



## RESEARCH ARTICLE

### Protective Effect of Astaxanthin on Histopathologic Changes Induced by Bisphenol A in the Liver of Rats

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#### ABSTRACT

Bisphenol A (BPA) has several potential uses, including in polycarbonate plastics and epoxy resins, which could expose humans to it. Recognized for its hepatotoxicity and ability to accumulate in organs. We prompted this study to explore the hepatoprotective potential of astaxanthin (ASTX), an antioxidant against BPA toxicity. We used 32 male Wistar Albino rats and randomly assigned them as: Control, Sham (olive oil), BPA, and BPA+ASTX. At the end of the experiment, Native Thiol, Total Thiol, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were measured in serum samples. Histopathological scoring was performed to evaluate the changes caused by ASTX in the liver. Caspase 3 and caspase 9 expression in liver tissues was demonstrated immunohistochemically and by PCR. Collagen I (COL1A1) and collagen III (COL3A1) mRNA levels were measured by PCR in the tissue samples. The BPA group showed elevated AST and ALT with decreased Thiol levels. ASTX administration reversed these changes as observed by reduced AST and ALT levels and increased Thiol levels. Histopathology indicated increased liver damage and fibrosis in the BPA group which were alleviated in the BPA+ASTX group. Gene expression analyses revealed upregulated COL1A1 and COL3A1 in BPA, which was downregulated with ASTX. Immunohistochemistry and PCR confirmed BPA-induced caspase 3 and caspase 9 expression, which were attenuated by ASTX. This study underscores ASTX's hepatoprotective efficacy against BPA-induced hepatotoxicity which ultimately attributed to its antioxidant and antiapoptotic properties. Consequently, ASTX emerges as a promising therapeutic agent for preventing and treating BPA-related liver diseases.

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#### INTRODUCTION

Originally developed in the 1890s as a synthetic estrogen, bisphenol A (BPA) is a monomer. In a manner akin to the observed stimulation of the female reproductive system in rats by estrone, it was determined during the 1930s that BPA may possess a comparable effect (Rochester, 2013). In synthesizing epoxy resins and polycarbonate plastics, BPA is frequently being utilized as a plasticizer, an intermediate, and also added to remove surplus hydrochloric acid while manufacturing polyvinyl chloride (PVC).

BPA is frequently employed in the manufacturing of consumer goods, including food packaging, powders, drinking glasses, bowls, and cups (Hamed & Li, 2022;

Konieczna *et al.*, 2015; Lambré *et al.*, 2023), including the coating of recycled papers, toys, and water pipes (Farrugia *et al.*, 2021; Sonavane & Gassman, 2019). Additionally, BPA can be found in dental materials, medical equipment, thermal paper, compact discs, electronics (Brown *et al.*, 2022; Neufeld *et al.*, 2015), canned goods, and food products in cardboard boxes, as well as in fresh products such as meat, milk, or eggs when animals are raised in dirty environments or when contaminated water is used for irrigation (Konieczna *et al.*, 2015). Therefore, people can be exposed to it by mouth, inhalation, or through the skin (Konieczna *et al.*, 2015).

BPA exposure is associated with growth impairment, arrest of normal development, infertility, endocrine system disruption, immunosuppression, and carcinogenicity

(Divakaran, 2014; Gerona *et al.*, 2013). Numerous in vivo and in vitro experiments have shown that BPA can accumulate and affect the function of many important organs, including the testis, brain, heart, liver, and pancreas (Jiang *et al.*, 2020). Consequently, there has been significant attention in the field of public health towards BPA and its impact on human health (Wang *et al.*, 2019). BPA is recognized for its ability to generate reactive oxygen species (ROS), which cause harm to several organs, including the brain, kidneys, and liver. Cytotoxic ROS causes oxidative damage to cell membranes and DNA (Can *et al.*, 2016). As scavengers, antioxidants protect cells and tissues from free radicals, which can cause cell damage and illness (Çölçimen *et al.*, 2020; Şahin *et al.*, 2018). Cellular endogenous antioxidant defense systems, which include reduced glutathione (GSH), catalase (CAT), and superoxide dismutase (SOD), scavenge ROS (Temiz & Daye 2024; Serel *et al.*, 1997). Experimental and clinical research has shown that the xanthophyll carotenoid Astaxanthin (ASTX), primarily marine in origin, has powerful antioxidant and anti-inflammatory benefits.

ASTX (3,3'-dihydroxy- $\beta,\beta$ -carotene-4,4'-dione), is a carotenoid compound characterized by a chemical formula of C<sub>40</sub>H<sub>52</sub>O<sub>4</sub>. Its molecular structure consists of two hydroxyl groups and two carbonyl groups. The hue of ASTX ranges from orange to deep-red because it has 13 conjugated double bonds. It is noteworthy to mention that ASTX when in its crystalline state, exhibits a lustrous black-purple hue (Nishida *et al.*, 2023).

