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Research Article

AMELIORATING POTENTIAL OF *CASSIA ABSUS* SEED-POWDER AGAINST CADMIUM-INDUCED ALTERATIONS IN ZEBRAFISH AND IDENTIFICATION OF FLAVONOIDS IN DIFFERENT EXTRACTS OF SEED

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ABSTRACT: The study was conducted to evaluate the ameliorating potential of *Cassia absus* L. (CA) seed-powder against cadmium-induced alterations in zebrafish (*Danio rerio* L.). Fish were divided into five groups (C1: received normal feed, C2: received normal feed along with CA powder, C3: normal feed along with cadmium exposure (1 ppm), C4: Vitamin E-enriched feed along with cadmium exposure and T1: Feeding of powder of CA (50% of total feed) along with cadmium exposure comprising of eighteen fish in each group. The fish of the treatment group were offered with powder of CA (10 mg /fish in the morning for 21 days along with exposure to cadmium chloride. Whole fish was used to evaluate oxidative stress markers and histopathological evaluation of organs. After 21 days of exposure to cadmium chloride to fish, a significant reduction in catalase activity, non-significant reduction in reduced glutathione level (GSH) along with a significantly higher level of malondialdehyde was observed. The treatment of CA seed-powder partially ameliorated alterations in oxidative stress markers. Cadmium chloride exposure for 21 days led to pathological changes in the liver, kidney, gills and brain of zebrafish. The microscopic changes in the organs of fish treated with CA powder were mild as compared to the toxicity group. Phytochemical analysis of the methanolic and n-butanol fraction of CA seed revealed the presence of various flavonoids. It can be concluded that *Cassia absus* seeds have the ability to control the toxic potential of Cadmium and it may be due to phytochemicals like flavonoids present in the seed powder.

Key words: *Cassia absus* seed, Zebrafish, Oxidative stress, Histopathological evaluation.

INTRODUCTION

Oxidative stress is the process that occurs due to stimulation of the production of free radicals like superoxide anion, hydrogen peroxide, hydroxyl radical, and lipid peroxides by xenobiotics and also occurs in most disease conditions. Various synthetic antioxidants are being used to combat oxidative stress in humans and animals. However, plant-based active principles having the antioxidant effect are in demand due to less chance of side effects. Medicinal plants can be considered the best source of free radical scavengers (Liu *et al.* 2009). Many plants have been evaluated so far for having an antioxidant effect and numerous phytochemicals have been identified and are being used to protect the body against continuously occurring oxidative insult in the body. The seed-powder, paste and juice of the herb can be effectively administered orally to produce the immunomodulatory effects (Pattanayak 2020).

Cassia absus (CA) is a plant from the family Caesalpiniaceae and is known as *Chimed* in Gujarat (India). The seed of this plant contains an abundant number of alkaloids named chaksine and isochaksine and also contains flavonoids (Khare 2008). Aqueous and ethyl acetate extracts of CA extract have been reported to have a reversal effect against oxidative stress-mediated alterations in diabetic rats (Rashid *et al.* 2017). Previously, we evaluated an *in vitro* antioxidant activity of extract of CA seed and observed that the extract of CA seed has the potency to scavenge the free radicals (Bhatt *et al.* 2019). The evaluation of *in vivo* antioxidant effects of CA seed

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has not been carried out so far and such study in the lower vertebrate animal model can be useful to explore its medicinal value.

Due to pollution in air, water and soil, animals including aquatic species are continuously exposed to various xenobiotics which cause oxidative stress-mediated alterations in the body. Therefore, it is important to protect the major organs from such oxidative insults. On the other hand, zebrafish (*Danio rerio* L.) is considered a popular model organism for understanding biological processes, effects of drugs and xenobiotics. Because of homology to humans, the zebrafish model is commonly used for research in the field of pharmacology and toxicology in the present scenario. The oxidative stress model can be easily developed by exposing laboratory animal-like zebrafish to stress inducers such as heavy metal. Amongst all heavy metals, cadmium is having a long biological half-life and damages most major organs through oxidative stress. Considering all these facts, the present study was planned to evaluate an *in vivo* antioxidant effect of CA seed in cadmium-induced oxidative stress model of zebrafish along with chromatographic analysis of an extract of CA for the presence of flavonoids.

MATERIALS AND METHODS

Collection, authentication and preservation of plant material

Seeds of CA were purchased from the local market of Junagadh, Gujarat, India. The plant specimen was authenticated by a Botanist. The powder was made from seeds of CA and stored in an air-tight container at refrigerator temperature for further use.

