JKMU
Journal of
Kerman University of Medical Sciences
https://jkmu.kmu.ac.ir
10.34172/jkmu.4039
JKMU. 2025;32:4039

Original Article

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Hepatoprotective effects of extracted polysaccharide from brittle star (*Ophiocoma erinaceus*) against carbon tetrachloride-induced acute liver injury in rats

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Abstract

Background: The exploration of marine-derived natural products, particularly polysaccharides, has highlighted their significant hepatoprotective effects, showcasing their therapeutic potential in liver-related studies. This research concentrated on the extraction of total polysaccharides from the brittle star species *Ophiocoma erinaceus* (*O. erinace*us). This study investigated the role of Persian Gulf brittle star polysaccharide (BSP) in immunotherapy and chemotherapy within a rat model of acute liver injury induced by carbon tetrachloride (CCl4).

Methods: The dried brittle stars underwent mechanical grinding to facilitate the extraction of the polysaccharide. Subsequent to the CCl4 induction, the rats assigned to the BSP treatment group received intragastric administration of BSP at dosages of 12.5, 25, and 37.5 mg/kg body weight daily for seven days. We meticulously evaluated the levels of various biochemical markers. Moreover, Western blot analysis was conducted to ascertain the expression levels of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, and adenosine A2A receptors. The rats were subjected to treatment with a 0.1% CCl4 solution (0.2 mL/10 g) mixed with soybean oil over the span of seven days.

Results: The methodologies implemented for polysaccharide detection effectively isolated crude polysaccharides from the brittle star indigenous to the Persian Gulf. The treatment with BSP yielded a significant reduction in the expressions of IL-6 (5.75), TNF-α (2.115), and IL-1β (4.465) among rats subjected to CCl4 intoxication. Furthermore, the administration of BSP resulted in an upregulation of the adenosine A2A receptor (2.05) in these CCl4-intoxicated rats. Additionally, rats administered with BSP displayed notable enhancements in the activities of superoxide dismutase (SOD) (87.5%), catalase (CAT) (72.5%), glutathione peroxidase (GPx) (81.25%), and glutathione S-transferase (GST) (83.8%), accompanied by a significant decrease in reduced glutathione (GSH) levels (81.3%).

Conclusion: The therapeutic efficacy of BSP is expected to extend to rats subjected to CCl4 intoxication. Recent investigations suggest that BSP may function as a therapeutic agent for these CCl4-intoxicated rats. Accordingly, these findings contribute novel insights into the hepatoprotective potential of BSP, establishing it as a promising candidate for hepatic treatment.

Keywords: Carbon tetrachloride, Brittle star, Proinflammatory cytokines, Polysaccharides, Antioxidants, Stress oxidative

Citation: Pourmohammad M, Baharara J, Khayatzadeh J, Afshari S, Lavi Arab F. Hepatoprotective effects of extracted polysaccharide from brittle star (*Ophiocoma erinaceus*) against carbon tetrachloride-induced acute liver injury in rats. *Journal of Kerman University of Medical Sciences*. 2025;32:4039. doi:10.34172/jkmu.4039

Received: August 12, 2024, **Accepted**: December 21, 2024, **ePublished**: December 7, 2025

Introduction

The increasing awareness of liver dysfunction highlights its significance as a widespread medical issue linked to diverse chronic injuries. The causes of this condition are manifold, encompassing toxic substance exposure, high levels of alcohol consumption, viral infections such as hepatitis B and C, metabolic disorders, certain pharmaceuticals, autoimmune reactions that target liver tissues or bile ducts, nonalcoholic steatohepatitis, and genetic defects (1). Evidence indicates that the pathological transformations occurring within the liver tissue are integral to the progression of portal hypertension. If left unaddressed, this condition can escalate into serious health issues such as liver fibrosis, liver failure, and the risk of cancer development (2). The recognition of liver diseases as a significant threat to public health worldwide underscores the importance of developing effective strategies for prevention, diagnosis, and treatment. This

growing challenge demands the concerted effort of healthcare professionals and policymakers alike (3).

The potential of various xenobiotic substances to cause liver toxicity and acute hepatic failure is well-documented. In experimental settings, carbon tetrachloride (CCl4) is often utilized to elicit liver damage in animal models, highlighting its role as a significant hepatotoxic agent. As a powerful hepatotoxin, CCl4 promotes the production of free radicals, leading to cellular necrosis, damage to cell membrane lipids, and inflammatory reactions, which ultimately result in liver impairment (4). During the early phase of hepatotoxicity induced by carbon tetrachloride (CCl4), the enzyme cytochrome P450 plays a crucial role in converting CCl4 into two types of trichloromethyl radicals ($\bullet CCl_3$ and $CCl_3OO\bullet$). These radicals are thought to trigger lipid peroxidation through mechanisms involving free radicals. This biochemical reaction leads to the accumulation of oxidative byproducts, which contribute to the peroxidation of cellular membrane lipids, ultimately resulting in cell death (5).

Polysaccharides represent a category of biological macromolecules that are noted for their antioxidant, antimicrobial, and anticoagulant characteristics. Recent studies have increasingly focused on the potential anticancer effects associated with naturally occurring polysaccharides (6). These substances function through various mechanisms, such as suppressing the growth of tumors, triggering programmed cell death, and hindering the spread of cancer to other parts of the body (7).

The marine ecosystem boasts a wealth of structurally bioactive compounds that exhibit a wide range of pharmacological effects. Both marine flora and fauna are remarkable sources of natural substances that possess diverse biological activities, such as antimicrobial, antiviral, antioxidant, and anticancer effects. This rich biodiversity provides significant opportunities for the discovery of new therapeutic agents. The exploration of marine life, particularly echinoderms, highlights the potential of the ocean as a reservoir of bioactive compounds. As scientists continue to investigate these natural resources, the implications for health and medicine are profound, paving the way for innovative treatments and enhancing our understanding of biological processes. The ongoing research in this area underscores the importance of marine biodiversity in contributing to human health (8).

In the realm of biomedical research, echinoderms, which encompass various groups, including *Crinoidea* (crinoids), *Asteroidea* (sea stars), *Echinoidea* (sea urchins), *Ophiuroidea* (brittle stars), and *Holothuroidea* (sea cucumbers), are particularly noteworthy. Their distinctive bioactive compounds have not been explored to the same extent as those found in *Asteroidea* (starfish) and *Holothuroidea* (sea cucumbers). Over the past three decades, there has been a significant gap in research dedicated to revealing the therapeutic possibilities of these intriguing organisms. *Ophiuroidea* (brittle stars) are captivating marine invertebrates recognized for their extraordinary regenerative capabilities, particularly their ability to regrow lost arms. Scientific investigations have identified a diverse array of bioactive compounds within brittle stars, including naphthoquinones, carotenoid sulfate, phenylpropanoids, sulfated sterols, and terpenes. These compounds may possess considerable potential for the advancement of anticancer therapies (9).

Research into marine invertebrates offers promising opportunities for discovering novel polysaccharides that could have therapeutic applications. The unique properties of these natural compounds may provide valuable insights into their potential use in medical treatments, particularly in the context of cell therapy (10). Sea cucumbers, scientifically classified as holothurians and part of the phylum *Echinodermata*, are significant for their production of sulfated polysaccharides. Fucoidan, a key sulfated polysaccharide present in both brown seaweed and these marine organisms, is associated with multiple beneficial biological effects. These include its roles as an antioxidant, its protective qualities for the liver, and its capacity to lower cholesterol levels (11).

Furthermore, the unique composition of these components contributes to the overall functionality of sea cucumbers in their natural habitat. The presence of these bioactive substances suggests potential applications in biomedical fields, particularly in developing therapeutic agents. Their ability to influence various biological processes makes them a subject of interest for further research (12).