In addition to lobster, the primary natural sources of ASTX include microalgae (*Haematococcus pluvialis*, *Chlamydomonas nivalis*), bacteria (*Agrobacterium aurantiacum*, *Paracoccus carotinifaciens*), yeasts (*Xanthophyllomyces dendrorhous*, *Phaffia rhodozyma*), and various marine organisms such as trout, shrimps, krill, and crayfish. The green microalga *H. pluvialis* has been recognized as a highly abundant source of ASTX, owing to its propensity to accumulate significant quantities of this compound, reaching up to 4% per dry weight (Davan *et al.*, 2023).

Multiple studies have indicated that this chemical possesses the capacity to neutralize singlet oxygen and eliminate free radicals. The compound exhibits antioxidant activity that surpasses that of zeaxanthin, lutein, canthaxanthin, and  $\beta$ -carotene by a factor of ten, and surpasses that of  $\beta$ -tocopherol by a factor of 100 (Davan *et al.*, 2023).

Many abnormal disorders necessitate the use of ASTX, a potent antioxidant that shields cells and organs from oxidative harm. Recent research has demonstrated that ASTX can prevent mitochondrial dysfunction brought on by oxidative stress. However, it is unclear if ASTX can prevent the tissue damage caused by BPA (Jiang *et al.*, 2020).

BPA induces liver injury by affecting the rat liver's oxidant/antioxidant balance (Hassan *et al.*, 2012). As such, ASTX represents a potentially effective treatment approach for treating BPA-induced illnesses and preventing oxidant/antioxidant imbalance.

Our main goal in doing this research was to find out if ASTX could reduce the oxidative stress and related damages to liver tissue induced by BPA.

## MATERIALS AND METHODS

The study's ethical permission was granted by the Animal Experiments Local Ethics Committee at Necmettin Erbakan University with the decision dated 07.05.2021, numbered 2021-023.

**Animals and experimental groups:** In our study, 32 4-month-old male Wistar Albino rats weighing 250-300 g were divided into 4 groups with 8 rats in each group.

Animals were fed a combination of tap water and normal laboratory diets ad libitum. In the room where the animals were kept in had the temperature of 24 $\pm$ 1 °C, humidity level of 45 $\pm$ 5% and light/dark cycle of 12 hours.

**Group 1: Control;** No treatment was performed.

**Group 2: Sham (Olive Oil);** 20 mg/kg olive oil was administered by gavage for 2 weeks.

**Group 3: BPA;** 250 mg/kg BPA was dissolved in olive oil and administered by gavage for 2 weeks (Mahdavinia *et al.*, 2019).

**Group 4 BPA+ASTX (20 mg/kg);** 250 mg/kg BPA was dissolved in olive oil and administered by gavage for 2 weeks, and 2 hours after this administration, ASTX was dissolved in 20 mg/kg olive oil and administered by gavage (Kınal *et al.*, 2021). The LD<sub>50</sub> dose of BPA in the rat has been reported to be 3250 mg/kg orally (Michałowicz, 2014).

BPA ( $\geq$ 99% CAS No. 80-05-7 Sigma Aldrich) and ASTX (Pure 98% CAS No. 472-61-7 Sigma Aldrich) were freshly prepared before each application.

At the end of 14 days, blood samples were taken under anesthesia with 50 mg/kg Ketamine HCl+10 mg/kg Xylazine HCl injection.

**Biochemical analysis:** Following the investigation, the blood of each experimental animal was collected and transferred into a biochemistry tube filled with gel. The serum fraction was isolated by subjecting the blood samples to centrifugation at 1500 g for a duration of 10 minutes following collection. After that, the serum samples were divided into portions, labeled, and kept at -80°C for analysis in eppendorf tubes. Before being examined, each serum sample was concurrently thawed and brought to room temperature. Then, all sera were mixed with the help of a vortex device and prepared for biochemical analysis.

Native thiol, total thiol is a new generation oxidant-antioxidant marker to determine oxidative stress from blood samples (Solakhan *et al.*, 2019), aspartate aminotransferase (AST), and Alanine aminotransferase (ALT) levels were also measured to indicate organ function and inflammation.

The amino group from alanine to 2-oxoglutarate is transferred by ALT, resulting in the production of pyruvate and glutamate. The reaction between pyruvate and NADH is catalyzed by LDH, leading to the formation of lactate and NAD<sup>+</sup>. The measurement of the decrease in absorbance at 340 nm is attributed to the consumption of NADH. The alteration in absorbance exhibits a direct correlation with the concentration of ALT (Talke & Schubert, 1965).