Thin-Layer Chromatography (TLC) of extracts of *C. absus* seed

Thin-Layer Chromatography (TLC) was performed to evaluate the presence of various flavonoids compounds in methanolic extract and an n-butanol fraction of CA seed-powder. The thin layer chromatographic analysis of the extracts was carried out using 10 × 10 cm pre-coated aluminium-backed silica gel plates GF254 (Merck, Darmstadt, Germany). The samples were applied as a band using Linomat 5 HPTLC applicator (Camag, Germany). The extract was prepared by extracting 0.5 g of CA seed-powder with 5 mL of 50%, 70%, and absolute methanol for 15 minutes on the ultrasonic bath. The content was then centrifuged at 2500 rpm for 10 minutes. The supernatant was collected, concentrated and used as a sample. For the preparation of the n-butanol fraction, 70% methanol extract was dissolved in distilled water and fractionated with n-hexane, chloroform, ethyl acetate and n-butanol in a separatory funnel. An n-butanol fraction was collected, dried in vacuum. The dried n-butanol fraction was dissolved in methanol and used as a sample for TLC. Two different solvent systems were used for the detection of various flavonoids in CA seed *viz*. Ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:26) (Wagner 2007) and n-butanol: glacial acetic acid: water (4:1:5) (Ali *et al.* 2011). The samples were applied at 1 cm from the edge of the plate as an 8 mm band and allowed to run up to 8 cm. Derivatization was achieved by spraying natural product reagents (1% diphenylboryloxyethylamine in methanol followed by 5% polyethylene glycol-4000 in methanol). The plates were then observed in the UV cabinet (Camag, Germany) at 366 nm. Quercetin (a flavonoid compound) (Sigma-Aldrich, India) was dissolved in methanol at a concentration (0.5 mg/mL) and used as a reference standard.

Procurement, housing and husbandry of adult zebrafish

Zebrafish were purchased from a local fish vendor. The fish were kept in a 9-litre glass tank for 10 days for acclimatization at laboratory animal house at department.

Table 1. Groups and types of treatments.

Groups	Type of treatments for 21 days
C1	Fish in RO water and fed with 10 mg normal feed /fish twice a day
C2	Fish in RO water and fed with 10 mg *C. absus* powder /fish in the morning + 10 mg normal feed /fish in the evening
C3	Fish in RO water containing $CdCl_2$ (1 ppm) + 10 mg normal feed /fish twice a day
C4	Fish in RO water containing $CdCl_2$ (1 ppm) + 500 mg/kg Vitamin-E-enriched feed 10 mg/fish in the morning + 10 mg normal feed /fish in the evening
T1	Fish in RO water containing $CdCl_2$ (1 ppm) and fed with 10 mg *C. absus* powder /fish in the morning + 10 mg normal feed /fish in the evening

during the entire study, the fish were kept at 27 ± 2°C temperature with 14:10 hour's light-dark cycle. Reverse osmosis water was used during the whole experiment. The water was changed every 48 hours during the experiment. Water quality parameters were evaluated every week for 21 days. Fish were fed with commercial aquarium fish feed (nutrition values: crude protein 28%; crude fat 3% and crude fibre 4%) during the study. Vitamin-E-enriched feed (500 mg Vitamin E/kg feed) was prepared by mixing thoroughly the required quantity of vitamin E with a fine powder of commercial feed and used in the experiment.

Grouping and treatment to zebrafish

Post acclimatization, ninety (90) male zebrafish were divided into 5 groups of eighteen (18) fish in each group. These fish were offered different types of treatments, as mentioned in Table 1. The experimental protocol was approved by the Institutional Animal Ethics Committee.

Evaluation of oxidative stress parameters

After 21 days of the treatment period, 12 zebrafish from each group were sacrificed humanely using the ice-cold method. The whole fish homogenate was prepared with double volume of cold phosphate buffer saline (pH 7.4) against the fish weight (mg) for CAT, GSH, and MDA analysis (n=6); and in Tris-EDTA buffer (pH 8.5) for SOD analysis (n=6). The homogenate was centrifuged at 5000 rpm for 10 minutes and used to evaluate the oxidative stress parameters. Oxidative stress parameters like superoxide dismutase (SOD) activity (Sigma-Aldrich kit), catalase activity (Aebi 1984), and estimation of reduced-glutathione level (Ellman 1959) and malondialdehyde level (Lykkesfeldt 2001) were evaluated from whole fish homogenate. Estimation of protein content in samples was carried out using the Bradford method (Bradford 1976) and was used to calculate the catalase activity.

