability and reproducibility.

2.8 Statistical analysis

Data were expressed as mean ± standard error (SE). Statistical analyses were performed using two-way ANOVA, followed by a Tukey hoc test, with IBM-SPSS software. A probability value $p < 0.05$ was considered statistically significant. Additionally Principal Component Analysis (PCA) was employed to determine the primary parameter contributing to individual variability in histopathological changes.

3. Results and discussion

3.1 Phytochemical screening of *Pinus roxburghii* needle aqueous extract

The aqueous needle extract of *Pinus roxburghii* analysed in this study exhibited the presence of wide range of bioactive phytochemical compounds, including flavonoids, alkaloids, tannins, steroids, carbohydrates, terpenoids, glycosides, and phenolics compounds. The chromatogram obtained from the Lipid Chromatography- Mass Spectrometry (LC-MS) analysis confirmed the presence of these phytoconstituents, reflecting the chemical complexity of the extract (Figure 3.1).

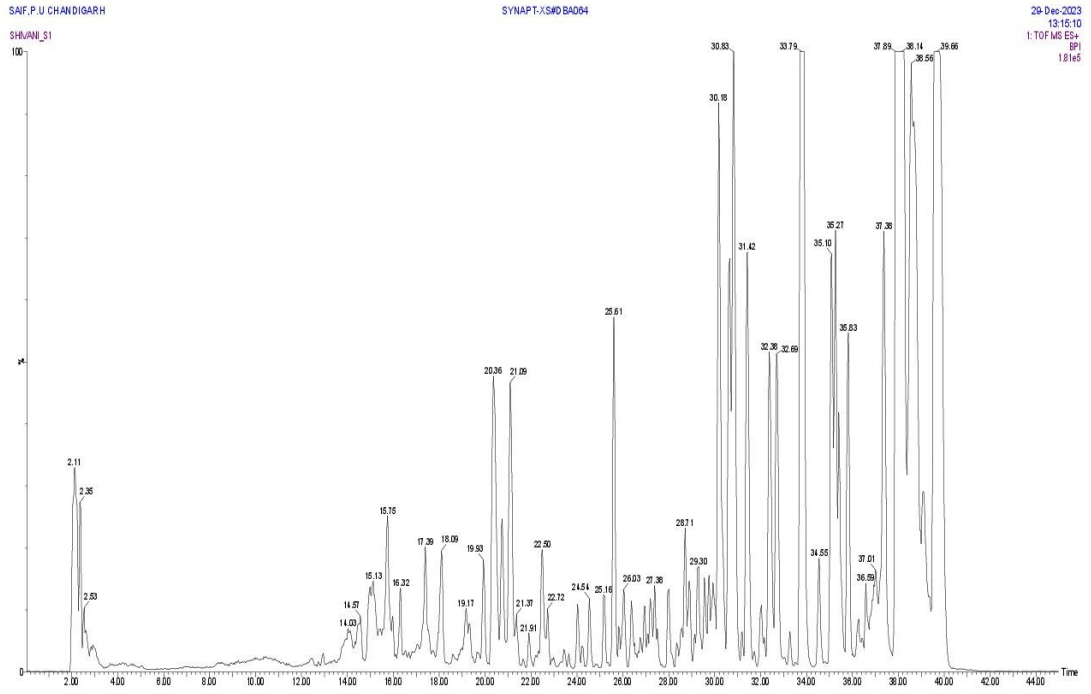


Fig. 3.1: Chromatogram of aqueous extract of *Pinus roxburghii* needle aqueous extract.

Comparative phytochemical screening of methanolic, hexane, and aqueous extracts of *P. roxburghii* needles further revealed higher concentration of xanthoproteins, phenols, sterols, saponins, and alkaloids in the methanolic and aqueous extract. These findings indicate that *P. roxburghii* needles are rich in diverse secondary metabolites with potential antioxidant and therapeutic properties.

Qualitative phytochemical analysis was performed to identify the presence bioactive compounds using standard phytochemical tests, as described by Singh et al. 2022. The analysis included test for major classes of phytochemicals such as tannins, flavonoids, alkaloids, phenolic compounds, glycosides, and saponins, all of which are known for their medicinal and biological significance (Yu et al., 2021). The variation in extract yield among different solvents was primarily attributed to differences in solvent polarity, with more polar solvents generally exhibiting higher extraction efficiencies compared to less polar solvents. Additionally, phytochemical composition and extraction efficiency may be influenced by factors such as the geographical origin, climatic conditions, plant species, organ type, developmental stage, collection period, preservation method, and extraction techniques (Bouzenna et al., 2021).

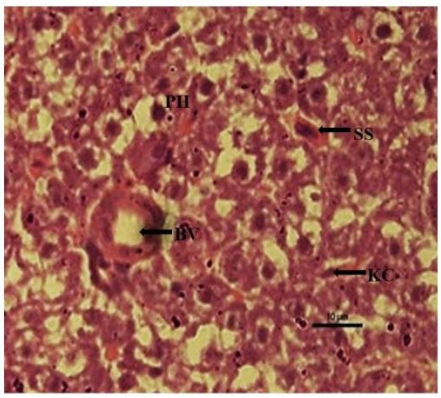
Previous studies have reported that distilled water serves as an effective solvent for extracting a broad range of phenolic compounds (Bouzenna et al., 2021). However, investigations on *Pinus* species have indicated that compounds exhibiting antioxidant activity, particularly those involved in free-radicals scavenging and reducing mechanisms, can be efficiently extracted using aqueous and moderately polar solvent systems (Barros et al., 2025).

LC-MS analysis of *P. roxburghii* needle extract in the present study identified numerous bioactive constituents belonging to diverse chemical classes, including fatty acids, flavonoids, aldehydes, and other secondary metabolites such as alkaloids and phenols. Previous studies have demonstrated that both needle and bark extracts of *P. roxburghii* possess growth-inhibitory activity against *Escherichia coli* and *Staphylococcus aureus* (Kanchan et al., 2020; Singh et al., 2020). Similarly, aqueous extract of *Pinus massoniana* needles (PNAE) have been shown to inhibit the growth of several spoilage and pathogenic bacteria, including *Bacillus subtilis*, *Proteus vulgaris*, *Bacillus cereus*, *Micrococcus luteus*, *E. coli*, and *S. aureus* (Zhang et al., 2023).

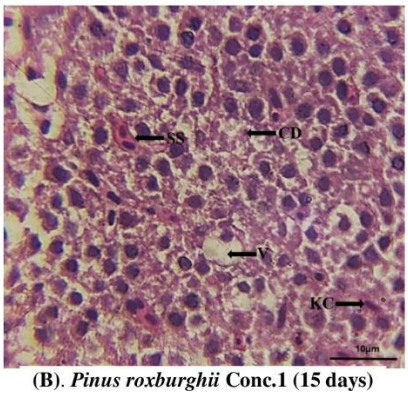
The secondary metabolites identified in *P. roxburghii* can be broadly categorized into three major groups: 1) aliphatic compounds- including terpenes, resin acids, terpenoids, sterols, waxes, fatty acids, and fats; 2) phenolic compounds: such as phenols, tannins, flavonoids, and stilbenes; 3) including sugars and alkaloids (Metsämuuronen and Sirén, 2019). Among these, flavonoids are of particular interest due to their well-established clinical, biological, and nutraceutical importance (Li et al., 2012; Stachelska et al., 2025). Plant species from temperate regions, including *P. roxburghii,* are known to synthesize a wide array of secondary metabolites with fungicidal, fungistatic, or bactericidal properties, contributing to their ecological resilience and pharmacological potential (Ganthaler et al., 2017; Routa et al., 2017).

3.2 Histopathological analysis

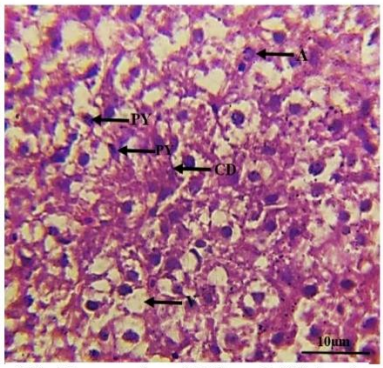
Three liver samples from each experimental tank were collected after 15 and 30 days of exposure for histopathological examination. Representative photomicrographs of haematoxylin and eosin (H&E)-stained liver sections are presented in Fig. 3.2. Histopathological evaluation of the liver was performed as it is a central organ involved in metabolism, detoxification, and nutrient processing and is highly sensitive to environmental and dietary stressors, making it a reliable biomarker for toxicological assessment in fish (Moussa et al., 2022; Abdallah et al., 2024).