2-oxoglutarate + L-alanine  $\rightarrow$  (in the presence of ALT) L-glutamate + Pyruvate  
Pyruvate + NADH  $\rightarrow$  (in the presence of LDH) L-lactate + NAD<sup>+</sup>

As to the guidelines set by the International Federation of Clinical Chemistry (IFCC), AST facilitates the transamination reaction between aspartate and 2-oxoglutarate, resulting in the formation of glutamate and oxaloacetate. Malate dehydrogenase (MDH) catalyzes the reduction of oxaloacetate to L-malate, whereas NADH undergoes conversion to NAD<sup>+</sup>. The observed reduction in absorbance at a wavelength of 340 nm is attributed to the consumption of NADH. The variation in absorbance is directly correlated with the concentration of AST (Bergmeyer *et al.*, 1986).

2-oxoglutarate + L-aspartate → (in the presence of AST) L-glutamate + Oxaloacetate  
Oxaloacetate + NADH + H<sup>+</sup> → (in the presence of MDH) L-malate + NAD<sup>+</sup>

ALT and AST measurement was performed using a commercial kit from Beckman Coulter (USA) on a Beckman Coulter AU 5800 model (USA) autoanalyzer, employing a colorimetric method. Results were reported in U/l.

**Histopathological analysis:** After the blood samples were taken, the abdominal cavities of the sacrificed rats were opened and the livers were examined grossly and then representative liver samples were placed in 10% buffered formalin to evaluate the histopathological lesions.

After undergoing standard tissue processing, liver tissues were fixed in paraffin and sections measuring 4 μm in thickness were extracted from each paraffin block using a microtome (Leica RM2125RTS).

4 μm thick sections were passed through xylene (3x20 min) and decreasing alcohol series (90%, 80%, 70% and 50%) for deparaffinization. They were then stained with Harris hematoxylin for 5 minutes and counterstained with 1% aqueous eosin for 1 minute. Sections were then dehydrated in alcohol, cleared with xylene, and mounted on Entellan®. Histopathological scoring was performed using the hematoxylin-eosin staining method to evaluate sinusoidal dilation, congestion, pyknotic nuclei, and mononuclear cell infiltration. Histopathological changes were graded as absent (0), mild (1), moderate (2) and severe (3) (Sozen *et al.*, 2024). Histopathological analysis was conducted under double-blind histopathological examination using a Zeiss Lab.A1 light microscope and a Zeiss AxioCam ERc 5s camera.

Sections of 4 μm thickness were taken from the liver blocks of the experimental groups for fibrosis assessment. They were deparaffinized by passing through xylene and decreasing alcohol series (concentrations of 90%, 80%, 70%, and 50%). Subsequently, Masson's Trichrome Stain Kit (Lot: 072022.036) was applied to the sections. After rinsing with alcohol series and xylene, they were covered with Entellan®.

**Immunohistochemistry (IHC) analysis:** Caspase 3 (ab184787) and caspase 9 (ab210611) antibodies were used for immunohistochemistry studies. Paraffin was extracted from 4 μm slices by immersing them in xylene for a duration of 30 minutes. Sections were incubated for 10 minutes in Super Block (ScyTek Laboratories, Logan, UT), followed by 5 minutes of PBS washing, overnight primary antibody incubation, 5 minutes of PBS washing, 20 minutes of secondary antibody incubation, and 5 minutes

of PBS washing. After that, streptavidin-peroxidase was added and incubated for 20 minutes, after which it was washed with PBS for five minutes. After adding and incubating for 15 minutes, the AEC (Sigma Alderich AEC101-1KT) chromogen was rinsed for 5 minutes with distilled water.

Mayer's hematoxylin was utilized as a counter stain once the immunological response was visible under a microscope. The sections were covered using a water-based sealing medium and examined under a Zeiss Lab.A1 light microscope and evaluated with a Zeiss AxioCam ERc 5s camera imaging system. At the end of the evaluations, we evaluated the immunohistochemistry staining for each primary antibody according to the following criteria (Cuce *et al.*, 2011): 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining.

**RT-qPCR analysis:** RT-qPCR analysis was performed to determine the expression of apoptotic cell death pathway (CASP3, CASP9) and collagen synthesis (COL1A1, COL3A1)-related genes at the mRNA level in liver tissues obtained from the control group, sham group, bisphenol A (BPA) alone treatment group, and BPA and AST-treated experimental groups. RiboEX total RNA isolation reagent (GeneAll, 301-001) was used for total RNA isolation as the first step of this analysis. The RNA of each isolated sample was treated with the DNase I enzyme to remove DNA contamination for use in the polymerase chain reaction (Thermo Fisher Scientific, #EN0521). After enzyme treatment, the iScript™ cDNA Synthesis Kit (Bio-Rad, 170-8891) was used to translate RNA samples into cDNA. The primers of the target (CASP3, CASP9, COL1A1, and COL3A1) and reference genes (GAPDH) analyzed in the study were designed with the IDT PrimerQuest program (<https://www.idtdna.com/PrimerQuest/Home/Index>). 5x HOT FIREPol® EvaGreen® qPCR Master Mix Plus (ROX) (Solis BioDyne) was used to determine the mRNA expression level of the designed genes. Following the enzyme activation step at 95 C0 for 12 min, an amplification step of 15 s at 95 C0, 20 s at 60 C0, and 20 s at 72 C0 for 40 cycles was performed in the Biorad CFX Connect device. The GAPDH gene was used as a reference for normalization.