Despite the potential benefits, the study of brittle stars and their regenerative processes is still in its infancy. Further research is essential to unlock the secrets of their arm regeneration and to explore how these insights could be translated into practical biomedical solutions (13). The diverse array of compounds found in brittle stars highlights their potential as a source of novel therapeutic agents. The presence of these bioactive molecules suggests that further exploration of brittle stars may lead to significant breakthroughs in cancer treatment (14).

In this investigation, the focus is placed on understanding how BSP can mitigate the harmful effects of oxidative stress in the liver, which is often exacerbated by toxic substances like CCl4. By utilizing various assessment techniques, the study aims to provide a comprehensive evaluation of the protective mechanisms that BSP may offer against liver damage.

Methods

Animals and Experimental Design

Female Wistar rats, weighing between 200 and 250 grams, were obtained from the animal facility associated with Mashhad University of Medical Sciences in Mashhad, Iran. These animals were maintained in a controlled

environment with a 12:12-hour light/dark cycle, with the temperature carefully regulated at 22 ± 2 °C. They had continuous access to food and water throughout the study period. Prior to the experimental procedures, the rats underwent a fasting period of 24 hours, during which they were deprived of food but allowed to drink water freely. The care and handling of these laboratory animals adhered strictly to the guidelines set forth by the National Institutes of Health (NIH). This ensured that all practices were in line with established ethical standards for animal research. Furthermore, all experimental protocols were approved by the Bioethics Committee, with the reference number IR.IAU.MSHD.REC.1401.149, ensuring that the welfare of the animals was prioritized throughout the study. The adherence to these ethical guidelines reflects a commitment to responsible research practices in the field of biomedical sciences.

To induce acute liver injury, CCl4 was administered using a modified protocol based on the work of Shyur et al. (2008). The normal rats were randomly divided into five groups, each consisting of ten rats:

1. CTRL: The normal control group, which received distilled water;
2. CCl4 + vehicle: The model group, which received CCl4;
3. CCl4 + BSP 12.5 mg/kg: The treatment group that was administered BSP intragastrically at doses of 12.5 mg/kg body weight per day;
4. CCl4 + BSP 25 mg/kg: The treatment group that was administered BSP intragastrically at doses of 25 mg/kg body weight per day;
5. CCl4 + BSP 37.5 mg/kg: The treatment group that was administered BSP intragastrically at doses of 37.5 mg/kg body weight per day;

The dosage range was delineated based on previous investigations concerning the ramifications of brittle star-extracted polysaccharides. The experimental model and treatment groups were administered 0.1% CCl4 (0.2 mL/10 g) in conjunction with soybean oil, whereas the control group was provided only with soybean oil over a duration of seven days. Each rat underwent intragastric administration once per day, with a dosage meticulously modulated based on its body weight throughout the duration of the experiment (15).

At the conclusion of the study period on day 14, and following a 12-hour pause after the last treatment, each rat was anesthetized using chloroform to facilitate blood collection and liver removal. Blood samples were obtained via cardiac puncture and allowed to clot for 45 minutes at room temperature. The serum was then separated through centrifugation at 600 × g for 15 minutes, preparing it for analysis of various biomarkers. The liver tissues that were excised underwent homogenization and were subsequently evaluated for biochemical markers, immunological factors, and histopathological characteristics. This comprehensive assessment aimed to provide insights into the physiological changes induced by the experimental conditions. The combination of serum analysis and liver tissue evaluation allowed for a thorough investigation of the effects observed during the study. By employing these methodologies, the research aimed to elucidate the underlying mechanisms and potential implications of the findings concerning the treatment administered.

Preparation of the Polysaccharide

The initial morphometric analysis of *O. erinaceus* was conducted at the Pharmaceutical Research Center of Mashhad University of Medical Sciences. After the specimens were collected, they underwent a thorough washing process and were then dried in a light-free environment. Following these preparatory steps, 500 grams of dried brittle star was combined with 100 milliliters of water and boiled for three hours, after which the mixture was subjected to centrifugation and filtration. In the next stage of the process, three volumes of 95% (v/v) ethanol were added to the mixture, which was then incubated at 4 °C overnight. This incubation facilitated the extraction of compounds from the brittle star. Afterward, the mixture was centrifuged again, and the resulting precipitate was dissolved in distilled water. A subsequent centrifugation for 20 minutes was performed to separate the aqueous supernatant, which was then lyophilized to obtain the total polysaccharides. The entire procedure culminated in the extraction of the polysaccharide, as illustrated in Figure 1. This meticulous approach ensured that the morphometric examination yielded reliable and significant results in order to facilitate our understanding of the biological characteristics of *O. erinaceus*. The careful handling and processing of the specimens were crucial for the integrity

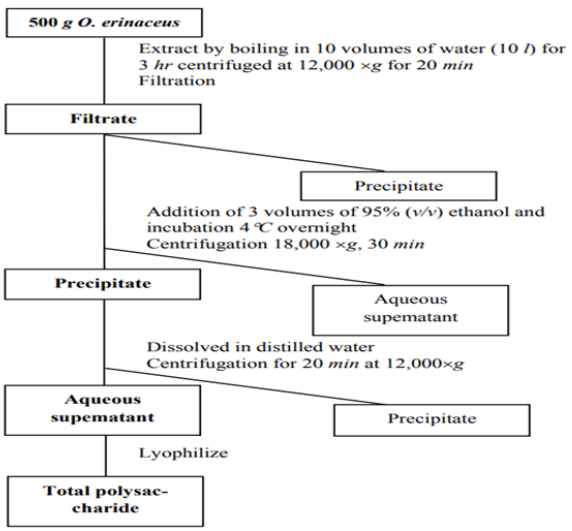

Figure 1. Schematic depiction of polysaccharide extraction method from *O. erinaceus*

of the research findings (16).

Preparation of the Hepatic Homogenate

The homogenization of liver tissues was carried out using a lysis buffer formulated with 100 mM NaF, 150 mM NaCl, and 50 mM Tris-HCl (pH 8.0), supplemented with 1 mM Na2VO4, 1 mM phenylmethylsulfonyl fluoride,1.5 mM MgCl2•6H2O, 1 mM ethylene glycol tetraacetic acid,1% Triton X-100, and 10% glycerol. After the homogenization step, the mixture was centrifuged at 14,000×g for 30 minutes.

Histological Assessment of Hepatic Damage

Liver samples were obtained from the right lobe and were subsequently stored in buffered formalin for one week. After the fixation process, the specimens were dehydrated through a gradient of increasing alcohol concentrations. Following this, the samples were cleared with xylene. Once the paraffin blocks were created, serial sections of 5 µm thickness were made. These sections were stained with Harris hematoxylin for five minutes at room temperature, followed by two minutes staining with 0.5% eosin yellowish at the same temperature, facilitating the evaluation of histological alterations via optical microscopy. Samples were treated with a 0.5 µg/ml solution of Nile red. This fluorescent stain, which is lipophilic, serves as an indicator of lipid hydrophobicity. When excited at a wavelength of 515 nm, the dye emits three separate bands at 635 nm, 580 nm, and 530 nm, which are associated with polar, total, and nonpolar lipids, respectively. Imaging was performed using a confocal microscope, and the FV-10 ASW 2.1 software enabled efficient handling and analysis of the images. This approach ensured that the evaluation adhered to the necessary standards for accuracy and dependability.

Cytometric Analysis

Evaluation of Oxidative Stress Through the Application of Flow Cytometry

To assess oxidative stress using flow cytometry, hepatic tissue samples measuring 5 mm^2 were collected. These samples were then disaggregated, filtered, and centrifuged at 1500×g for 5 minutes at a temperature of 4 °C. The resulting pellet, which contained the hepatic cells, was prepared for further analysis. The pellet was incubated with 0.4 µM of 2', 7'-dichlorofluorescein-diacetate (DCFH-DA) along with 0.0001% propidium iodide (PI) for 30 minutes at 37 °C in a phosphate-buffered saline (PBS) solution. This step was crucial for the subsequent evaluation of oxidative stress levels in the hepatic cells. To evaluate the production of reactive oxygen species (ROS), the fluorescence of 20, 70-dichlorofluorescein (DCF) was measured, reflecting the oxidized state of DCFH-DA. Control samples were created with phosphate-buffered saline (PBS) as the medium (17).