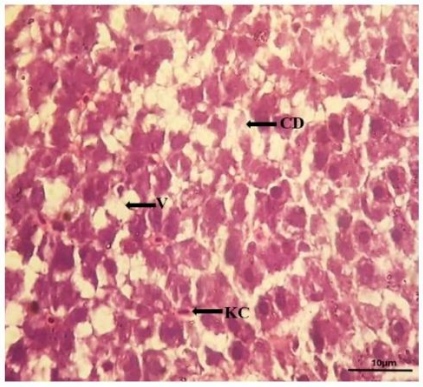
(A) Control



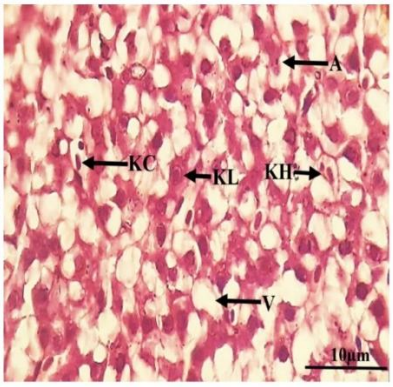
(B). *Pinus roxburghii* Conc.1 (15 days)



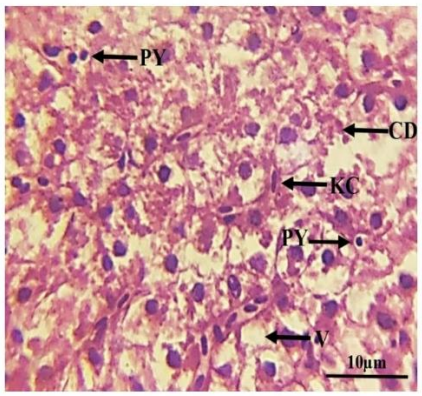
(C). *Pinus roxburghii* Conc.1 (30 days)



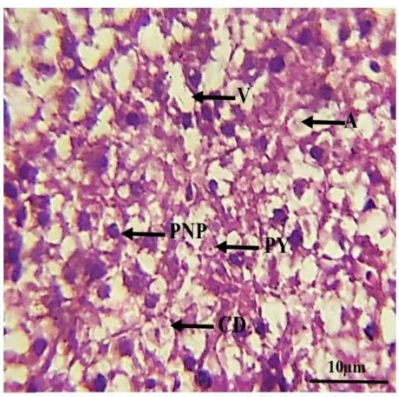
(D). *Pinus roxburghii* Conc.2 (15 days)



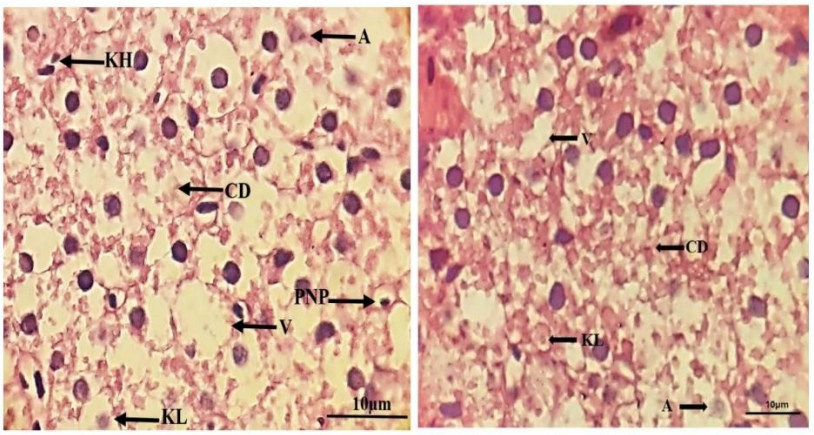
(E). *Pinus roxburghii* Conc.2 (30 days)



(F). Negative control (15 days)

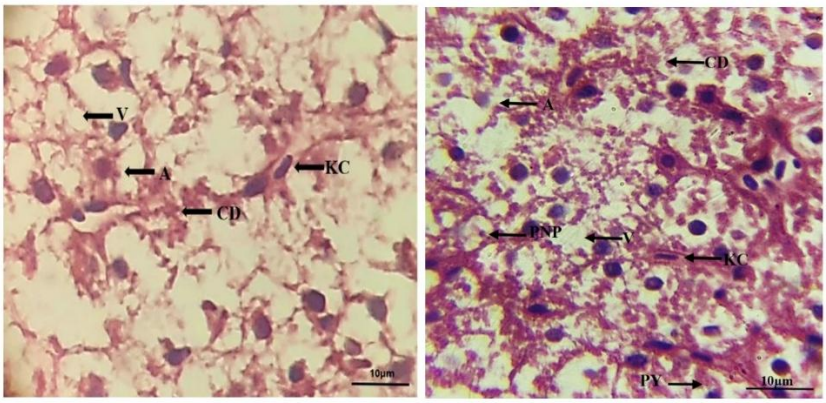


(G). Negative control (30 days)



(H). *Pinus roxburghii* Conc.1+ Crowding stress (15 days)

(I). *Pinus roxburghii* Conc.1+ Crowding stress (30 days)



(J). *Pinus roxburghii* Conc.2+ Crowding stress (15 days)

(K). *Pinus roxburghii* Conc.2+ Crowding stress (30 days)

Fig. 3.2 : A-K: Represents the histopathological changes in the liver of Zebrafish (*Danio rerio*) exposed to different concentrations of *Pinus roxburghii* needle aqueous extract on 15- and 30-days exposure; BV- Blood vessels; PH- Polygonal hepatocytes; KC- Kupffer cells; PNP- Pushing of nucleus to the periphery; KH- Karyorrhexis; KL- Karyolysis; PY- Pyknosis; SS- Sinusoidal Space; A- Apoptosis; V- Vacuolation; CD- Cytoplasmic degeneration.

The liver of control zebrafish exhibited normal histological architecture, characterized by well-organised polygonal hepatocytes arranged in branching and anastomosing cords, separated by well-defined blood sinusoids (Fig. 3.2A). Hepatocytes displayed granular cytoplasm with centrally located vesicular nuclei, indicating active metabolic and synthetic functions (Ahmed, 2022). The hepatic sinusoids were clearly visible and connected to the central vein, facilitating efficient microcirculation of blood derived from the portal vein and hepatic artery (Miyai, 2020).

Kupffer cells, the resident hepatic macrophages located within the sinusoidal lumen, were also observed and appeared normal in morphology (Fig. 3.2A-B). These cells play a critical role in the immune surveillance and maintenance of hepatic homeostasis by phagocytizing foreign particles and cellular debris (Santa, 2023). Overall, the liver histology of control fish reflected a healthy and functionally intact hepatic tissue (AbouShabana et al., 2025).

Zebrafish exposed to *Pinus roxburghii* needle aqueous extract exhibited dose-dependent hepatic alterations. At the lower concentrations (1%), mild histological changes were observed, including slight distortion of hepatocyte shape and mild sinusoidal irregularities. These changes were generally reversible and did not markedly disrupt tissue organization. However, exposure to the higher concentration (10%), particularly for 30 days, resulted in more pronounced alterations, including hepatocellular vacuolization, nuclear displacement toward the peripheral nuclear positioning (PNP), and activation of kupffer cells (Fig. 3.2B-E).

Fish subjected to crowding stress alone exhibited clear pathological alterations including extensive cytoplasmic vacuolation, hepatocyte degeneration, and sinusoidal dilution. These changes suggest impaired metabolic activity and compromised hepatic integrity under prolonged stress conditions (Anshar et al., 2025). Similar stress-induced hepatic degeneration has been reported in zebrafish and other teleost species exposed to high stocking densities (Omnes et al., 2024).

Combined exposure to *P. roxburghii* extracts and crowding stress resulted in severe hepatic lesions, including extensive vacuolization, cytoplasmic disintegration, necrosis, disrupted blood sinusoids, and apoptotic features such as nuclear condensation and apoptotic body formation (Fig. 3.2H-K). Several hepatocytes exhibited pyknotic nuclei, indicating irreversible cellular damage and ongoing cell death (Myers et al., 1987; Shaw et al., 2019). Mild inflammatory infiltrates were also observed in necrotic regions, reflecting the liver's immune response to tissue injury (Lu et al., 2025).