Histopathological evaluation of organs

On the 22nd day, six fish from each group were sacrificed humanely and fixed in 10% buffered formalin. After seven days, fish were removed from formalin and rinsed with phosphate buffer saline (pH 7.4) and immersed into 0.35M disodium EDTA (pH 7.8) for seven days for the decalcification process (Copper *et al.* 2018). The decalcified fish were subjected to paraffin wax embedding for tissue sectioning. The sectioning and staining were done as per the standard method for histopathological examination (Luna 1968).

Statistical analysis

All the numerical data were expressed as Mean ± Standard Error of Mean (SEM). Data were analyzed by one-way Analysis of Variance (ANOVA) followed by Duncan Multiple Range Test (DMRT) to compare the difference in means, and $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

HPTLC analysis of methanolic extracts and an n-butanol fraction of CA revealed the presence of various flavonoid compounds with different Rf values, as shown in Table 2. The detected bands of flavonoids with both mobile phases are shown in Fig. 1. The n-butanol: glacial acetic acid: water (4:1:5) was quite a more efficient mobile phase for the separation of flavonoids from extracts than ethyl acetate: formic acid: glacial acetic acid: water (100: 11: 11: 26).

Table 2. Rf values of flavonoids detected in *C. absus* seed.

Extract/fraction/compound	Rf values [a]	Rf values [b]
C. absus methanolic extract (50%)	0.21, 0.27, 0.33, 0.41, 0.52, 0.77, 0.92	0.15, 0.35, 0.41, 0.47, 0.52, 0.58, 0.67, 0.77,0.86
C. absus methanolic extract (70%)	0.21, 0.27, 0.33, 0.41, 0.52, 0.60, 0.77, 0.92	0.15, 0.35, 0.41, 0.47, 0.52, 0.58, 0.77, 0.86
C. absus methanolic extract (100%)	0.15, 0.21, 0.27, 0.33, 0.41, 0.52, 0.60	0.22, 0.35, 0.47, 0.52, 0.58, 0.91
n-butanol fraction	0.15, 0.21, 0.27, 0.52, 0.60, 0.77, 0.88	0.15, 0.22, 0.41, 0.47, 0.58, 0.70, 0.77
Quercetin as a standard flavonoid	0.99	0.90

a = Rf value for the solvent system ethyl acetate: formic acid: glacial acetic acid: water (100: 11: 11: 26).
b = Rf value for the solvent system n-butanol: glacial acetic acid: water (4:1:5).

Table 3. Oxidative stress parameters determined from the whole fish homogenate.

	Treatment Groups				
Parameters	**C1**	**C2**	**C3**	**C4**	**T1**
SOD (% inhibition)	92.02±0.99^{a}	95.88±0.65^{bc}	94.25±0.60^{b}	98.31±0.63^{d}	97.30±0.61^{cd}
Catalase (U/mg protein)	0.09±0.02^{b}	0.06±0.01^{ab}	0.05±0.01^{a}	0.05±0.005^{ab}	0.07±0.01^{ab}
GSH (µg/mg tissue)	4.74±2.42^{a}	6.58±1.51^{a}	2.71±0.33^{a}	4.74±1.27^{a}	4.60±0.71^{a}
MDA (nM/mg tissue)	1.14±0.14^{a}	5.30±0.78^{a}	34.40±4.00^{b}	32.86±4.35^{b}	30.44±3.52^{b}

*Data are expressed as mean ± SEM. Means with different superscripts in a row differ significantly ($p< 0.05$).

The mean values of SOD and CAT activities and levels of GSH and MDA of all treatment groups are shown in Table 3. The activity of SOD in the tissue of fish treated with vitamin E, as well as the CA, was significantly higher compared to that of the normal control, the feed control and the toxicity group. The catalase activity in the tissue of cadmium-exposed fish was significantly lower than that of normal fish. Reduction of catalase activity due to cadmium exposure was prevented by the CA, which was similar to that of fish offered the vitamin E-enriched feed. The GSH level in the toxicity group was slightly lowered ($p> 0.05$) as compared to that observed in the normal control group. The tissue GSH level of fish of the feed control group was slightly higher than that of normal fish. Similarly, the levels of GSH were also high in vitamin E treatment and CA treatment groups compared to that of the toxicity group, indicating the glutathione stimulating effect of both treatments. The level of MDA in the toxicity group was significantly higher ($p< 0.05$) than that of the normal control group. The MDA level in fish treated with CA and vitamin E-enriched feed was lower ($p > 0.05$) compared to the level observed in the toxicity group.