Apoptosis Measurement

The procedure involved immersing hepatic cells in a 70% ethanol solution at a controlled temperature of 20 °C with agitation for one minute. Afterward, the cells were washed with PBS and resuspended in an identical buffer for further analysis. The resultant solution was incubated for 60 minutes at a temperature of 37 °C in the presence of 100 µg/mL RNAse and 0.003% propidium iodide (PI). Following this, flow cytometry analysis was executed.

Determination of Biochemical Markers

Assessment of the Enzymatic Activities of Lactate Dehydrogenase (LDH)

A sample of 100 µL of serum was combined with 100 µL of 20 mM NADH and 100 µL of 10 mM sodium pyruvate. This combination was then incubated in a 100 mM sodium phosphate buffer, which was carefully maintained at a pH of 7.4, resulting in a total volume of 3 mL. The enzymatic activity was evaluated by measuring the change in absorbance at a wavelength of 340 nm. For this analysis, a molar extinction coefficient of 6220 M-1 cm-1 was utilized for NAD+, allowing for accurate quantification of the reaction. The results were expressed as nanomoles of NAD+ produced per minute per milligram of protein, providing a clear metric for assessing the enzymatic activity within the serum sample. This methodology ensures precise and reproducible measurements critical for further biochemical analysis.

Assessment of the Enzymatic Activities of Glutamate Pyruvate Transaminase (GPT) and Glutamate Oxaloacetate Transaminase (GOT)

The formulation of reactants intended for evaluating GOT activity included 15 mM α-ketoglutarate, 290 mM L-aspartate, 100 mM Tris–HCl, 386 U/L of malate dehydrogenase, and 0.26 mM NADH, adjusted to a pH of 7.8, culminating in a total volume of 1 mL. This particular arrangement was essential for the precise quantification of the enzymatic activity linked to GOT. For the evaluation of GPT activity, a similar reaction mixture was utilized, with the notable modification of replacing L-aspartate with 600 mM L-alanine and substituting malate dehydrogenase with 1080 U/L of lactate dehydrogenase. These adjustments were essential to tailor the reaction conditions for the specific enzymatic activity being measured. In both experimental setups, the enzymatic activity was determined by observing the changes in absorbance at a wavelength of 340 nm. The results obtained from these measurements were expressed in terms of U/g protein, providing a standardized metric for comparing the enzymatic activities of GOT and GPT.

Assessment of GSH

To isolate proteins, a solution consisting of 10% trichloroacetic acid was mixed with hepatic homogenate

in equal volumes, followed by centrifugation to separate the components. Subsequently, 10 µL of the supernatant obtained from this process was mixed with 1 mL of a 100 mM phosphate buffer at pH 8.4, which included 0.165 mL of distilled water and 0.025 mL of a 5 mM solution of 5,5'-dithio-bis-(2-nitrobenzoic acid). After 15 minutes incubation, the absorbance of the mixture was measured at a wavelength of 412 nm. The reduced glutathione concentration was then determined and expressed as micrograms of GSH per milligram of protein.

Assessment of Hydrogen Peroxide Concentration

The formulation for the reaction included a homogenized sample along with 3.5 µM of phenol red, 180 units of horseradish peroxidase, a phosphate buffer at 50 mM, and 0.370 mM of dextrose, which was adjusted to a pH of 7.6. This mixture resulted in a total volume of 1.2 ml. The sample was then incubated for 30 minutes at a controlled temperature of 37 °C. Following the incubation, an alkalinization step was performed using 0.3 M NaOH, after which the sample underwent centrifugation at 14,000 × g for 3 minutes. This process allowed for the separation of the supernatant, which was subsequently analyzed for absorbance at a wavelength of 610 nm. The concentration of hydrogen peroxide present in the supernatant was quantified and expressed in terms of nanomoles of H2O2 per milligram of protein. This measurement provides insight into the enzymatic activity and the efficiency of the reaction under the specified conditions.

Assessment of Lipid Peroxidation

The formation of chromogen-thiobarbituric acid reactive substance (TBARS) occurs through the interaction of malondialdehyde, which is a metabolic byproduct resulting from lipid peroxidation, with thiobarbituric acid. To synthesize TBARS, it is essential to combine equal volumes of 15% 2-thiobarbituric acid and 15% trichloroacetic acid in a 0.25 M hydrochloric acid solution. This preparation is crucial for the subsequent analytical evaluation of the sample. For the analytical procedure, two volumes of the prepared reactive mixture are added to a specified volume of the sample. This combined mixture is then heated to 100 °C for 20 minutes, allowing the reaction to proceed effectively. After the heating phase, the mixture is allowed to cool before undergoing centrifugation at 10,000 × g for 15 minutes to separate the components. The supernatant obtained from the centrifugation process is analyzed spectrophotometrically at a wavelength of 532 nm. The absorbance measured at this wavelength is directly proportional to the concentration of TBARS produced in the reaction. The final results are reported as nanomoles of malondialdehyde equivalents per milligram of protein, providing a quantitative assessment of lipid peroxidation in the sample.

Assessment of the Enzymatic Activities of CAT

The experimental setup involved a homogenate sample combined with 50 mM phosphate buffer and 10 mM hydrogen peroxide, adjusted to a pH of 7.0, resulting in a final volume of 1 mL. The assessment of catalase (CAT) activity was conducted by measuring the rate of hydrogen peroxide breakdown. Absorbance readings were taken at a wavelength of 240 nm, and the results were quantified in terms of nanomoles of hydrogen peroxide decomposed per minute per milligram of protein, utilizing an extinction coefficient of 32.54 M-1 cm-1 specific to hydrogen peroxide.

Assessment of the Enzymatic Activities of SOD

The assessment of superoxide dismutase (SOD) enzymatic activity was conducted by measuring absorbance at a wavelength of 420 nm. The reaction mixture included the homogenate of the sample, 1 mM ethylenediaminetetraacetic acid (EDTA), 50 mM Tris–HCl buffer, and 0.2 M pyrogallol, which was adjusted to a pH of 8.2, culminating in a total volume of 1 mL. SOD activity was quantified as the enzyme quantity required to achieve a 50% reduction in the auto-oxidation of pyrogallol, thereby defining one unit of activity. This method provides a reliable means of evaluating the enzymatic function of SOD within the specified experimental conditions.

Assessment of the Enzymatic Activities of GST

The assessment of glutathione S-transferase (GST) activity was conducted following established protocols. The reaction mixture included the homogenate sample, 20 µL of 50 mM 1-chloro-2,4-dinitrobenzene (CDNB), 0.1 M phosphate buffer, and 0.1 mL of 1 mM glutathione (GSH) adjusted to a pH of 6.5, resulting in a final volume of 3.0 mL. Absorbance changes were recorded at a wavelength of 340 nm, allowing for the quantification of enzymatic activity expressed as nanomoles of CDNB–GSH conjugate formed per minute per milligram of protein, based on a molar extinction coefficient of 9.6 $\mu M^{-1}\ cm^{-1}$.

Assessment of the Enzymatic Activities of GPx

The evaluation of glutathione peroxidase (GPx) activity commenced with the addition of 100 µL of 12 mM hydrogen peroxide (H2O2) to a reaction mixture that included 400 µL of 0.25 M potassium phosphate buffer at pH 7.0, 200 µL of sample homogenate, 100 µL of glutathione reductase, 100 µL of 2.5 mM NADPH, and100 µL of 10 mM glutathione (GSH) at a concentration of 6 U/mL. The determination of GPx activity was based on the changes in absorbance measured at 366 nm, with results expressed in milliunits per milligram of protein, employing an extinction coefficient for NADPH of $6.22 \times 10^3\ M^{-1}\ cm^{-1}$.