**Statistical analysis:** Statistical analysis and calculations were performed in GraphPad Prism 8.

The mean and standard deviation were used to express histologic changes and biochemical results. The normality of the groups' data was assessed using the Shapiro-Wilk and One-Sample Kolmogorov-Smirnov tests. It was determined that all groups showed normal distribution. One-way analysis of variance (ANOVA) in independent groups, which is a parametric test, was applied to the study groups. Post-hoc Tukey's HSD test was preferred for pairwise comparisons after ANOVA. Immunohistochemical data not showing normal distribution were compared by the Kruskal-Wallis test and differences between paired groups were compared by Dunn's test. Statistical analysis of the differences in the mRNA level of each gene between the groups was performed by the 2-ΔΔCT method. The acceptance of a significant value of p<0.05 was seen.

## RESULTS

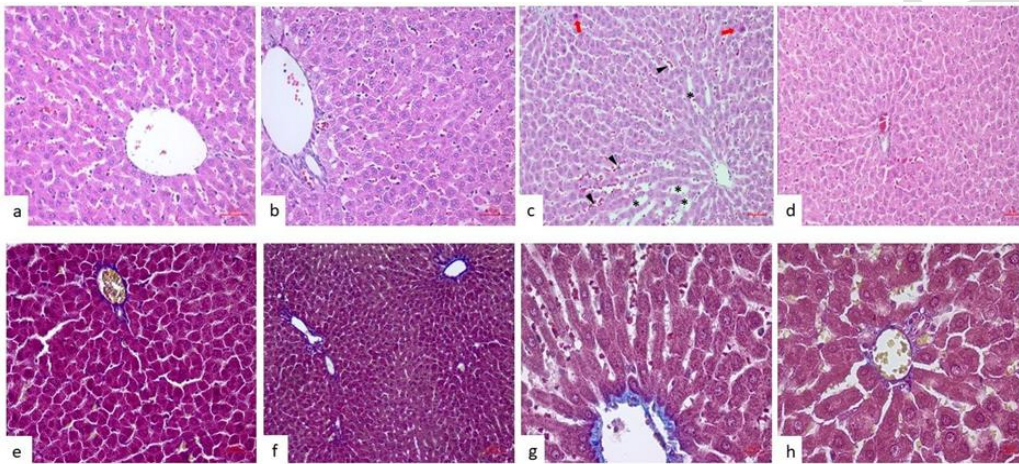
**Hematoxylin and eosin staining:** Light microscopic examination revealed that the control group had normal hepatic cells and cords, and the arrangement of the structure of the central veins, portal areas, and sinusoids was normal almost in all livers. The sham group's liver sections showed typical histological liver structure, just like the control group's of all livers. In the BPA group, sinusoid dilatation, congestion, pyknotic nucleus, and mononuclear cell infiltration were observed in the liver (Fig. 1). ASTX administration was found to reduce BPA-induced side effects in the liver (Fig. 2A).

**Masson's trichrome staining of liver tissues:** There was no increase in fibrosis or histologic alteration noted in the control group. Comparing the BPA group to the control group, more fibrosis was seen in and around the portal

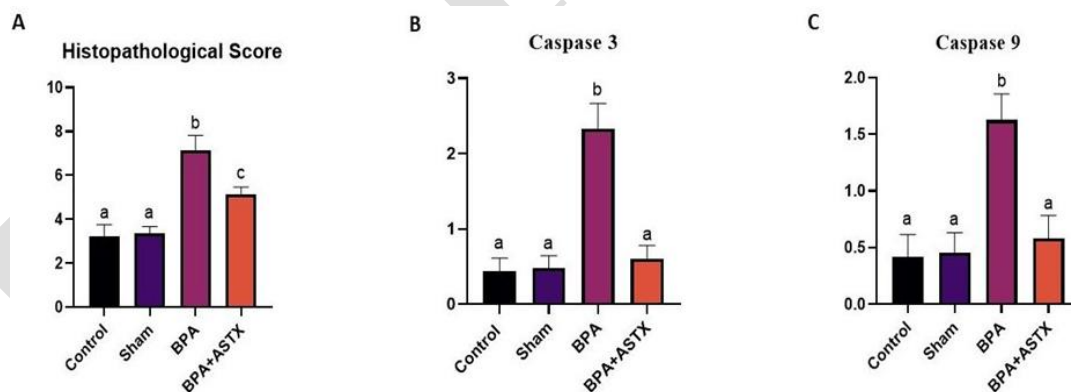
region. In comparison to the BPA group, less fibrosis observed in the ASTX-treated groups (Fig. 1).