Upon microscopic histological examination of the liver of fish exposed to cadmium, focal areas of necrosis with vacuolization were observed. However, mild degenerative changes in hepatocytes with less vacuolization compared to the toxicity group were observed in the liver of zebrafish treated with vitamin E-enriched feed along with cadmium exposure and also in the liver of fish treated with CA (Fig. 2).

Histological sections of the kidney of fish exposed to cadmium were found with atrophied and detached glomerulus with loss of tubular architecture. However, vitamin E-enriched feed and powder of CA had partially

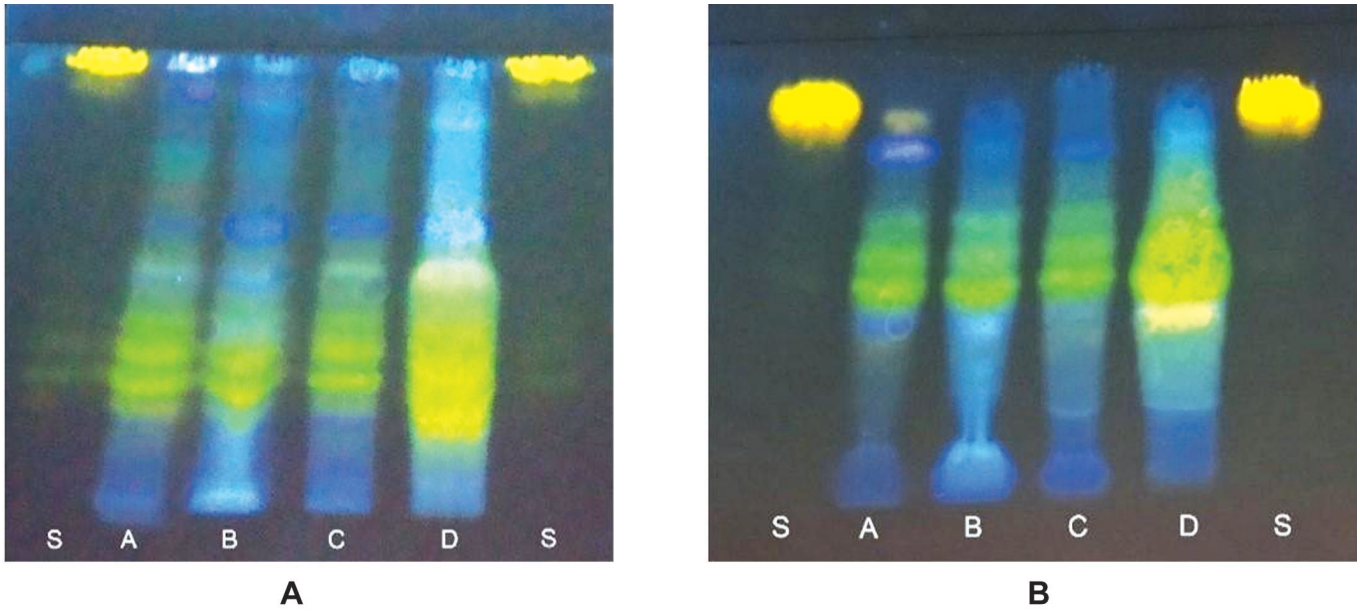

Fig. 1. Detection of various flavonoids from various extracts and an n-butanol fraction of *C. absus* seed.
[S = standard (Quercetin); A = 50% methanol extract; B = 70% methanol extract; C = 100% methanol extract; D = n-butanol fraction. A) Detection with ethyl acetate: formic acid: glacial acetic acid: water (100: 11: 11: 26). B) Detection with n-butanol: glacial acetic acid: water (4:1:5)].