Assessment of the Enzymatic Activities of Lipoxygenase (LOX)

The activity of hepatic lipoxygenase was assessed by quantifying the rise in absorbance at 234 nm, which is indicative of the formation of hydroperoxide derivatives derived from arachidonic acid.

Western Blot Analysis

The primary antibodies chosen for this study comprised rabbit antibodies targeting interleukin-1 beta (IL-1β) (1:1,000; Santa Cruz Biotechnology), rabbit antibodies against the adenosine A2A receptor (1:1,000; Santa Cruz Biotechnology), goat antibodies against tumor necrosis factor-alpha (TNF-α) (1:1,000; Santa Cruz Biotechnology), goat antibodies for interleukin-6 (IL-6) (1:1,000; Santa Cruz Biotechnology) and rat antibodies for β-actin (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). For the secondary antibodies, a horseradish peroxidase-conjugated anti-rat antibody (1:2,000; Vector Laboratories, Burlingame, CA, USA) was employed for detecting β-actin, while horseradish peroxidase-conjugated anti-rabbit antibodies (1:3,000; Vector Laboratories) were utilized for both IL-1β and the adenosine A2A receptor. Additionally, horseradish peroxidase-conjugated anti-goat antibodies (1:2,000; Vector Laboratories) were used for the detection of IL-6 and TNF-α. The transfer of membranes was performed at a temperature of 4 °C, utilizing a cold pack along with pre-cooled buffer solutions. To determine the intensity of the protein bands, an enhanced chemiluminescence detection kit (Santa Cruz Biotechnology) was employed. The quantified bands were then analyzed comparatively, focusing on the relative expression of proteins, using the Molecular Analyst software version 1.4.1 (Bio-Rad). This methodological approach ensured a rigorous assessment of protein expression levels in the samples under investigation (18).

Statistical Analysis

Each group's data were presented as mean ± standard deviation (SD). Statistical evaluation was performed using a one-way ANOVA in SPSS 16, followed by Tukey's multiple comparison test. A significance threshold of $P \leq 0.05$ was considered statistically significant for all comparisons.

Result

Liver Weight Variation Analysis

The findings indicated that exposure to CCl4 toxicity correlates with a substantial increase in liver weight in rats relative to the control group. However, the administration of BSP for seven days post-CCl4 injection reduced liver weight significantly, with the degree of this reduction varying according to the dosage applied ($P < 0.05$), as shown in Table 1.

Histology of Liver

The data presented in Figure 2 demonstrates that exposure to CCl4 led to significant inflammation characterized by the infiltration of neutrophils, alongside observable vacuolation and necrosis in the liver tissue. In contrast, the administration of BSP at a dosage of 37.5 mg/kg body weight markedly mitigated liver damage and suppressed necrotic changes, indicating a protective effect against the toxicity induced by CCl4. To evaluate the levels of polar and nonpolar lipids, we employed confocal microscopy with Nile red staining. The findings summarized in Table 2 reveal that treatment with CCl4 resulted in a substantial 48% increase in nonpolar lipid concentration when compared to the control samples. On the other hand, BSP treatment demonstrated a dose-dependent improvement in the nonpolar/polar lipid ratio, achieving levels comparable to the control group at 37.5 mg/kg body weight. It is important to note that Nile red is specifically designed to stain neutral and polar lipids, making it an effective tool for examining lipid alterations in hepatocytes following exposure to xenobiotics. Our findings suggest that BSP treatment plays a crucial role in reversing the oxidative alterations to liver lipids that are typically associated with CCl4 exposure, thereby highlighting its potential as a hepatoprotective agent.

Assessment of Oxidative Stress Levels in the Liver Utilizing Flow Cytometry

The impact of BSP treatment on reactive oxygen species (ROS) production in rats is illustrated in Figure 3, which employs DCFH-DA as a fluorescent indicator. The

Table 1. Effects of BSP on relative liver weight in CCl4-treated rats.

Groups	Liver weight
CTRL	4.10 ± 0.13^{a}
CCl_4 + vehicle	6.27 ± 0.35^{b}
CCl_4 + BSP 12.5 mg/kg	5.15 ± 0.29^{c}
CCl_4 + BSP 25 mg/kg	4.75 ± 0.14^{d}
CCl_4 + BSP 37.5 mg/kg	4.33 ± 0.15^{a}

Data are reported as mean ± SEM ($n = 8$). CTRL (control), CCl4 (carbon tetrachloride treated), and BSP (BSP doses). Means with different letters in the same column are significantly different ($P < 0.05$).

Table 2. Effects of BSP doses on the polar and nonpolar lipids in hepatocytes from controls and CCl4-intoxicated rats.

Groups	Polar lipids (%)	Apolar lipids (%)	Apolar/ Polar ratio
CTRL	100 ± 3.2^{a}	100 ± 4.6^{a}	$1:1^{a}$
CCl_4 + vehicle	58 ± 3.7^{b}	146 ± 6.4^{b}	$2.52:1^{b}$
CCl_4 + BSP 12.5 mg/kg	74 ± 4.7^{c}	130 ± 4.8^{c}	$1.76:1^{c}$
CCl_4 + BSP 25 mg/kg	82 ± 5.5^{d}	115 ± 5.2^{a}	$1.40:1^{d}$
CCl_4 + BSP 37.5 mg/kg	98 ± 4.1^{a}	100 ± 4.5^{a}	$1.02:1^{a}$

Data are reported as mean ± SEM ($n = 10$). CTRL (control), CCl4 (carbon tetrachloride treated), and BSP (BSP doses). Means with different letters in the same column are significantly different ($P < 0.05$).