**Caspase 3 and caspase 9 immunohistochemical staining:** In comparison to all other groups, it was discovered that the BPA group had higher expression levels of caspase 3 and caspase 9. ASTX administration was found to result in a decrease in caspase 3 and caspase 9 expression when compared to the BPA group (Fig. 2B, C and Fig. 3).

**mRNA expression analysis:** The effects of BPA alone and BPA plus ASTX treatment on the mRNA expression of apoptosis (Caspase 3, Caspase 9) and collagen (COL1A1, COL3A1) related genes in liver tissues were analyzed by RT-qPCR. According to the results obtained, the expression of the Caspase 3 gene in the BPA-treated group increased 2.009-fold ( $p=0.0074$ ) compared to the control



**Fig. 1:** H&E staining of liver section specimens a; control, b; sham c; BPA, d; BPA+ASTX black arrowhead; congestion, asterisk; dilatation, red arrow; pyknotic nucleus. Masson staining of liver sections e; control, f; sham, g; BPA, h; BPA+ASTX



**Fig. 2A:** Preparations were evaluated for sinusoid dilatation, congestion, pyknotic nuclei, and mononuclear cell infiltration. Histopathologic changes were graded as absent (0), mild (1), moderate (2) and severe (3). The maximum score was determined as 12. Oneway ANOVA was evaluated with the Tukey Test ( $p<0.05$ ). a, b, c indicate statistical significance between groups ( $p<0.05$ ). B; Caspase 3 and C; caspase 9 expression immunohistochemical scoring a, b, c, and d indicate statistical significance between groups ( $p<0.05$ ).

**Table 1:** Primer sequences of target and reference genes used in RT-qPCR analysis

Gene	Forward primer (5' - >3')	Reverse primer (5' - >3')
CASP3	CCCTGAAATGGGCTTGTGTA	GAGGTTAGCTGCATCGACAT
CASP9	GAAGAACGACCTGACTGCTAAG	ATGAGAGAGGATGACCACCA
COL1A1	CCAATGGTGCTCCTGGTATT	GTTACCACCTGTTGCCCTTTG
COL3A1	GTGTGATGATGAGCCACTAGAC	TGACAGGAGCAGGTGTAGAA
GAPDH	GCATTGCAGAGGATGGTAGAG	GCGGGAGAAGAAAGTCATGATTAG

group and 1.97-fold ( $p=0.0088$ ) compared to the sham group. When the group in which ASTX was administered together with BPA was compared with the group treated with BPA alone, a 1.72-fold ( $p=0.0277$ ) decrease in Caspase 3 gene mRNA expression was determined (Fig. 4).

When compared to the control group and sham group, the mRNA expression of the Caspase 9 gene increased 2.9 ( $p=0.0229$ ) and 2.81 ( $p=0.0256$ ) fold in the group treated with BPA alone, respectively. When the BPA+ASTX group was compared with the BPA alone group, the expression of the Caspase 9 gene decreased 2.51 fold ( $p=0.0395$ ) (Fig. 4).

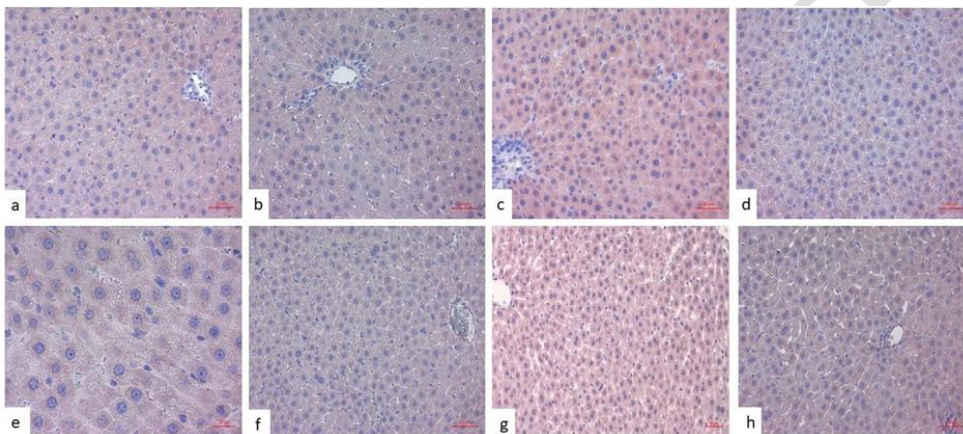
When the mRNA expression level results of the COL1A1 gene were analyzed, it was found that it increased 2.67-fold ( $p=0.0679$ ) and 2.43-fold ( $p=0.0920$ ) in the BPA alone treatment group compared to the control and sham groups, respectively, and decreased 2-fold ( $p=0.1804$ ) in the BPA+ASTX treatment group compared to the BPA group (Fig. 4).