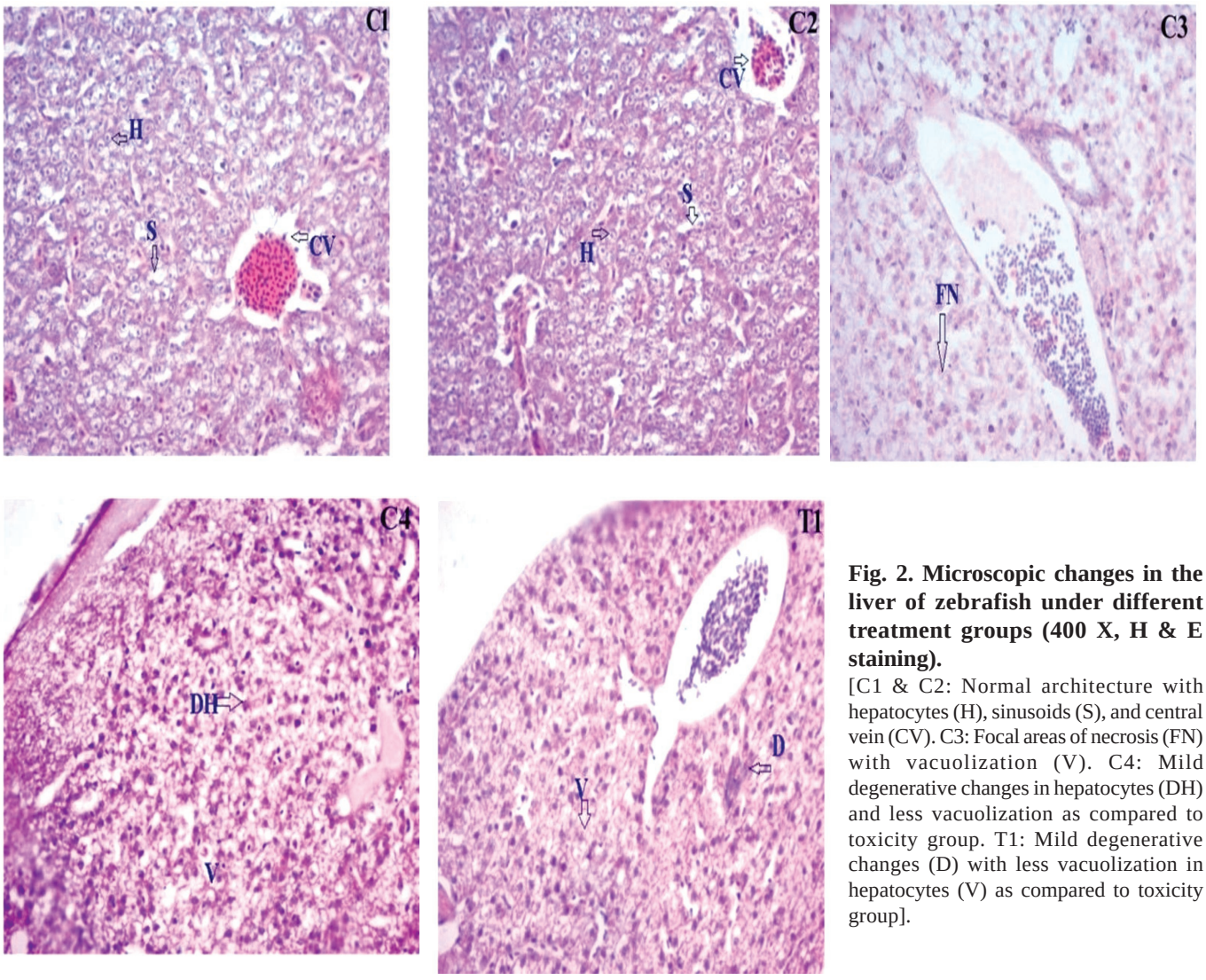

Fig. 2. Microscopic changes in the liver of zebrafish under different treatment groups (400 X, H & E staining).
[C1 & C2: Normal architecture with hepatocytes (H), sinusoids (S), and central vein (CV). C3: Focal areas of necrosis (FN) with vacuolization (V). C4: Mild degenerative changes in hepatocytes (DH) and less vacuolization as compared to toxicity group. T1: Mild degenerative changes (D) with less vacuolization in hepatocytes (V) as compared to toxicity group].

protected the kidney from cadmium-induced damage as evidenced by the normal architecture of glomerulus with only mild tubular degeneration in the kidney compared to the toxicity group (Fig. 3).

Gills of normal control fish and fish treated with vitamin E-enriched feed had shown the normal architecture of pillar cells and lamellae. Gills of Cd-exposed fish were found with degenerated pillar cells and lamellae. Gills of fish treated with CA powder had shown the normal primary lamellae with blunting and fusion of secondary lamellae at few places (Fig. 4).

The brains of fish exposed to cadmium had shown reduced neuron density with congested capillaries. Treatment with vitamin E-enriched feed and CA powder protected the brain from cadmium toxicity (Fig. 5). Sections of the brain of fish treated with CA were found with mild degeneration of neurons (MDN) with normal neuropils which indicated the protection against Cd-induced damage.