Cytoplasmic vacuolization observed in treated fish is indicative of metabolic disturbances, potentially associated with reduced glycogen reserves and increased lipid accumulation-common cellular responses to oxidative stress and impaired energy metabolism. Hepatocellular necrosis and apoptosis further suggest that excessive reactive oxygen species (ROS) generation may disrupt cellular membranes and organelles integrity, ultimately impairing liver function (Wolf and Wheeler, 2018; Li et al., 2023).

Overall, the histopathological findings demonstrated that both *P. roxburghii* extract concentration and crowding stress significantly influence hepatic integrity in zebrafish. The observed alterations-ranging from mild vacuolar degeneration to severe necrosis and apoptosis-highlight the liver's vulnerability to combined dietary and environmental stressors. These results reinforce the importance of dose optimization when evaluating plant-based bioactive compounds and confirm the utility of liver histopathology as a sensitive tool for assessing sub-lethal toxicity in aquatic organisms.

3.3 Antioxidant parameters

In healthy fish, a well-coordinated enzymatic and non-enzymatic antioxidant defence systems is essential for neutralizing reactive oxygen species (ROS) and maintaining cellular redox homeostasis. Key enzymatic antioxidants, including catalase (CAT), superoxide dismutase (SOD), and glutathione-S-transferase (GST) play pivotal roles in this process, with the SOD-CAT pathway constituting the primary line of defence against oxidative stress-induced cellular damage.

3.3.1 Catalase (CAT) enzyme activity

CAT is a key antioxidant enzyme present in nearly all aerobic organism and catalyses the decomposition of hydrogen peroxide into water and molecular oxygen, thereby preventing oxidative injury to cellular components. In this present study, variations in hepatic catalase activity were evaluated in zebrafish exposed to the aqueous needle extract of *Pinus roxburghii*, as illustrated in Fig. 3.3.

A significant increase ($p < 0.05$) in hepatic catalase activity was observed in fish treated with both *P. roxburghii* concentration (Conc.1 and Conc.2) after 15 days of exposure compared to the control group. Following 30 days of treatment, catalase activity further increased in the higher-dose group (*P. roxburghii* Conc.2), indicating a clear dose-dependent enhancement of antioxidant defence.

Moreover, when compared with the crowding stress group (negative control), zebrafish co-exposed to *P. roxburghii* extract and crowding stress (*P. roxburghii* Conc. 1 + stress and *P. roxburghii* Conc. 2 + stress) exhibited a significant elevation in catalase activity at both 15 and 30 days. This enhanced enzymatic response suggests that *P. roxburghii* needle extract effectively strengthens hepatic antioxidant defence mechanism, thereby, alleviating oxidative stress induced by crowding conditions.

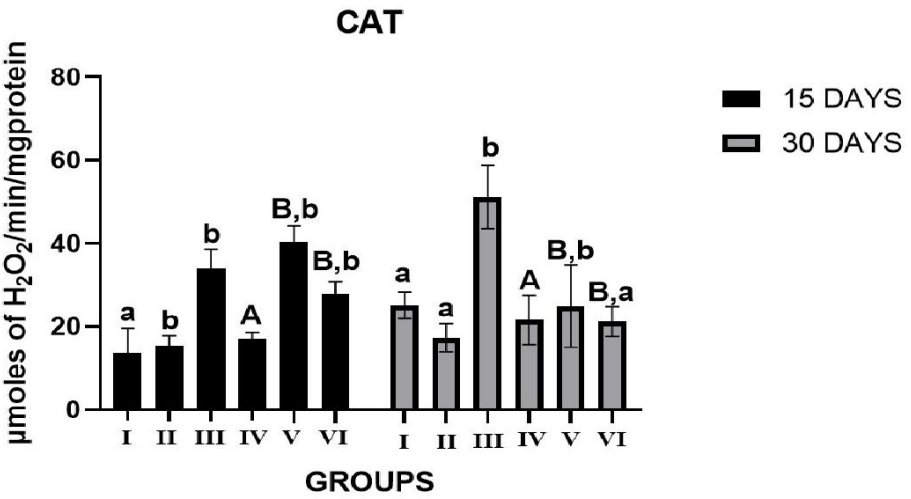


Fig. 3.3: Graph depicting the variations in CAT activity at the end of 15 and 30 days; where Group I: control group; Group II: *Pinus roxburghii* (Conc.1); Group III: *Pinus roxburghii* (Conc.2); Group IV: Crowding Stress (Negative control); Group V: *P. roxburghii* (Conc.1) + Crowding Stress; Group VI: *P. roxburghii* (Conc.2) + Crowding Stress. Values were analysed by two-way ANOVA followed by Tukey Post hoc Test. Small letters (a,b) indicate results with respect to control and capital letters (A,B) indicate results with respect to negative control. Different letters (b,B) were used to express significant variations ($p < 0.05$).

3.3.2 Superoxide dismutase (SOD) enzyme activity

This study evaluated variations in hepatic superoxide dismutase (SOD) activity in liver of zebrafish exposed to the aqueous needle of *Pinus roxburghii*, as illustrated in Fig. 3.4. SOD. Superoxide dismutase is a key antioxidant enzyme that catalyses the dismutation of superoxide radicals into hydrogen peroxide and molecular oxygen, thereby constituting an essential component of the cellular defence mechanism against oxidative stress.

A significant reduction ($p < 0.05$) in hepatic SOD activity was observed in the zebrafish treated with *P. roxburghii* extract at both concentrations-Group II (Conc. 1) and Group III (Conc. 2) after both 15 and 30 days of exposure, when compared with the control group. Similarly, in Group IV (negative control), SOD activity was markedly reduced, indicating enhanced oxidative stress under high-density rearing conditions.

Notably, zebrafish co-exposed to *P. roxburghii* extract and crowding stress exhibited concentration and time-dependent responses. In Group V (*P. roxburghii* Conc. 1 + Crowding Stress), a significant decline ($p < 0.05$) in SOD activity was recorded after 15 days of exposure.

In contrast, Group VI (*P. roxburghii* Conc. 2 + Crowding Stress) showed a significant increase ($p < 0.05$) in SOD activity relative to the negative control, supporting an enhanced antioxidant response at higher extract concentration. After 30 days, Group V demonstrated a notable recovery, with a significant elevation ($p < 0.05$) in SOD activity compared to the negative control group.

These findings indicate that higher concentrations of *P. roxburghii* needle extract may effectively enhance the antioxidant defence capacity of zebrafish under stress conditions, whereas lower concentrations may exert a transient or comparatively weaker effect on SOD activity.

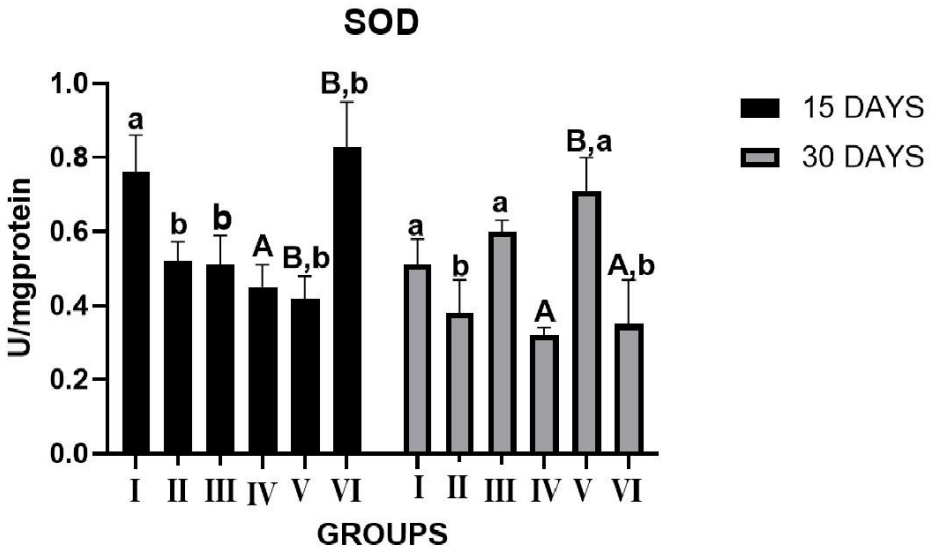


Fig. 3.4: Graph depicting the variations in SOD activity at the end of 15 and 30 days, where Group I: control group; Group II: *Pinus roxburghii* (Conc.1); Group III: - *Pinus roxburghii* (Conc.2); Group IV: Crowding Stress (Negative control); Group V: *P. roxburghii* (Conc.1) + Crowding Stress; Group VI: *P. roxburghii* (Conc.2) + Crowding Stress. Values were analysed by two-way ANOVA followed by Tukey Post hoc Test. Small letters (a,b) indicate results with respect to control, and capital letters (A,B) indicate results with respect to the negative control. Different letters (b,B) were used to express significant variations ($p < 0.05$).