When the mRNA expression level of the COL3A1 gene was evaluated, a 2.61 ( $p=0.017$ ) and 2.76 ( $p=0.0132$ ) fold increase was observed in the group treated with BPA alone compared to the control and sham groups, respectively. When the group treated with BPA+ASTX was compared with the group treated with BPA alone,

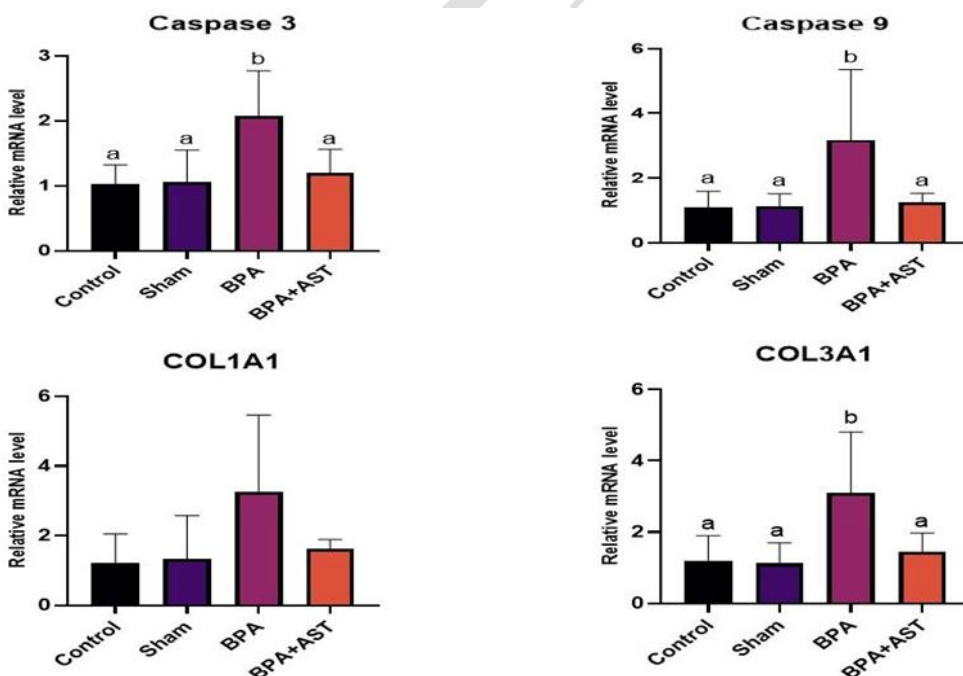
COL3A1 mRNA expression decreased 2.143 fold ( $p=0.0442$ ) (Fig. 4).

**Biochemical analyses:** BPA application caused a significant increase in disulphide compared to the control group. Hence, it has been discovered that exposure to bisphenol A (BPA) may lead to an elevation in oxidative stress, thereby resulting in oxidative damage. The delivery of oral gavage may have contributed to the disulphide increase in the sham group, which was less than that in the BPA group. It was demonstrated that the administration of ASTX resulted in a reduction in disulphide elevation induced by BPA to a level comparable to that of the control group, as well as a significant decrease in oxidative stress (Fig. 5).

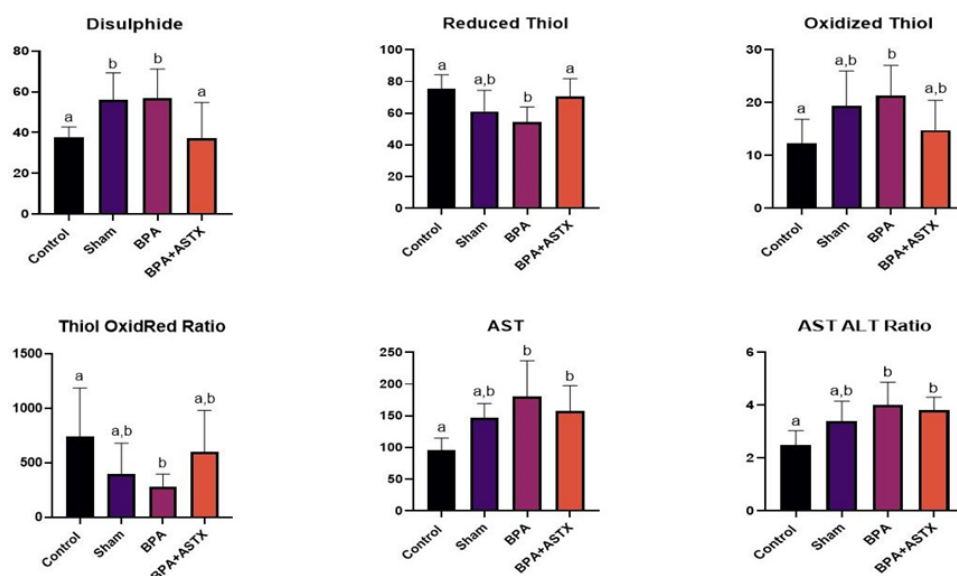
Injecting BPA significantly decreased the reduced thiol level relative to the control group; this decline in reduced thiol results in a reduction in antioxidant capability. ASTX administration significantly increased the decrease in reduced thiol level caused by BPA and provided a reduced thiol level close to the control group and increased the antioxidant capacity. The partial reduced thiol decrease in the sham group may be attributed to the stress (Fig. 5).