The preliminary detection of active phytochemicals in plant extract is a crucial step in ethnopharmacological research. In this study, for evaluation of the suitability of the mobile phase for separation and detection of various flavonoids using TLC was also carried out. It was noticed that the use of ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:26) and n- butanol: glacial acetic acid: water (4:1:5) as mobile phase resulted in the good separation of flavonoids from the extract of CA. Other than flavonoids, various phytochemicals have been found in *C. absus* seed. The phytosterol like β-sitosterol has also been reported to be present in *C. absus* seed (Pandita and Vaidya 2016).

Long-term exposure to heavy metals like cadmium can lead to the production of reactive oxygen species (ROS) beyond the typical function of the antioxidant system, which oxidize the active sites of proteins, resulting in the loss of function of the enzymes (Zhou *et al.* 2019). Superoxide dismutase and catalase are considered as the

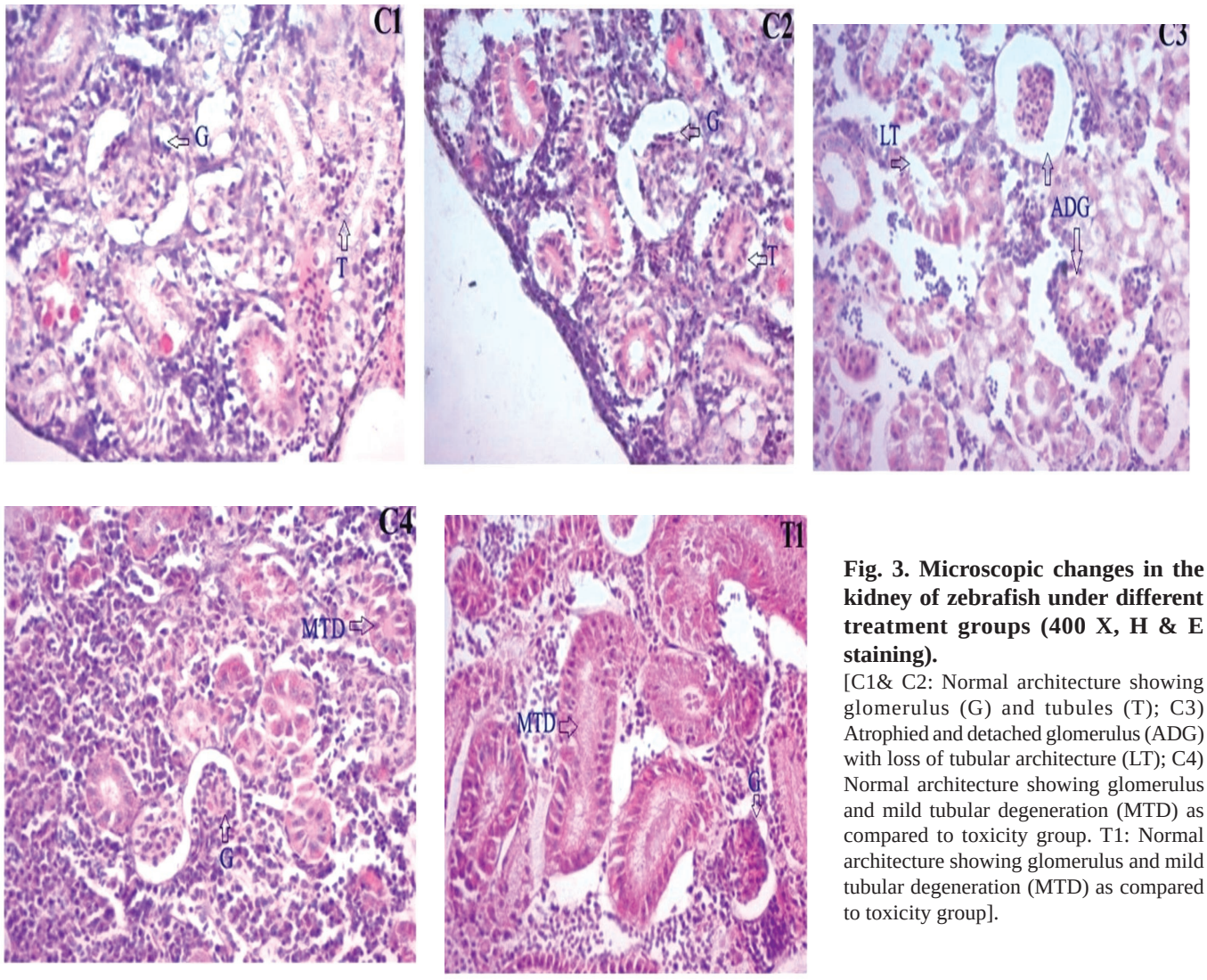

Fig. 3. Microscopic changes in the kidney of zebrafish under different treatment groups (400 X, H & E staining).