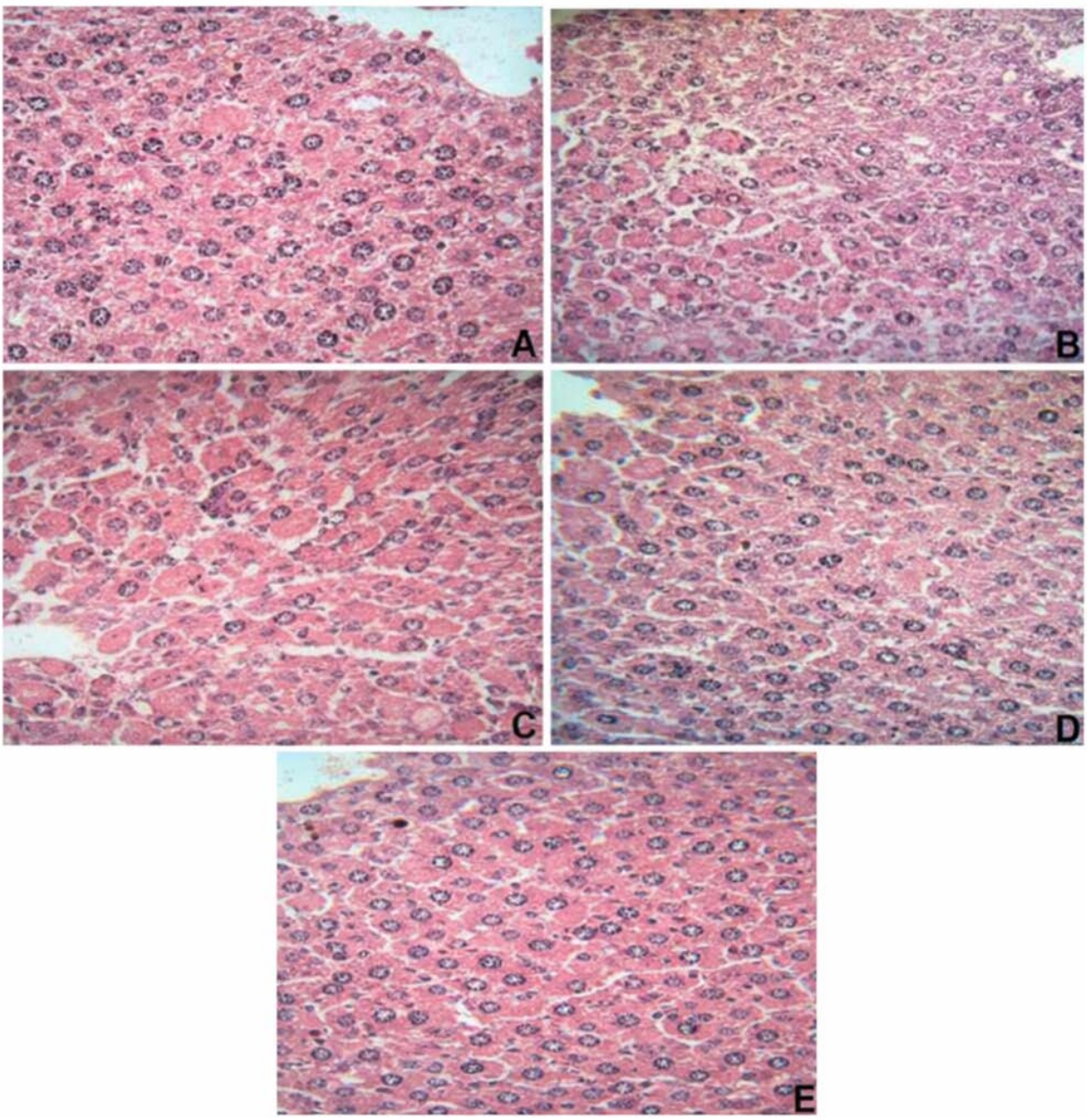

Figure 2. Hematoxilin and eosin-stained liver slices. (A) Histology of the liver of control rats showing normal hepatic cell architecture. (B) Histology of liver from rats treated with CCl4. (C) Histology of liver from BSP-treated rats at a dose of 12.5 mg/kg b.w. (D) Histology of liver from BSP-treated rats at a dose of 25 mg/kg body weight. (E) Histology of liver from BSP-treated rats at a dose of 37.5 mg/kg body weight

data indicated a significant increase in ROS levels, with an escalation of up to 80% observed in rats subjected to CCl4 toxicity. Conversely, the BSP-treated group exhibited a gradual dosage-dependent reduction in ROS levels, ultimately achieving concentrations comparable to those of the control group at a dosage of 37.5 mg/kg body weight. These results are consistent with our earlier confocal microscopy investigations, which employed Nile red to assess the effects of oxidative stress on the lipid profiles of hepatocytes. The correlation between the reduction in ROS levels following BSP treatment and the lipid profile alterations underscores the potential of BSP as a therapeutic agent in mitigating oxidative stress-related damage in liver cells.

The Role of BSP Intervention in Hepatocyte Apoptosis

The information illustrated in Figure 3 reveals a considerable increase in the number of apoptotic hepatocytes in rats subjected to CCl4 treatment compared to the control group. The administration of BSP resulted in a dose-dependent decrease in the number of apoptotic cells, with the most significant reduction observed at a dosage of 37.5 mg/kg body weight. Furthermore, the occurrence of early apoptosis, evaluated through Annexin V staining, was significantly reduced by 15–20% in the BSP-treated group compared with the CCl4 group. These findings imply that BSP plays a crucial role in alleviating oxidative stress, which is consistent with the results obtained from our histological evaluations presented

in Table 3. The data collectively support the hypothesis that BSP may offer protective effects against hepatocyte apoptosis induced by CCl4, highlighting its potential therapeutic benefits in managing oxidative damage in liver cells.

Assessment of BSP Treatment Effect on Serum Biochemical Levels

Our research indicates that rats subjected to CCl4 exhibited statistically significant ($P<0.05$) elevation in the activities of LDH (169.2%), GOT (1050.2%), and GPT (417.8%) compared to the control group. In contrast, the administration of BSP after CCl4 exposure led to a dose-dependent decrease in serum concentrations of LDH (61.5%), GOT (435.5%), and GPT (195.2%), reaching statistical significance at a dosage of 37.5 mg/kg body weight ($P<0.05$), as demonstrated in Figure 4. The results of this study highlight the detrimental effects of CCl4 on enzyme activities in rats and the potential therapeutic benefits of BSP. The significant reduction in enzyme levels following BSP treatment suggests its efficacy in mitigating the biochemical alterations induced by CCl4 exposure, reinforcing the importance of further investigation into its protective mechanisms.

Table 3. Effects of BSP treatment on apoptosis in hepatocytes from CCl4-intoxicated rats.

Groups	Apoptotic cells (%)
CTRL	5.3 ± 0.22^{a}
CCl_4 + vehicle	24.5 ± 0.15^{b}
CCl_4 + BSP 12.5 mg/kg	15.5 ± 0.70^{c}
CCl_4 + BSP 25 mg/kg	10.5 ± 0.45^{d}
CCl_4 + BSP 37.5 mg/kg	6.1 ± 0.25^{a}

Data are reported as mean ± SEM ($n=10$). CTRL (control), CCl4 (carbon tetrachloride treated), and BSP (BSP doses). Means with different letters in the same column are significantly different ($P<0.05$).

Assessment of BSP Treatment Effect on Oxidative Stress Indicators TBARS, GSH, and H2O2

Under conditions of oxidative stress, there is a significant decline in glutathione (GSH) levels, which leads to lipid peroxidation and the formation of hydrogen peroxide. As depicted in Figure 5, the concentration of hepatic GSH in rats exposed to CCl4 toxicity decreased to 53% of control group levels. However, the administration of BSP to these rats resulted in a marked increase in GSH levels, rising as much as 81.3%. Notably, at a BSP dosage of 37.5 mg/kg body weight, GSH levels were nearly equivalent to those of the control group, reaching 97.1% ($P<0.05$). In addition to enhancing GSH levels, BSP treatment also significantly mitigated lipid peroxidation, as evidenced by a 34.4% reduction in TBARS in the intoxicated rats. This effect was found to be dose-dependent, ultimately approaching the normal oxidative status ($P<0.05$). The results indicate that BSP not only restores GSH levels but also plays a crucial role in protecting cellular integrity under oxidative stress conditions.

Furthermore, the elevated levels of hepatic hydrogen peroxide resulting from CCl4 exposure, which was recorded at 60%, showed a substantial decrease following BSP treatment, dropping to 20.8%. At the 37.5 mg/kg body weight dosage, the hydrogen peroxide levels approached those of the control group, measuring 2.08% ($P<0.05$). These findings underscore the potential of BSP as a