**Fig. 3:** Caspase 3 expression of liver section samples a; control, b; sham c; BPA, d; BPA+ASTX. Caspase 9 expression of liver sections e; control, f; sham, g; BPA, h; BPA+ASTX



**Fig. 4:** mRNA expression analysis of Caspase 3, Caspase 9, COL1A1, and COL3A1 genes a and b indicate statistical significance between groups ( $p<0.05$ ).



**Fig. 5:** Biochemical analysis of Disulphide, reduced thiol, oxidized thiol, thiol oxidred ratio, AST, AST ALT ratio. a and b indicate statistical significance between groups ( $p < 0.05$ ).

BPA administration caused a significant increase in oxidised thiol levels. ASTX administration significantly decreased the BPA-induced oxidised thiol levels and brought it to a level close to the control group, thereby reducing the increase in oxidative stress. The partial increase in oxidised thiol in the sham group may be attributed to the stress (Fig. 5).

BPA administration caused a significant increase in AST and AST/ALT levels. ASTX administration decreased the BPA-induced increase in AST and AST/ALT levels, and thus, it was observed that the possible liver damage and dysfunction regressed. The partial increase in AST and AST/ALT in the sham group may be attributed to stress-induced oxidative damage (Fig. 5).

Although there were negative changes in total thiol, native thiol, thiol oxidation/reduction, and ALT levels with BPA exposure and positive improvements with ASTX, the results did not reach statistical significance. The fact that the significant parameters clearly reveal the oxidative stress caused by BPA, the increase in antioxidant capacity and liver dysfunction, and that ASTX application clearly reveals the corrective aspect of the negative effects of BPA supports the numerical changes in total thiol, native thiol, thiol oxidation/reduction and ALT levels.

## DISCUSSION

Many sectors, including dentistry, employ bisphenol A, an endocrine disruptive chemical. BPA has attracted attention for its threat to human health (Zhang *et al.*, 2020). The toxic effects of BPA in the liver have been reported in many studies. Manifestations such as cellular damage, degeneration, necrosis, and fibrosis in the liver have been observed in rats exposed to BPA (Melzer *et al.*, 2010; Vandenberg *et al.*, 2007). The present investigation aims to assess the possible impact of ASTX on the histopathological alterations generated by bisphenol A (BPA) in the liver.

ASTX is a carotenoid naturally found in seafood and has antioxidant properties (Hussein *et al.*, 2007). The ability of ASTX to reduce oxidative stress is attributed to its mechanism of preventing cellular damage caused by free radicals (Park *et al.*, 2010). When there is too much

ROS generation and not enough antioxidant defense, oxidative stress occurs. DNA oxidative damage and aberrant protein expression can occur when the body is under oxidative stress due to increased ROS and reactive nitrogen that exceeds the body's ability to scavenge free radicals properly. Evidence from the past indicates that oxidative stress is a key mechanism through which BPA affects mammalian cells (Jiang *et al.*, 2020).

In a study conducted by Hassan *et al.* (2012), it was shown that male Wistar albino rats, when orally supplied at a dosage of 50 mg/kg of bisphenol A (BPA) through gavage once daily for a duration of four weeks, exhibited a notable elevation in biochemical markers such as alanine aminotransferase (ALT), alkaline phosphatase (ALP), and total bilirubin levels (Hassan *et al.*, 2012). In a study conducted by Mahdavinia *et al.* (2019), it was shown that the administration of 250mg/kg BPA for a duration of 14 days resulted in a notable elevation in serum AST and ALT activity, indicating liver injury (Mahdavinia *et al.*, 2019). In another study, exposure to 100 mg/kg BPA for 30 days significantly decreased the activities of CAT, SOD, peroxidase (POD), glutathione reductase (GSR), glutathione S-transferase (GST), and GSH content, while significantly increasing the levels of thiobarbituric acid reactive substances (TBARS) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). It has been reported that BPA produces free radicals and generally impairs the antioxidant enzyme defense system (Ijaz *et al.*, 2022). Li *et al.* (2022) showed that oxidative stress damage in follicles induced by BPA (0.2 to 17.6 µg/g) significantly increased the expression levels of mitochondrial membrane potential, antioxidant genes (CAT, SOD1, and SOD2), and anti-apoptotic gene Bcl-2 in oocytes in the BPA+ASTX group (Li *et al.*, 2022).