[C1& C2: Normal architecture showing glomerulus (G) and tubules (T); C3) Atrophied and detached glomerulus (ADG) with loss of tubular architecture (LT); C4) Normal architecture showing glomerulus and mild tubular degeneration (MTD) as compared to toxicity group. T1: Normal architecture showing glomerulus and mild tubular degeneration (MTD) as compared to toxicity group].

first line of defence mechanism against oxidative stress via catalyzing the dismutation of intracellular oxygen free radicals (O_2^-) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2), which is subsequently transformed into H_2O and O_2 (Wang *et al.* 2020). In the present study, the activity of catalase was significantly reduced by cadmium in the toxicity group and increased in the CA treatment group (T1). This finding indicated that CA treatment may be useful to modulate the oxidative stress induced by cadmium. Similar to our finding, the feeding of powder of a medicinal plant like *Gracilaria gracilis* had shown an effect on growth performance, oxidative stress and immune system of zebrafish (Hoseinifar *et al.* 2018).

The GSH, a non-enzymatic antioxidant compound that modulates oxidative stress-induced lipid peroxidation and provides secondary protection against oxidative stress (Ullah *et al.* 2018, Wang *et al.* 2018). Previous studies have demonstrated that exposure to cadmium had decreased the GSH level in *Sparus Aurata* (Souid *et al.* 2013). In the present study, the GSH level was higher in fish supplemented with the vitamin E-enriched feed and CA seed-powder as compared to that of the toxicity group which indicates that CA seed-powder had shown a significant antioxidant effect against Cd-induced free radicals. The MDA is considered the marker of the lipid peroxidation process and an increased level of MDA indicates the lipid peroxidation (Ayala *et al.* 2014). In the present study, fish treated with CA seed-powder had shown to have lower levels of MDA compared to the toxicity group, which indicates that CA seed-powder might protect the body from lipid peroxidation caused by cadmium exposure. Similarly, the active constituents from Chinese herbal medicine have been reported to have protection against free radicals, which could result in a reduced level of MDA (Cui *et al.* 2018).

Cadmium has been reported to produce the most conspicuous changes in the liver like darker nucleoli,