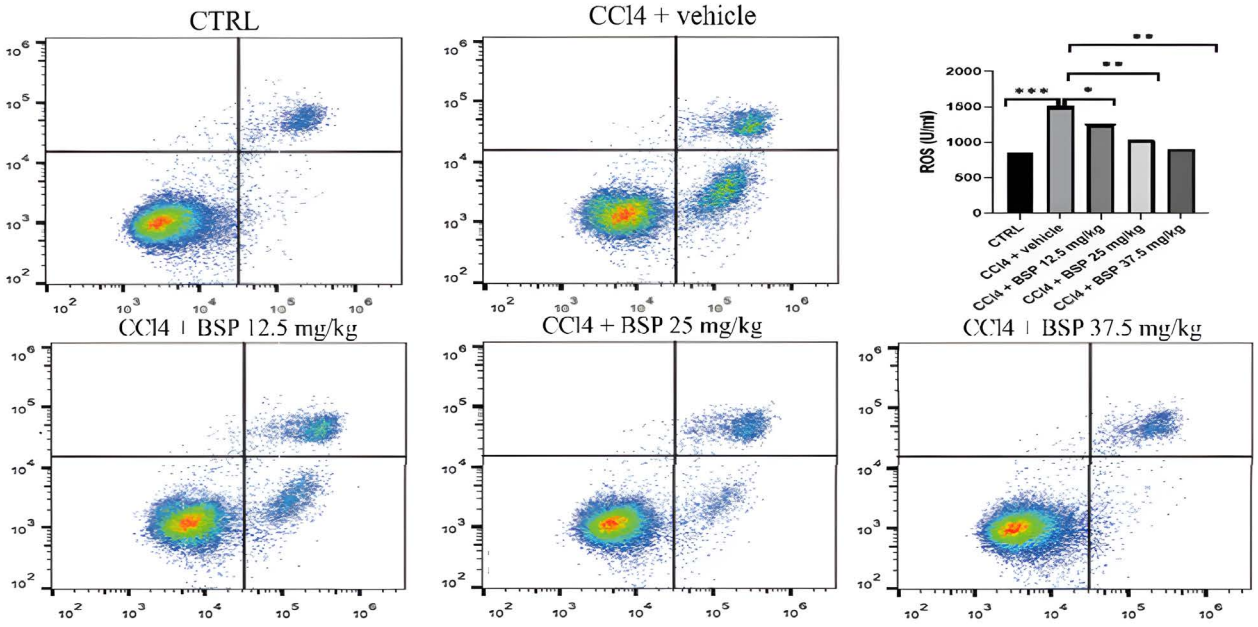

Figure 3. Effects of BSP treatment with doses of 12.5, 25, and 37.5 mg/kg body weight on apoptotic cells. In each panel, data are reported as the mean ± SD of triplicate analysis ($n=10$). Bars with different letters in the same panel are significantly different ($P<0.05$)

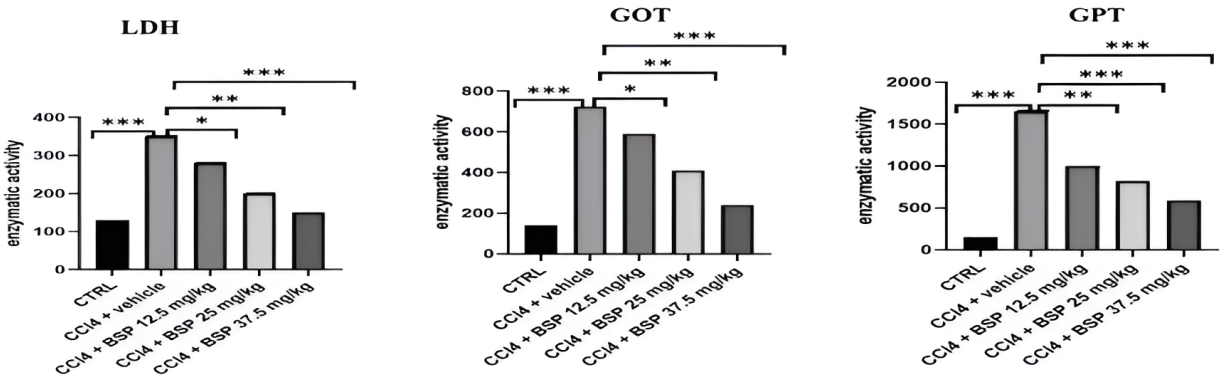

Figure 4. Effects of BSP treatment with doses of 12.5, 25, and 37.5 mg/kg body weight on LDH, GOT, and GPT activity. 100% LDH activity corresponds to 0.018 μM NADH/min/mg protein. 100% GOT activity corresponds to 17 mM NADH/min/mg protein. 100% GPT activity corresponds to 21 mM NADH/min/mg protein. In each panel, data are the mean ± SD of triplicate analyses ($n = 10$). Bars with different letters in the same panel are significantly different ($P < 0.05$)

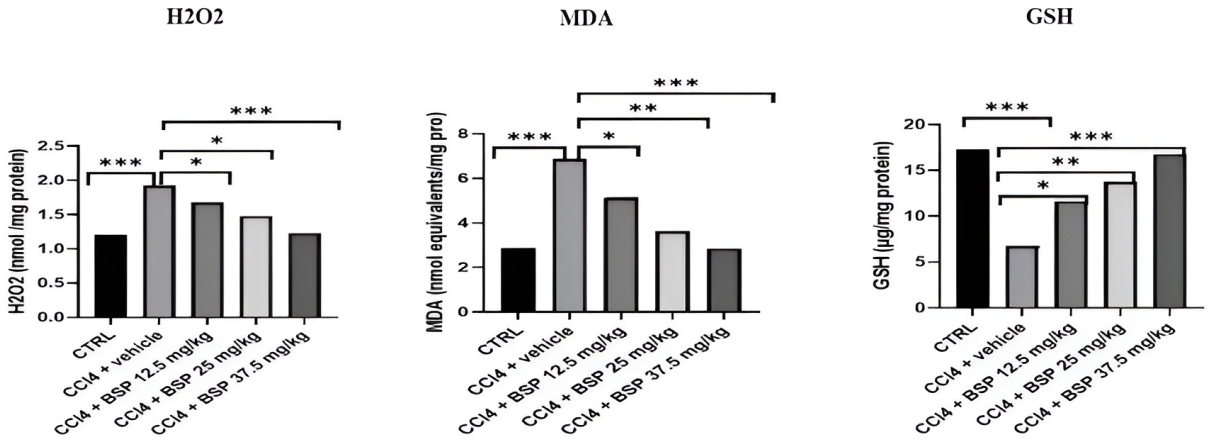

Figure 5. Effects of BSP treatment with doses of 12.5, 25, and 37.5 mg/kg body weight on GSH, MDA, and H_2O_2 concentrations. Data are reported as mean ± SD of triplicate analyses ($n = 10$). Bars with different letters in the same panel are significantly different ($P < 0.05$)

therapeutic agent in counteracting oxidative stress and its associated cellular damage.

Assessment of BSP Treatment Effect on CAT, SOD, GST, GPx, and LOX Enzymatic Activities

In order to evaluate the role of BSP in reducing oxidative liver damage in rats exposed to CCl4, we analyzed the activities of several critical antioxidant enzymes, including SOD, CAT, GPx, and GST (Figure 6). The results indicated that in rats treated with CCl4, there was a significant reduction ($P < 0.05$) in hepatic SOD and CAT activities, which fell to 37% and 65% of the control levels, respectively. Conversely, rats administered BSP exhibited a marked increase in hepatic SOD (87.5%) and CAT (72.5%) activities, approaching those of the control group, with the most significant effects observed at a dosage of 37.5 mg/kg body weight. The metabolism of CCl4 resulted in an increased inactivation of lipoxygenase (LOX) by 92.5%, which contributed to a decrease in GPx (52.5%) and GST (57.4%) activities in the CCl4-treated rats, correlating with reduced availability of GSH ($P < 0.05$). In contrast, the intoxicated rats that received BSP treatment showed a substantial enhancement in GPx (81.25%) and GST (83.8%) activities, approaching control group levels ($P < 0.05$). These observations suggest that BSP effectively counteracts the detrimental effects of CCl4 on these antioxidant enzymes. Our findings reveal an almost 100% increase in lipoxygenase activity in rats intoxicated with CCl4 compared to the control group. However, following treatment with BSP, LOX activity was significantly reduced to 77% ($P < 0.05$). These results indicate that BSP exerts a protective effect on the liver, primarily by inhibiting the lipoxygenase pathway, thereby mitigating oxidative stress and enhancing the overall antioxidant defense mechanisms in the liver.

Assessment of BSP Treatment Effect on the Expression Level of IL-6, TNF-α, IL-1β, and Adenosine A2A Receptor Expression

The data presented in Figure 7 indicates a significant increase in the concentrations of IL-6 (7.85%), TNF-α (4.125), and IL-1β (8.245%) in the liver tissue of rats subjected to carbon tetrachloride (CCl4) intoxication ($P < 0.05$). Following treatment with BSP, there was

a significant ($P<0.05$) decrease in the levels of these inflammatory markers, with TNF-α at 2.115, IL-1β at 4.465, and IL-6 at 5.75. Furthermore, the exposure to CCl4 resulted in a significant reduction in the expression of the adenosine A2A receptor in hepatic tissue, recorded at 0.29 ($P<0.05$). However, the administration of BSP effectively reversed this downregulation, leading to a significant increase in the expression of the adenosine A2A receptor to 2.05 ($P<0.05$), demonstrating the therapeutic potential of BSP in mitigating the effects of CCl4 exposure.