3.3.3 Reduced glutathione (GSH) enzyme activity

Variations in hepatic glutathione (GSH) levels in zebrafish exposed to the aqueous needle extract exposed to *Pinus roxburghii* are presented in Fig. 3.5. Glutathione is a major non-

enzymatic antioxidant that plays a central role in cellular redox regulation and protection against oxidative damage.

A significant increase ($p < 0.05$) in hepatic GSH concentration was observed in Group II and Group III (*P. roxburghii* Conc. 1 and Conc. 2) after 15 days of exposure compared with the control group. Prolonged exposure for 30 days further elevation of GSH levels, with the higher concentration group (Conc. 2) showing a more pronounced response.

Relative to the negative control (Group IV, crowding stress), zebrafish treated with *P. roxburghii* extract exhibited significantly higher GSH levels at 15 days, indicating a protective antioxidant response under stress conditions. However, after 30-day of exposure, GSH levels declined significantly compared to the negative control, suggesting depletion of glutathione reserves under sustained oxidative stress.

Collectively, these results indicate that *P. roxburghii* needle extract enhances the hepatic antioxidant capacity during early exposure, whereas prolonged treatment under combined stress conditions may disrupt glutathione homeostasis.

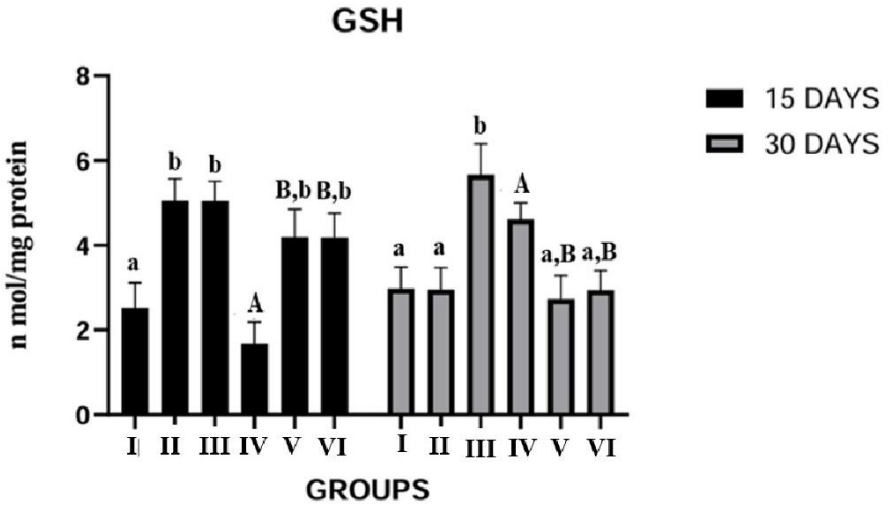


Fig. 3.5: Graph depicting the variations in GSH activity at the end of 15 and 30 days; where Group I: control group; Group II: *Pinus roxburghii* (Conc.1); Group III: *Pinus roxburghii* (Conc.2); Group VI: Crowding Stress (Negative control); Group V: *P. roxburghii* (Conc.1) + Crowding Stress; Group VI: *P. roxburghii* (Conc.2) + Crowding Stress. Values were analysed by two-way ANOVA followed by Tukey Post hoc Test. Small letters (a,b) indicate results with respect to control and capital letters (A,B) indicate

results with respect to negative control. Different letters (b,B) were used to express significant variations ($p < 0.05$).

3.3.4 Glutathione s-transferase (GST) enzyme activity

Variations in hepatic glutathione-S-transferase (GST) activity in zebrafish exposed to the aqueous needle extract of *Pinus roxburghii* are presented in Fig. 3.6. GST is a key phase II detoxification enzyme involved in the conjugating of reduced glutathione with electrophilic compounds, thereby facilitating the elimination of reactive metabolites and xenobiotics.

A significant increase ($p < 0.05$) in hepatic GST activity was observed in Group II (*P. roxburghii* Conc. 1) after 15 days of exposure compared with the control group. After 30 days, GST activity was further elevated ($p < 0.05$) in both Group II (*P. roxburghii* Conc. 1) and Group III (*P. roxburghii* Conc. 2) relative to the corresponding control, indicating a dose- and duration-dependent induction of GST activity.

In contrast, under crowding stress conditions, GST activity declined significant ($p < 0.05$) in Group V and Group VI (*P. roxburghii* + crowding stress) at both 15 and 30 days when compared with the negative control (Group IV). This reduction suggests that prolonged or combined stress may impair hepatic detoxification capacity, potential due depletion of glutathione reserves and sustained oxidative conditions.

Overall, these findings indicate that *P. roxburghii* needle extract enhances hepatic GST activity under normal physiological conditions, whereas its detoxification-supporting efficacy appears to be attenuated during chronic or combined stress exposure.

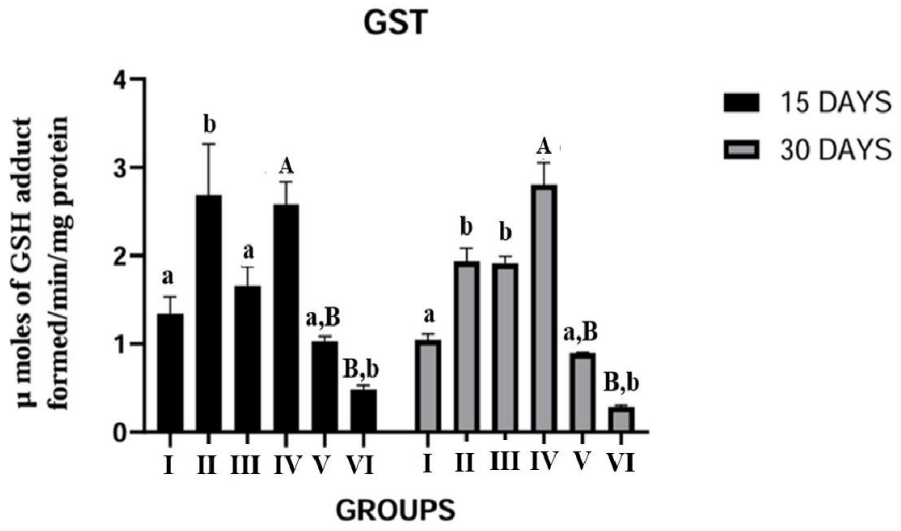


Fig. 3.6: Graph depicting the variations in GST activity at the end of 15 and 30 days; where Group I: control group; Group II: *Pinus roxburghii* (Conc.1); Group III: *Pinus roxburghii* (Conc.2); Group IV: Crowding Stress (Negative control); Group V: *P. roxburghii* (Conc.1) + Crowding Stress; Group VI: *P. roxburghii* (Conc.2) + Crowding Stress. Values were analysed by two-way ANOVA followed by Tukey Post hoc Test. Small letters (a,b) indicate results with respect to control and capital letters (A,B) indicate results with respect to negative control. Different letters (b,B) were used to express significant variations ($p < 0.05$).

3.3.5. Lipid peroxidation (LPO) enzyme activity

Fig. 3.7. illustrates the variations in hepatic lipid peroxidation (LPO) levels in zebrafish exposed to the aqueous extract of *Pinus roxburghii*. LPO is a well-established biomarker of oxidative stress, reflecting the extent of membrane lipid damage caused by reactive oxygen species (ROS).

No significant changes ($p < 0.05$) in hepatic LPO levels were observed in zebrafish treated solely with *P. roxburghii* extract after either 15 or 30 days of exposure compared with the control group, indicating that the extract did not induce lipid oxidative damage under normal physiological conditions.