Native Thiol and Total Thiol levels were measured in all groups in our study. Native Thiol and Total Thiol values are expected to increase with an increase in antioxidant capacity. Considering our study results, it could be evaluated that BPA exposure resulted in a decrease in antioxidant capacity, and ASTX supplementation resulted in an increase in antioxidant capacity per our hypothesis regarding oxidant-antioxidant balance. These statistically significant values are similar to our calculation of oxidative stress, which is a very good indicator of oxidant-

antioxidant balance and excludes reactive increases ( $p < 0.05$ ). Furthermore, the BPA group exhibited a statistically significant elevation in AST levels compared to the control group, whereas the ASTX-treated group had a decrease in these levels.

In a study conducted by Vahdati *et al.* (2017), it was observed that male Wistar rats exposed to a dosage of 0.5 mg/kg BPA for a duration of 30 days exhibited fragmented necrosis, condensed nuclei, and dense infiltration of leukocytes or periportal inflammation (Vahdati *et al.*, 2017). Additionally, in the study by Amin *et al.* (2023), after oral administration of 50 mg/kg BPA daily for 8 weeks to male albino rats, it was reported that the expression of X protein associated with Bcl-2 (Bax) and transforming growth factor beta 1 (TGF B1) significantly increased, and fibrosis was also observed (Amin *et al.*, 2023).

In our investigation, we found that administering ASTX preserved the liver tissue and lessened the histological alterations brought on by BPA. Based on our findings, it is believed that liver damage may result from oxidative stress caused by BPA, and ASTX application is thought to assist in reducing this damage through its potent antioxidant effect. Additionally, the Masson trichrome staining data showed that the BPA+ASTX group had less fibrosis whereas the BPA group had more. In addition, it was observed that the group subjected to bisphenol A (BPA) had increased levels of COL1A1 gene expression linked to fibrosis when compared to the control group, however no statistically significant difference was found between the two groups. Additionally, there was a statistically significant increase in COL3A1 gene expression in the BPA group. In the ASTX-treated group, COL3A1 gene expression decreased in a statistically meaningful way. These findings support that ASTX has the potential to reduce BPA-induced liver fibrosis.

Wang *et al.* (2021) showed that apoptosis increased in the liver of mice receiving a single dose of 500 mg/kg BPA every day for 2 weeks by TUNEL staining method. This effect was significantly reduced by oridonin (Wang *et al.*, 2021). In another study, oral administration of 50 mg/kg BPA for 8 weeks caused an increase in the level of pro-apoptotic protein caspase-3 and a decrease in anti-apoptotic protein Bcl-2 immunoreactivity in the liver of male rats (Elswefy *et al.*, 2016). Administration of BPA to pregnant rats (0.036 and 3.42 mg/kg b.w. daily) caused liver damage in lactating females. Additionally, it was discovered to have perinatal effects on female postnatal day 6 (PND6) pups through apoptotic pathways in the liver, increased oxidative stress levels, and an inflammatory response (Linillos-Pradillo *et al.*, 2023). In another study, Sprague Dawley rats were administered BPA by gavage at different doses (0, 30, 90 and 270 mg/kg bw) for 30 days. It was reported that high doses of BPA decreased the amounts of Bcl-2 in the liver and increased the levels of Bax, cleaved-Caspase3, and cleaved-PARP1 proteins, which impaired the mitochondrial activity of hepatocytes and encouraged cell death (Liu *et al.*, 2022). In the study conducted by Amin *et al.* (2023), after 8 weeks of oral administration of 50 mg/kg BPA to male albino rats, the expression of Bax protein and TGF-B1, along with apoptosis and fibrosis, was significantly demonstrated (Amin *et al.*, 2023). In our

study, by the above data, it was observed that the expression of caspase 3 and caspase 9 increased with BPA application as a result of immunohistochemistry staining and PCR analysis, ASTX application decreased this increase and thus reduced BPA-induced apoptosis. All of these data support that the strong apoptotic effect of BPA in the liver can be mitigated by the protective effects of ASTX application.

**Conclusion:** This study demonstrated that ASTX supplementation can attenuate BPA-induced histopathological changes and protect liver tissue. These results suggest that the use of AST as a potential treatment or protective agent could be considered. But more investigation is necessary, as is a deeper comprehension of ASTX's mode of functioning.

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