Discussion

The use of CCl4 in experimental settings has provided valuable insights into liver toxicity and has become a standard approach for studying potential treatments. This model allows for a comprehensive understanding of the mechanisms underlying liver injury and the potential benefits of different pharmacological agents (19). The production of trichloromethyl peroxyl radicals is a critical factor in the pathophysiology of liver injury associated with carbon tetrachloride exposure. The cleavage of the carbon-chlorine bond is a pivotal step that initiates a cascade of biochemical events, ultimately resulting in adverse effects on liver function and structure (20). The assessment of liver weight changes post-CCl4 treatment is essential for gauging the severity of liver injury. Our research highlights the protective role of BSP, particularly at a dosage of 37.5 mg/kg body weight. By employing Nile red staining, which is effective in identifying neutral and polar lipids, we were able to examine the modifications in lipid composition within the liver cells triggered by the presence of xenobiotics (21). Overall, our findings suggest that BSP could play a significant role in preserving liver health by preventing lipid peroxidation associated with CCl4 exposure.

Oxidative stress induced by exposure to carbon tetrachloride (CCl4) is recognized for its cytotoxic effects, leading to either apoptosis or necrosis in liver tissues (22). The findings of the analysis suggest that BSP plays a significant role in mitigating oxidative stress, aligning well with our histological observations.

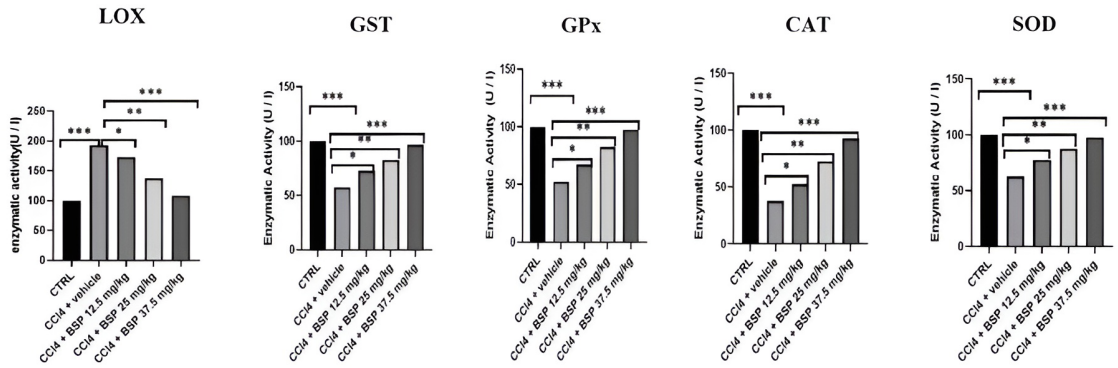

Figure 6. Effect of BSP treatment on SOD, CAT, GPx, GST, and LOX activity in CCl_4 intoxicated rats. 100% SOD activity corresponds to 24.1 U/mg protein. 100% CAT activity corresponds to 0.83 nM/min/mg protein. 100% GPX activity corresponds to 300 nM/min/mg protein. 100% GST activity corresponds to 700 nM/min/mg protein. 100% LOX activity corresponds to 17.0 μM arachidonic acid/min/mg protein. Data are reported as mean ± SD of triplicate analyses ($n=10$). Bars with different letters in the same panel are significantly different ($P<0.05$)

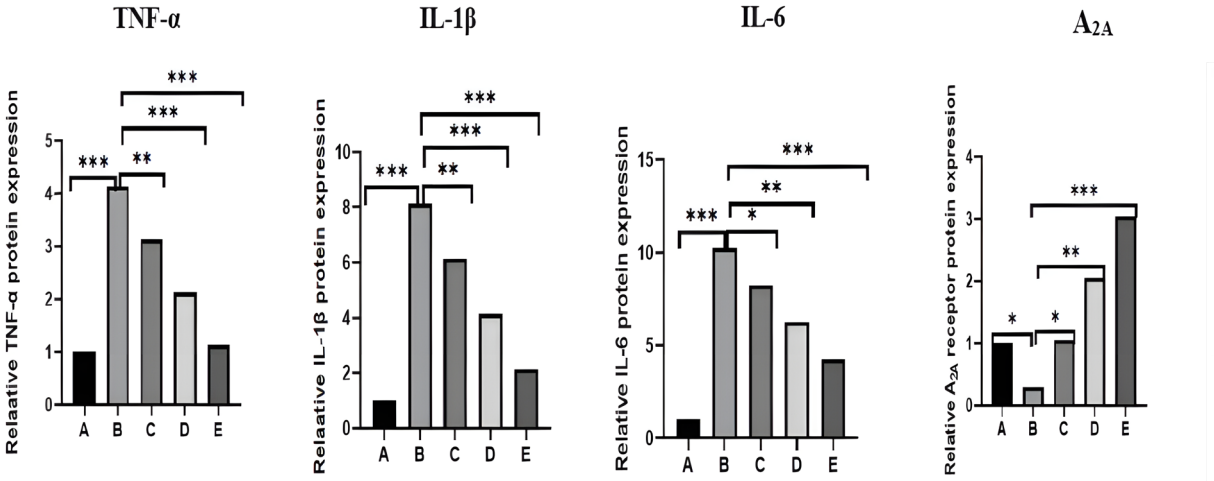

Figure 7. Effect of BSP treatment on TNF-α, IL-1β, IL-6, and adenosine A_{2A} receptor expression in the liver tissue. Upper panel: representative expressions. Lower panel: data of expressions. A, control group; B, CCl4 + vehicle group; C, CCl4 + BSP 12.5 mg/kg group; D, CCl4 + BSP 25 mg/kg group; E, CCl4 + BSP 37.5 mg/kg group. Data are reported as mean ± SD of triplicate analyses ($n=10$). Bars with different letters in the same panel are significantly different ($P<0.05$)

The oxidative damage that occurs due to free radicals produced during the bioactivation of CCl4 leads to a disruption of cellular membrane integrity. This disruption ultimately affects membrane permeability and facilitates the leakage of enzymes, including GOT, GPT, and LDH, into the bloodstream (23). This observation suggests that compounds able to lower the serum levels of GOT, GPT, and LDH may function as protective agents for the liver. The implications of these results highlight the potential therapeutic role of BSP in mitigating the effects of CCl4-induced liver damage (24).

The depletion of GSH in oxidative stress conditions plays a critical role in cellular damage, as it disrupts the balance of antioxidants and pro-oxidants. This imbalance not only promotes lipid peroxidation but also contributes to the accumulation of harmful byproducts, such as hydrogen peroxide. The increase in malondialdehyde levels serves as a biomarker for oxidative damage, indicating the extent of lipid peroxidation occurring in the organism (25). Our research indicates that the polysaccharide extracted from the brittle star (BSP) demonstrates significant effectiveness as a radical scavenger in vivo. This antioxidant capability of BSP is linked to a notable decrease in hydrogen peroxide levels in rats that were exposed to CCl4 and subsequently treated with BSP. Glutathione peroxidase (GPx) and glutathione S-transferase (GST) play crucial roles in the antioxidant defense system of mammalian cells. The increased lipid peroxidation and the inactivation of glutathione reductase caused by radical species from CCl4 metabolism contribute to a decline in the activities of GPx and GST in the treated rats, primarily due to the reduced availability of glutathione (GSH). Since GPx is essential for the reduction of hydrogen peroxide and lipid hydroperoxides by utilizing GSH as a co-substrate, the inhibition of this enzyme during oxidative stress may arise from negative feedback mechanisms related to substrate levels or from oxidative modifications induced by reactive oxygen species (ROS) produced during the metabolism of CCl4. This interplay highlights the importance of maintaining adequate GSH levels to support the activity of antioxidant enzymes and mitigate the effects of oxidative stress in biological systems (26). The findings align with the noted reduction in GSH levels in rats subjected to CCl4 exposure; this reduction was later normalized after treatment with BSP. This observation underscores the impact of CCl4 intoxication on GSH levels and highlights the potential of BSP to restore these levels (27). Our research confirms the hepatoprotective properties of brittle star-extracted polysaccharide, which is attributed to its ability to inhibit the inactivation of glutathione S-transferase (GST) caused by carbon tetrachloride (CCl4). This protective mechanism highlights the potential of brittle star-extracted polysaccharide in mitigating oxidative stress, reinforcing findings from earlier studies that have demonstrated the protective effects of the brittle star (*Ophiocoma Erinaceus*) extract against liver damage induced by CCl4 in adult male Wistar rats (28).