In contrast, a significant reduction ($p < 0.05$) in LPO levels was recorded in Groups V and VI (*P. roxburghii* + crowding stress) at both 15 and 30 days relative to the negative control (Group IV). This decrease suggests a protective effect of *P. roxburghii* extract against crowding stress-induced oxidative damage, likely mediated through enhancement of endogenous antioxidant defences.

Collectively, these results indicate that *Pinus roxburghii* needle extract contributes to the stabilization of hepatic membrane lipids, thereby mitigating oxidative stress and preventing peroxidative damage in zebrafish under both normal and stress conditions.

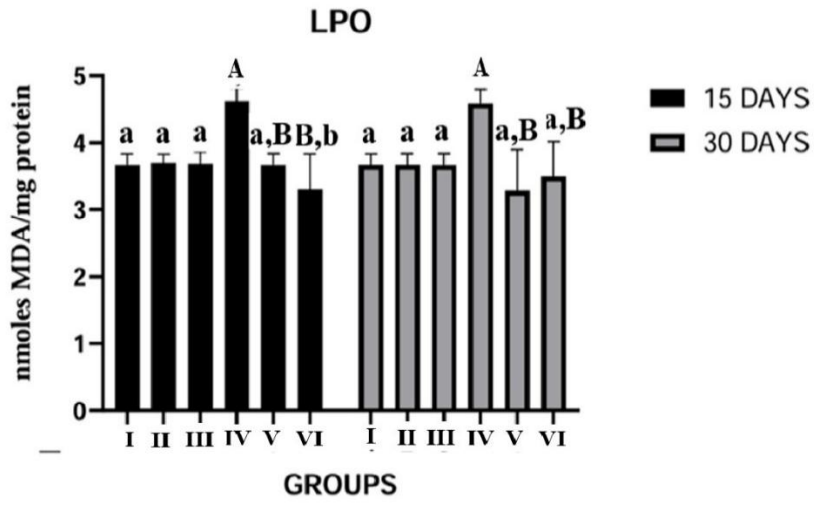


Fig. 3.7: Graph depicting the variations in LPO activity at the end of 15 and 30 days; where Group I: control group; Group II: *Pinus roxburghii* (Conc.1); Group III: *Pinus roxburghii* (Conc.2); Group IV: Crowding Stress (Negative control); Group V: *P. roxburghii* (Conc.1) + Crowding Stress; Group VI: *P. roxburghii* (Conc.2) + Crowding Stress. Values were analysed by two-way ANOVA followed by Tukey Post hoc Test. Small letters (a,b) indicate results with respect to control and capital letters (A,B) indicate results with respect to negative control. Different letters (b,B) were used to express significant variations ($p < 0.05$).

Reactive oxygen species (ROS), indicating hydrogen peroxide (H_2O_2), superoxide anions (O_2^-), and hydroxyl radicals (OH), are continuously generated during normal aerobic metabolism (Alam et al., 2025). While ROS play essential roles in cellular signalling, their excessive accumulation disrupts redox homeostasis and causes oxidative damage to lipids, proteins, and nucleic acids (Malhotra and Kaufman, 2007; Ansari et al., 2025). Environmental stressors, such as pollutants or xenobiotics, high stocking density exacerbate ROS production, overwhelming endogenous antioxidant defences (Rana and Shivanandappa, 2010; Latif et al., 2025). In the present study, exposure of zebrafish to different concentration of *P. roxburghii* needle aqueous extract resulted in statistically significant alterations in hepatic antioxidant biomarkers, indicating modulation of oxidative stress responses.

The initial increase in hepatic CAT activity observed after 15 days of extract exposure suggests an adaptive antioxidant response aimed at detoxifying excess H_2O_2 and limiting oxidative damage. Enhanced CAT activity improves the conversion of H_2O_2 into water and oxygen, thereby protecting hepatocellular integrity (Kaur and Kaur, 2015; Shehata et al., 2025). Similar

increases in CAT activity have been reported following administration of *Pinus massoniana* needle extract (PNE) in fish and cineole exposure in rainbow trout (Mirghaed et al., 2018; Guo et al., 2022). However, after prolonged exposure (30 days), a decline in CAT activity was evident in the combined extract and crowding stress group, likely reflecting enzyme exhaustion or inhibition under sustained oxidative stress (Xu et al., 1999; Cui et al., 2023). Comparable temporal reductions in CAT activity under crowding stress have been documented in other teleosts, supporting the sensitivity of this enzyme to chronic stress (Xie et al., 2008; Hou et al., 2019).

SOD activity exhibited an initial exhibited an initial decline in extract-treated and stressed groups at 15 days, which may be attributed to excessive superoxide radical accumulation or oxidative modification of enzyme thiol group during superoxide dismutation (Vijayavel et al., 2006). Reduced SOD activity can compromise downstream CAT function, as NADPH-dependant regeneration of CAT activity becomes impaired (Kono and Fridovich, 1982; Yousefi et al., 2020). Interestingly, prolonged exposure (15-30 days) resulted in recovery and enhancement of SOD activity in zebrafish receiving higher extract concentrations under crowding stress, suggesting physiological adaptation and reinforcement under antioxidant capacity (Yang et al., 2025). Similar compensatory responses have been reported in fish exposed to plant-derived antioxidants under chronic stress conditions (Bouzenna et al., 2016).

GSH, a central non-enzymatic antioxidant, plays a pivotal role in maintaining redox balance and facilitating detoxification (Bandodkar et al., 2025). The significant elevation of hepatic GSH levels observed after 15 days across extract-treated groups likely represents an adaptive mechanism to neutralize ROS and maintain cellular homeostasis (Türkoğlu et al., 2017). However, prolonged exposure under crowding stress resulted in GSH depletion, indicating exhaustion of antioxidant reserves under sustained oxidative demand (Zhang et al., 2005; Bi et al., 2023). Similar reductions in hepatic GSH levels have been reported in grass carp (*Ctenopharyngodon idella*) reared under high stocking densities (20 kg/m^3) (Li et al., 2023). As GSH acts as s critical cofactor in enzymatic ROS detoxification pathways, its depletion may compromise long-term oxidative defence (Ait Tayeb et al., 2023).

GST, a key phase II detoxification enzyme, showed increased activity in zebrafish treated with *P. roxburghii* extract under non-stressed conditions, reflecting enhanced conjugation and elimination of electrophilic metabolites (Ahmed et al., 2020). GSTs also regulate intracellular ROS levels and support cellular survival mechanisms (Teodoro et al., 2019). In contrast, GST

activity declined significantly under combined extract and crowding stress, suggesting depletion of glutathione pool and reduced detoxification efficiency during prolonged oxidative challenges. Similar reductions in GST activity have been reported following long-term exposure to bioactive plant extract in fish (Magara et al., 2022)

LPO levels, a direct indicator of oxidative membrane damage, was significantly reduced in extract-treated zebrafish under crowding stress. This reduction indicates effective scavenging of free radicals and stabilization of cellular membranes by *P. roxburghii* needle extract (Ramalingam et al., 2025). Comparable protective effects against lipid peroxidation have been reported in *Cyprinus carp* fed oak leaf extract under high stocking density stress (Paray et al., 2020).

Collectively, these findings demonstrated that *Pinus roxburghii* needle aqueous extract exerts a modulatory effect on hepatic antioxidant defence system in zebrafish (Gullilat et al., 2022). The extract enhanced enzymatic (CAT, SOD, GST) and non-enzymatic (GSH) antioxidant responses while suppressing lipid peroxidation, particularly during early exposure and under moderate stress conditions (Mishra et al., 2023; Vicidomini et al., 2024; Zhang et al., 2025). These antioxidative properties likely arise from the presence of bioactive phytochemicals such as flavonoids, phenolics, and fatty acids (Ayub et al., 2023; Rahman et al., 2025).

From an applied perspective, *P. roxburghii* needle extract shows promising potential as a natural antioxidant supplement in aquaculture to enhance stress resilience and liver health under intensive rearing conditions. Its use could reduce reliance on synthetic antioxidants and antioxidants and antibiotics, supporting more sustainable aquaculture practices. Nevertheless, further investigations focusing on optimal dosage, long-term safety, and species-species response are required prior to large-scale applications.

5. CONCLUSION

The present study demonstrates that *Pinus roxburghii* needle aqueous extract modulates hepatic antioxidant defence mechanisms in *Danio rerio* under both normal and crowding stress conditions. Exposure to the extract resulted in significant alterations in the antioxidant biomarkers, including catalase (CAT), superoxide dismutase (SOD), Glutathione (GSH), glutathione-S-transferase (GST), and lipid oxidations (LPO), indicating its capacity to influence oxidative stress responses in a dose- and duration dependant manner.

Under non-stressed conditions, the extract enhanced enzymatic (CAT and GST) and non-enzymatic (GSH) antioxidant activity without inducing lipid peroxidation, suggesting that it does not exert hepatotoxic effects at the tested concentrations. In contrast, crowding stress induced oxidative imbalance, reflecting by supressed antioxidant enzyme activities and altered redox status. Notably, co-administration of *P. roxburghii* extract under stress conditions mitigated lipid peroxidation and partially restored antioxidant enzyme activities, particularly at higher concentration, indicating protective role against stress-induced oxidative damage.

However, prolonged exposure under combined stress conditions led to partial depletion of antioxidant reserves, highlighting the importance of dose optimization and duration in practical applications. Histopathological findings further corroborated the biochemical data, revealing that the contributed to the preservation of hepatic architecture under moderate stress while preventing severe oxidative injury.

Overall, the findings suggest that *P. roxburghii* needle extract possesses significant antioxidative potential and may serve as a natural phytogenic supplement to enhance hepatic resilience and stress tolerance in aquaculture species. Its rich phytochemical composition, including phenolics and flavonoids, likely underpins its protective effects. Nevertheless, further studies focusing on long-term safely, molecular mechanisms, and species-specific responses are warranted before large-scale application in aquaculture systems.

List of abbreviations

HPI- Hypothalamic-pituitary-inter renal

ROS- Reactive oxygen species

LC-MS- Liquid chromatography- mass spectrometry

IAEC- Institutional Animal Ethical Committee

CPCSEA- Committee for the Purpose of Control and Supervision of Experiments on Animals

CDNB- 1-chloro-2,4-dinitrobenzene

MDA- Malondialdehyde

BV- Blood vessels

PH- Polygonal hepatocytes

KC- Kupffer cells

PNP- Pushing of nucleus to the periphery

KH- Karyorrhexis

KL- Karyolysis

PY- Pyknosis

SS- Sinusoidal Space

A- Apoptosis

V- Vacuolation

CD- Cytoplasmic degeneration

CAT- Catalase

SOD- superoxide dismutase

LPO- Lipid peroxidation

GSH- Reduced glutathione

GST- Glutathione-S-transferase

Declarations

Ethics approval/declaration: Ethical approval for the study was obtained from the Institutional Animal Ethical Committee (IAEC)/ Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), under reference no. **IAEC/SU/22/14.**

Data availability: Data will be made available on request by Shivani Guleria

Declaration of interests: The authors declare that they have no competing interests.

Funding: No funding was received from any source.

Author's contributions- Shivani Guleria: Conceptualization, Literature review, Data curation, Writing – original draft preparation.

Divya Jyoti: Supervision, Visualization, Writing – review & editing, Validation, Final approval of manuscript.

Acknowledgement: Authors are thankful of the Dr. Prem Kumar Khosla (Chancellor) and Prof. Atul Khosla (Vice Chancellor) of Shoolini university for providing necessary facilities for compilation of data. I would like to express my regards to my supervisor Prof. Ranbir Chander Sobti. I consider it a privilege to work under his guidance.

REFERENCES

1. Abdallah SM, Muhammed RE, Mohamed RE, El Daous H, Saleh DM, Ghorab MA, Chen S, El-Sayyad GS. Assessment of biochemical biomarkers and environmental stress indicators in some freshwater fish. Environmental Geochemistry and Health. 2024 Nov;46(11):464
2. AbouShabana NM, Aboseif AM, Taha MK, Ramadan EA, Elhammady AK, Ashour M, Van Doan H, El-Haroun E, Goda AM. Supplementing Nile tilapia (Oreochromis niloticus) (Linnaeus, 1758) larvae with dietary beta-glucan could improve their growth, survival, immune function, intestinal and liver histomorphology. Annals of Animal Science. 2025 Apr 1;25(2):679-93.
3. Ahmed, N. F., Sadek, K. M., Soliman, M. K., Khalil, R. H., Khafaga, A. F., Ajarem, J. S., Maodaa, S. N., & Allam, A. A. (2020). *Moringa oleifera* leaf extract repairs the oxidative misbalance following sub-chronic exposure to sodium fluoride in nile tilapia *Oreochromis niloticus. Animals*, *10*(4), 626.
4. Ahmed, R. A. (2022). Melano-macrophage Centers: Comparative Histological Insights among Three Reptilian Species. *Egyptian Academic Journal of Biological Sciences, B. Zoology*, *14*(2), 191–210.
5. Ait Tayeb, A. E. K., Poinsignon, V., Chappell, K., Bouligand, J., Becquemont, L., & Verstuyft, C. (2023). Major depressive disorder and oxidative stress: A review of peripheral and genetic biomarkers according to clinical characteristics and disease stages. *Antioxidants*, *12*(4), 942.
6. Alam P, Faizan M, Arif Y, Azzam MM, Hayat S, Afzal S, Albalawi T. Reactive oxygen species: balancing agents in plants. Frontiers in Plant Science. 2025 Dec 8;16:1713590.
7. Ansari WA, Srivastava K, Nasibullah M, Khan MF. Reactive oxygen species (ROS): Sources, generation, disease pathophysiology, and antioxidants. Discover Chemistry. 2025 Sep 1;2(1):191.
8. Anshar AR, Darusman HS, Manalu W, Yaqin K, Nurdin MA, Cordova MR. Acute styrene exposure induces hepatocellular injury and molecular stress responses in Oryzias

celebensis: Evidence for a tropical sentinel species in ecotoxicological monitoring. Veterinary World. 2025 Dec 1;18(12):3902-13.

9. Ayub H, Nadeem M, Mohsin M, Ambreen S, Khan FA, Oranab S, Rahim MA, Zubair khalid M, Zongo E, Zarlasht M, Ullah S. A comprehensive review on the availability of bioactive compounds, phytochemicals, and antioxidant potential of plum (Prunus Domestica). International Journal of Food Properties. 2023 Sep 22;26(1):2388-406.
10. Baker, R. D. (1945). The internal lesions in burns with special reference to the liver and to splenic nodules: An analysis of 96 autopsies. *The American Journal of Pathology*, *21*(4), 717.
11. Bandodkar VV, Moger VS, Baliga P. Antioxidant Defense Mechanisms: Enzymatic and Non-Enzymatic. InThe Role of Reactive Oxygen Species in Human Health and Disease 2025 (pp. 43-80). IGI Global Scientific Publishing.
12. Barros D, Grenho L, Fernandes MH, Gomes PS, Fernandes É. Correlation of Antioxidant and Antibacterial Activities of the Aqueous Pinus pinaster Aiton Bark Extract Within a Cytocompatible Concentration Range. Antioxidants. 2025 Nov 19;14(11):1377.
13. Beuge, J. A., & Aust, S. D. (1978). The thiobarbituric acid assay.
14. Beutler, E., Duron, O., & Kelly, B. M. (1963). Improved method for determination of blood glutathione.
15. Bi B, Yuan Y, Zhao Y, He M, Song H, Kong L, Gao Y. Effect of crowding stress on growth performance, the antioxidant system and humoral immunity in hybrid sturgeon. Aquaculture Reports. 2023 Feb 1;28:101468.
16. Bouzenna, H., Samout, N., Amani, E., Mbarki, S., Tlili, Z., Rjeibi, I., Elfeki, A., Talarmin, H., & Hfaiedh, N. (2016). Protective effects of *Pinus halepensis* L. essential oil on aspirin-induced acute liver and kidney damage in female Wistar albino rats. *Journal of Oleo Science*, *65*(8), 701–712.
17. Bouzenna, H., Samout, N., Fatma, G., Dhibi, S., & Saidi, I. (2021). Phytochemical, antioxidant and antibacterial activities of the aqueous and ethanol extracts of *Pinus halepensis*. *Discovery Phytomedicine*, *8*(1), 24–28.
18. Cai J, Chan HL, Yan X, Leung P. A global assessment of species diversification in aquaculture. Aquaculture. 2023 Nov 15;576:739837
19. CPCSEA (2021). Guidelines of CPCSEA for Experimentation on Fishes. Ministry of Fisheries, Animal Husbandry and Dairying Department of Animal Husbandry pp. 1-63.
20. Cui Z, He F, Li X, Li Y, Huo C, Wang H, Qi Y, Tian G, Zong W, Liu R. Response pathways of superoxide dismutase and catalase under the regulation of triclocarban-

triggered oxidative stress in Eisenia foetida: Comprehensive mechanism analysis based on cytotoxicity and binding model. Science of the Total Environment. 2023 Jan 1;854:158821.