Increased levels of these lipoxygenases have been linked to the progression of prostate cancer, highlighting their potential role in the disease's pathophysiology. The presence of 5-HETE, in particular, has drawn attention to its possible contributions to tumor growth and metastasis (29). Furthermore, studies indicate that the use of lipoxygenase inhibitors may provide a degree of protection to the liver, particularly in cases of hepatopathy induced by CCl4 (30). This protective effect highlights the therapeutic potential of targeting lipoxygenase in liver-related disorders (31). The findings of our study reveal a notable increase in lipoxygenase activity in rats exposed to CCl4 toxicity compared to the control group. The administration of brittle star-extracted polysaccharide led to a significant reduction in lipoxygenase activity, suggesting a potential therapeutic effect. Previous in vitro research has shown that lipoxygenase has direct interactions with natural antioxidants, including resveratrol, quercetin, and a range of phenolic compounds. These interactions may play a crucial role in modulating lipoxygenase activity and its associated pathways. The implications of these results highlight the importance of exploring natural compounds like brittle star-extracted polysaccharides as potential modulators of lipoxygenase activity, particularly in the context of oxidative stress and related conditions. Further investigation into these interactions could provide valuable insights into therapeutic strategies for managing oxidative damage (32).

The interference caused by CCl4 on these vital enzymes diminishes the body's ability to combat oxidative stress, which is crucial for maintaining cellular health. As a consequence, the inflammatory response is exacerbated, further contributing to the deterioration of tissue integrity (33). Given the role of adenosine A2A receptor activation in promoting an anti-inflammatory effect within immune cells, there is growing interest in developing agonists that act on this receptor (34). These agents are being considered as potential treatments for various disorders linked to inflammation (35). The evidence supporting the role of BSP in mitigating inflammation and preventing cell death in gastric ulcer scenarios suggests a significant therapeutic potential. By focusing on the adenosine A2A receptor, researchers may uncover new pathways for intervention that could enhance treatment options for patients suffering from gastric ulcers. This line of inquiry is particularly relevant given the need for innovative therapies in this area (36). Overall, the results underscore the capacity of BSP to enhance adenosine A2A receptor expression, highlighting its potential as a protective agent against the detrimental effects of CCl4 intoxication in rat models. This research contributes to a deeper understanding of the mechanisms involved in receptor regulation under toxic conditions.

Following the activation of toll-like receptor 4, there

was a notable increase in the levels of IL-6, IL-1, and TNF-α within Kupffer cells. This elevation indicates a significant response in these immune cells to the receptor's stimulation (37). The process of inflammation is central to understanding the liver injury that results from the administration of CCl4 (38). Upon administration of carbon tetrachloride, the body exhibits a pronounced inflammatory response characterized by heightened levels of proinflammatory mediators. This surge in mediators, particularly IL-6, IL-1β, and TNF-α, significantly contributes to the exacerbation of liver damage associated with CCl4 exposure (39). The roles of TNF-α, IL-1β, and IL-6 are essential in the development of inflammation and the resulting liver injury. Evidence suggests that inhibiting these inflammatory mediators can effectively mitigate liver damage (40). In the present study, the administration of CCl4 led to an increase in the expression levels of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6. Conversely, when BSP was administered to rats exposed to CCl4, a notable reduction in the expression of these cytokines was observed.

A research study conducted by Gamal-Eldeen in 2009 examined the anti-proliferative and apoptotic effects of different polysaccharide fractions derived from the brown algae *Sargassum latifolium* alongside the properties of naturally occurring polysaccharides from marine sources. The results revealed that the E1 and E4 fractions were capable of causing DNA damage, promoting macrophage proliferation, and exhibiting antimetastatic properties through their anti-inflammatory effects. In contrast, the E3 fraction demonstrated specific anticancer activity against 1301 leukemia cells. This distinction highlights the varying biological activities of the polysaccharide fractions, suggesting that certain components may be more effective in targeting specific cancer cell lines. Overall, the study underscores the potential of marine-derived polysaccharides as therapeutic agents, particularly in the context of cancer treatment. The findings contribute to the understanding of how these natural compounds can influence cellular processes related to cancer proliferation and metastasis (41).

Research into the therapeutic applications of marine invertebrates, particularly echinoderms, has revealed significant findings regarding ovothiol A, a sulfur-rich compound extracted from sea urchin eggs. This compound has shown promising anti-fibrotic properties in mouse models suffering from liver fibrosis caused by carbon tetrachloride (CCl4) exposure. The underlying mechanisms responsible for this anti-fibrotic effect seem to involve a decrease in the levels of key mediators that contribute to the development of liver fibrosis. Notable among these mediators are TGF-β, α-SMA, and tissue inhibitors of metalloproteinase (TIMP-1), all of which play critical roles in the fibrotic process. These findings underscore the potential of marine-derived compounds like ovothiol A in the treatment of liver fibrosis, highlighting the importance of further exploration into the therapeutic benefits of marine invertebrates. Continued investigation may lead to novel strategies for managing fibrotic diseases and improving patient outcomes (42). Furthermore, an ethanolic extract derived from *Hypnea muciformis*, a type of red algae, has been reported to exhibit a range of beneficial properties, including antioxidant, antitumor, and antimicrobial activities. This extract has also demonstrated hepatoprotective effects in rat models subjected to CCl4-induced toxicity (43). Astaxanthin (AST), derived from starfish and algae, has gained attention for its anti-fibrotic effects. It operates by modulating the TGF-β1/Smad3 signaling pathway within hepatic stellate cells (44). Spirulina liquid extract (SLE), which is a patented aqueous formulation of *Arthrospira platensis*, has shown promise in reducing hepatic fibrosis. Its mechanism involves the modulation of inflammation, oxidative stress, and insulin resistance in mice that are subjected to a Western diet, a dietary pattern linked to the onset of nonalcoholic steatohepatitis (NASH) (45). Nouroozzadeh and Tajaddini (46) provided evidence that fucoidan extracted from *C. okamuranus* Tokida has the potential to mitigate liver fibrosis caused by N-nitrosodiethylamine. Their research highlighted the compound's ability to exert an anti-fibrotic effect, which is primarily attributed to the suppression of CXCL12 and TGF-β1 expression levels.

Conclusion

In summary, the findings indicate that BSP demonstrates both protective and therapeutic effects against CCl4-induced liver toxicity in rat models. Numerous bioactive compounds and medicinal properties of the Persian Gulf brittle star (*O. Erinaceus*) have been recognized thus far. The results of this study could contribute to a deeper understanding of the medicinal capabilities of the Persian Gulf brittle star in the fields of medicine, pharmacology, and veterinary science. However, additional research is necessary to confirm these results and to clarify the potential molecular and cellular mechanisms that drive the effects of BSP, paving the way for future clinical applications.

Authors' Contribution
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Competing Interests
The authors declare that they have no competing interests.

Ethical Approval
The Ethics Committee of Mashhad Medical Science Islamic Azad University in Mashhad, Iran, granted ethical clearance for this study (ethical code: IR.IAU.MSHD.REC.1401.149).

Funding
This research was financially supported by the Young Researchers and Elites Club in Mashhad, Iran (grant number 62587).

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