21. Eachus H, Choi MK, Ryu S. The effects of early life stress on the brain and behaviour: Insights from zebrafish models. Frontiers in Cell and Developmental Biology. 2021 Jul 21;9:657591.
22. Fernando GC, Khansari AR, Tort L. Response to chronic crowding stress in shy and bold behavioral groups of male and female zebrafish. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 2024 Sep 1;327(3):R275-90.
23. Ganthaler, A., Stöggl, W., Mayr, S., Kranner, I., Schüler, S., Wischnitzki, E., Sehr, E. M., Fluch, S., & Trujillo-Moya, C. (2017). Association genetics of phenolic needle compounds in Norway spruce with variable susceptibility to needle bladder rust. *Plant Molecular Biology*, *94*(3), 229–251. https://doi.org/10.1007/s11103-017-0589-5
24. Gullilat H, Kumari R, Chandan G, Saini AK, Malik T, Saini RV. Immunomodulatory potential of the ethyl acetate fraction of Pinus roxburghii from the Himalayan region of India towards Ehrlich ascites carcinoma. South African Journal of Botany. 2022 Sep 1;149:878-86.
25. Guo, Y., Huang, S., Zhao, L., Zhang, J., Ji, C., & Ma, Q. (2022). Pine (*Pinus massoniana* Lamb.) needle extract supplementation improves performance, egg quality, serum parameters, and the gut microbiome in laying hens. *Frontiers in Nutrition*, *9*, 810462.
26. Habig, W. H., Pabst, M. J., & Jakoby, W. B. (1974). Glutathione S-transferases: The first enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry*, *249*(22), 7130–7139.
27. Hamed M, Abdel-Wahab M, Said RE, Sayed AE. Ecotoxicological Impacts of Perfluorooctane Sulfonate on the Freshwater Snail Lanistes carinatus: Oxidative Stress, Neurotoxicity, and Histopathological Alterations. International Journal of Molecular Sciences. 2025 Sep 12;26(18):8898.
28. Hou, Z.-S., Wen, H.-S., Li, J.-F., He, F., Li, Y., & Qi, X. (2019). Effects of long-term crowding stress on neuro-endocrine-immune network of rainbow trout (*Oncorhynchus mykiss*). *Fish & Shellfish Immunology*, *95*, 180–189.
29. Kanchan B, Prerna B, Simran K. Medicinal value of secondary metabolites of pines grown in Himalayan region of India. Res J Biotechnol. 2020 Jul;15:7.

30. Kaur, K., & Kaur, A. (2015). Fish erythrocytes as biomarkers for the toxicity of sublethal doses of an azo dye, basic violet-1 (CI: 42535). *Microscopy and Microanalysis*, *21*(1), 264–273.
31. Kono, Y. (1978). Generation of superoxide radical during autoxidation of hydroxylamine and an assay for superoxide dismutase. *Archives of Biochemistry and Biophysics*, *186*(1), 189–195.
32. Kono, Y., & Fridovich, I. (1982). Superoxide radical inhibits catalase. *Journal of Biological Chemistry*, *257*(10), 5751–5754.
33. Latif F, Ujan JA, Rind KH, Habib SS, Naz S, Al-Emam A. Effects of Lead Concentration and Exposure Time on Accumulation, Antioxidant Defense, and Genotoxicity in Indian Major Carps. Biological Trace Element Research. 2025 Nov 20:1-1
34. Li M, Yang L, Liu Y, Ma H. Long-term crowding stress disrupts intestinal homeostasis in largemouth bass (Micropterus salmoides). Aquaculture. 2025 Apr 15;599:742171.
35. Li, W., Li, D., Yang, Q., Liu, L., Liu, J., Lu, J., Wang, Y., Tang, R., Li, L., & Zhang, X. (2023). Long-term crowding stress induces chronic inflammatory response and declines the immunity of grass carp (*Ctenopharyngodon idella*). *Aquaculture*, *577*, 739976.
36. Lu D, Han Y, Xu R, Wang C, Qin M, Shi J, Ye F, Zhang J, Luo Z, Wang Y, Lin H. Respiratory transmission potential of severe fever with thrombocytopenia syndrome bunyavirus: evidence from intranasal exposure in a humanized mouse model. Emerging Microbes & Infections. 2025 May 26:2511134.
37. Lück, H. (1965). Catalase. In Methods of enzymatic analysis (pp. 885–894). Elsevier.
38. Lundin L, Keiter SH. Zebrafish as a Model to Investigate Nanoparticles. Nanosafety: A Comprehensive Approach to Assess Nanomaterial Exposure on the Environment and Health. 2025 Aug 12:365-400.
39. Ma W, Zeng W, Zhang D, Zhou Y, Huang Y, Hong Y. Oxidative stress in aquaculture: Pathogenic mechanisms and preventive strategies in farmed aquatic animals. Current Issues in Molecular Biology. 2025 Oct 22;47(11):873.
40. Magara, G., Prearo, M., Vercelli, C., Barbero, R., Micera, M., Botto, A., Caimi, C., Caldaroni, B., Bertea, C. M., & Mannino, G. (2022). Modulation of antioxidant defence in farmed rainbow trout (*Oncorhynchus mykiss*) fed with a diet supplemented by the waste derived from the supercritical fluid extraction of basil (*Ocimum basilicum*). *Antioxidants*, *11*(2), 415.

41. Malhotra, J. D., & Kaufman, R. J. (2007). Endoplasmic Reticulum Stress and Oxidative Stress: A Vicious Cycle or a Double-Edged Sword? *Antioxidants & Redox Signaling*, *9*(12), 2277–2294. https://doi.org/10.1089/ars.2007.1782
42. Metsämuuronen, S., & Sirén, H. (2019). Bioactive phenolic compounds, metabolism and properties: A review on valuable chemical compounds in Scots pine and Norway spruce. *Phytochemistry Reviews*, *18*(3), 623–664. https://doi.org/10.1007/s11101-019-09630-2
43. Mirghaed, A. T., Hoseini, S. M., & Ghelichpour, M. (2018). Effects of dietary 1, 8-cineole supplementation on physiological, immunological and antioxidant responses to crowding stress in rainbow trout (*Oncorhynchus mykiss*). *Fish & Shellfish Immunology*, *81*, 182–188.
44. Mishra N, Jiang C, Chen L, Paul A, Chatterjee A, Shen G. Achieving abiotic stress tolerance in plants through antioxidative defense mechanisms. Frontiers in Plant Science. 2023 Jun 2;14:1110622.
45. Miyai, K. (2020). Structural organization of the liver. *In Hepatotoxicology* (pp. 1–65).
46. Moussa MA, Mohamed HR, Abdel-Khalek AA. The antioxidant defense capacities and histological alterations in the livers and gills of two fish species, Oreochromis niloticus and Clarias gariepinus, as indicative signs of the Batts drain pollution. Environmental Science and Pollution Research. 2022 Oct;29(47):71731-41.
47. Myers, M. S., Rhodes, L. D., & McCain, B. B. (1987). Pathologic anatomy and patterns of occurrence of hepatic neoplasms, putative preneoplastic lesions, and other idiopathic hepatic conditions in English sole (*Parophrys vetulus*) from Puget Sound, Washington. *Journal of the National Cancer Institute*, *78*(2), 333–363.
48. Omnes, D. S., Xavier, J., & Suresh, A. (2024). Assessing oral acute toxicity and histopathological effects of *Strelitzia reginae* Aiton leaf extracts in Zebrafish (*Danio rerio* Hamilton). *Plant Science Today*, *11*(2). https://www.horizonepublishing.com/journals/index.php/PST/article/view/3092
49. Paray, B. A., Hoseini, S. M., Hoseinifar, S. H., & Van Doan, H. (2020). Effects of dietary oak (*Quercus castaneifolia*) leaf extract on growth, antioxidant, and immune characteristics and responses to crowding stress in common carp (*Cyprinus carpio*). *Aquaculture*, *524*, 735276.
50. Pearse, A. G. E. (1968). Histochemistry, theoretical and applied: Theoretical and applied. Churchill Livingstone.

51. Rahman AU, Iqbal A, Gul S, Kausar F, Tabassum S, ElSharkawy ER, Ali Khan Khalil A, Ali S. Phytochemical Analysis of Cupressus sempervirens stem bark and evaluation of its Antioxidant and Antimicrobial potentials. Journal of Pharmaceutical Innovation. 2026 Feb;21(1):32
52. Ramalingam S, Senthilkumar G, Saravanan R. Enhanced cytotoxic and antitumour properties of Cleome gynandra on Ehrlich ascites carcinoma in swiss albino mice. Medical Oncology. 2025 Dec 26;43(2):81.
53. Rana, I., & Shivanandappa, T. (2010). Mechanism of potentiation of endosulfan cytotoxicity by thiram in Ehrlich ascites tumor cells. *Toxicology In Vitro*, *24*(1), 40–44.
54. Routa, J., Brännström, H., Anttila, P., Mäkinen, M., Jänis, J., & Asikainen, A. (2017). Wood extractives of Finnish pine, spruce and birch–availability and optimal sources of compounds. https://jukuri.luke.fi/handle/10024/540829
55. Santa, K. (2023). Macrophages: Phagocytosis, Antigen Presentation, and Activation of Immunity. In *Phagocytosis-Main Key of Immune System*. IntechOpen. https://www.intechopen.com/chapters/86690
56. Sharma R, Sharma R, Thakur V, Randhawa A, Kumar R, Dutt B, Chaudhary A, Kumar S. Insights into Pinus species: phytochemistry, pharmacology and industrial potential of Indian Pinus species. Phytochemistry Reviews. 2025 Jan 23:1-31.
57. Shaw, P., Mondal, P., Bandyopadhyay, A., & Chattopadhyay, A. (2019). Environmentally relevant concentration of chromium activates Nrf2 and alters transcription of related XME genes in liver of zebrafish. *Chemosphere*, *214*, 35–46.
58. Shehata AI, Tefal E, Elmaghraby AM, Amer AA, Teiba II, Alhoshy M, Gewaily MS, Guo Z, Li S, El Basuini MF. Evaluation of Silymarin–L-Carnitine as a Dietary Supplement on Growth Performance, Antioxidants and Immunity, Gut/Liver Health, and Gene Expression in Nile Tilapia (Oreochromis niloticus). Fishes. 2025 Nov 11;10(11):580.
59. Singh L, Dixit P, Upadhyay AK, Srivastava RP, Pandey S, Verma PC, Saxena G. Assessment of total phenolic and flavonoid contents and potential biological efficacy of few Pinus species growing in Northern Himalayas. Agriculturae Conspectus Scientificus. 2020 Nov 10;85(4):335-44.
60. Singh, P. K., Singh, J., Medhi, T., & Kumar, A. (2022). Phytochemical Screening, Quantification, FT-IR Analysis, and *In-Silico* Characterization of Potential Bio-active Compounds Identified in HR-LC/MS Analysis of the Polyherbal Formulation from

Northeast India. *ACS Omega*, *7*(37), 33067–33078. https://doi.org/10.1021/acsomega.2c03117

61. Song W, Jiang Q, He Y, Ma B, Tang Y, Zhao J. Crowding-induced physiological stress, not aggression, makes high stocking density more worthy of attention in aquaculture. Aquaculture. 2026 Jan 27:743700.
62. Stachelska MA, Karpiński P, Kruszewski B. A comprehensive review of biological properties of flavonoids and their role in the prevention of metabolic, cancer and neurodegenerative diseases. Applied Sciences. 2025 Oct 9;15(19):10840.
63. Teodoro, J. S., Nunes, S., Rolo, A. P., Reis, F., & Palmeira, C. M. (2019). Therapeutic options targeting oxidative stress, mitochondrial dysfunction and inflammation to hinder the progression of vascular complications of diabetes. *Frontiers in Physiology*, *9*, 1857.
64. Türkoğlu, S., Celik, S., Keser, S., Türkoğlu, İ., & Yilmaz, Ö. (2017). The effect of *Pistacia terebinthus* extract on lipid peroxidation, glutathione, protein, and some enzyme activities in tissues of rats undergoing oxidative stress. *Turkish Journal of Zoology*, *41*(1), 82–88.
65. Vasile C, Tantaru G, Creteanu A. Recent Insights into the Research of (Bio) Active Additives for Advanced Polymer Materials. Polymers. 2025 Nov 26;17(23):3139.
66. Vicidomini C, Palumbo R, Moccia M, Roviello GN. Oxidative processes and xenobiotic metabolism in plants: mechanisms of defense and potential therapeutic implications. Journal of Xenobiotics. 2024 Oct 18;14(4):1541-69.
67. Vijayavel, K., Gopalakrishnan, S., Thilagam, H., & Balasubramanian, M. P. (2006). Dietary ascorbic acid and α-tocopherol mitigates oxidative stress induced by copper in the thornfish *Terapon jarbua*. *Science of the Total Environment*, *372*(1), 157–163.
68. Wolf, J. C., & Wheeler, J. R. (2018). A critical review of histopathological findings associated with endocrine and non-endocrine hepatic toxicity in fish models. *Aquatic Toxicology*, *197*, 60–78.
69. Xie, J., Liu, B., Zhou, Q., Su, Y., He, Y., Pan, L., Ge, X., & Xu, P. (2008). Effects of anthraquinone extract from rhubarb *Rheum officinale* Bail on the crowding stress response and growth of common carp *Cyprinus carpio* var. Jian. *Aquaculture*, *281*(1–4), 5–11.
70. Xu, L., Zheng, G. J., Lam, P. K. S., & Richardson, B. J. (1999). Relationship between Tissue Concentrations of Polycyclic Aromatic Hydrocarbons and DNA Adducts in

Green-Lipped Mussels (*Perna viridis*). *Ecotoxicology*, *8*(2), 73–82. https://doi.org/10.1023/A:1008910617843

71. Yang Z, Sun G, Tao J, Tang W, Li W, Wei Z, Yu Q. Dietary Tea Polyphenols Improve Growth Performance and Intestinal Microbiota Under Chronic Crowding Stress in Hybrid Crucian Carp. Animals. 2025 Jul 5;15(13):1983.

72. Yousefi, M., Shabunin, S. V., Vatnikov, Y. A., Kulikov, E. V., Adineh, H., Hamidi, M. K., & Hoseini, S. M. (2020). Effects of lavender (*Lavandula angustifolia*) extract inclusion in diet on growth performance, innate immunity, immune-related gene expression, and stress response of common carp, *Cyprinus carpio*. *Aquaculture*, *515*, 734588.
73. Yu, M., Gouvinhas, I., Rocha, J., & Barros, A. I. (2021). Phytochemical and antioxidant analysis of medicinal and food plants towards bioactive food and pharmaceutical resources. *Scientific Reports*, *11*(1), 10041.
74. Zhang Q, Xie Y, Zhang Y, Zhang Z, Zhou J, Liu D, Liu Y, Tong T. The Effects of Cadmium Exposure on Biochemical Indexes, Antioxidant Responses, Inflammation, and Anti-Stress Responses of Juvenile GIFT Tilapia (Oreochromis niloticus). Environmental Toxicology. 2025 Dec;40(12):1401-16.
75. Zhang Y, Chung WK, Moon SH, Lee JG, Om AS. Comparison of antibacterial activities of Korean pine (Pinus densiflora) needle steam distillation extract on Escherichia coli and Staphylococcus aureus focusing on membrane fluidity and genes involved in membrane lipids and stress. Molecules. 2023 Dec 27;29(1):165.
76. Zhang, J. F., Liu, H., Sun, Y. Y., Wang, X. R., Wu, J. C., & Xue, Y. Q. (2005). Responses of the antioxidant defences of the Goldfish Carassius auratus, exposed to 2, 4-dichlorophenol. *Environmental Toxicology and Pharmacology*, *19*(1), 185–190.
77. Zia-Ul-Haq M, AL-Huqail AA, Riaz M, Gohar UF, editors. Essentials of Medicinal and Aromatic Crops. Springer Nature; 2023 Oct 9.