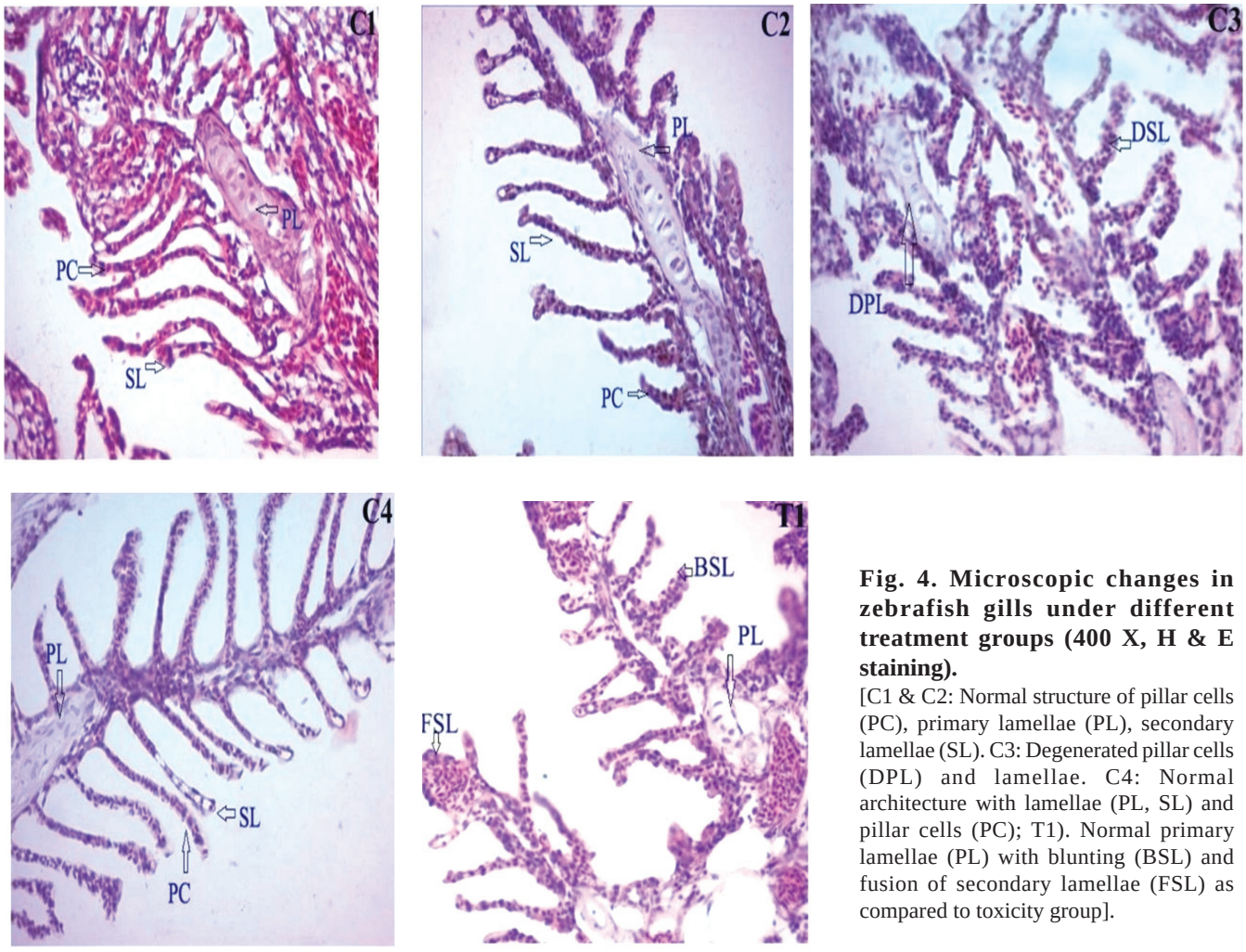

Fig. 4. Microscopic changes in zebrafish gills under different treatment groups (400 X, H & E staining).

[C1 & C2: Normal structure of pillar cells (PC), primary lamellae (PL), secondary lamellae (SL). C3: Degenerated pillar cells (DPL) and lamellae. C4: Normal architecture with lamellae (PL, SL) and pillar cells (PC); T1). Normal primary lamellae (PL) with blunting (BSL) and fusion of secondary lamellae (FSL) as compared to toxicity group].

irregularly shaped hepatocytes with dilated blood capillaries, focal and single necrosis in aquatic species (Ahmed *et al*. 2014). In the present study, the treatment group had shown mild degenerative changes with less vacuolization in hepatocytes compared to that of the toxicity group. It has also been reported that flavonoids can lower the cadmium load in the tissue (Abbas *et al.* 2019). Marked changes in the kidney of fish (*Oreochromis niloticus* L.) following exposure to cadmium chloride included the loosening of hemopoietic tissue, hemorrhagic conditions, and degeneration of hemopoietic tissue. Renal tubules with loss of their original appearance, wide spaces and oedema were also reported along with narrowing of the tubular lumen, vacuolated cytoplasm and damaged glomeruli (Nagar and Bhattacharya 2020). In the present study, the histological section of the kidney of fish treated with CA seed-powder had shown normal glomerulus architecture but having mild tubular degeneration compared to the toxicity group which indicated the nephroprotective role of the CA. *C. absus* is rich in various polyphenols like flavonoids which might be responsible for protecting the kidney from heavy metals like cadmium (Mohammadi *et al.* 2016). Similar to the findings in the present study, cadmium exposure (10 µg/L for 21 days) has been reported to cause histopathological changes in zebrafish gill (Lu *et al.* 2018). The gills of the fish treated with CA seed-powder had shown normal primary lamellae with blunting and fusion of secondary lamellae only. In line with the result obtained in the present study, Bhalerao and Kothari (2014) reported a protective effect of herbal treatment against mercuric chloride-induced histopathological changes in gills of freshwater teleost. In the brain, cadmium has been reported to cause gliosis and degeneration of Purkinje cells with loss of neuropils in the zebrafish brain at 30 ppm exposure (Al-sawafi *et al.* 2017, Monaco *et al.* 2017). A previous report related to the damage of the brain by cadmium supports the findings of the present study. However, treatment of CA seed-powder to fish during exposure of cadmium had shown

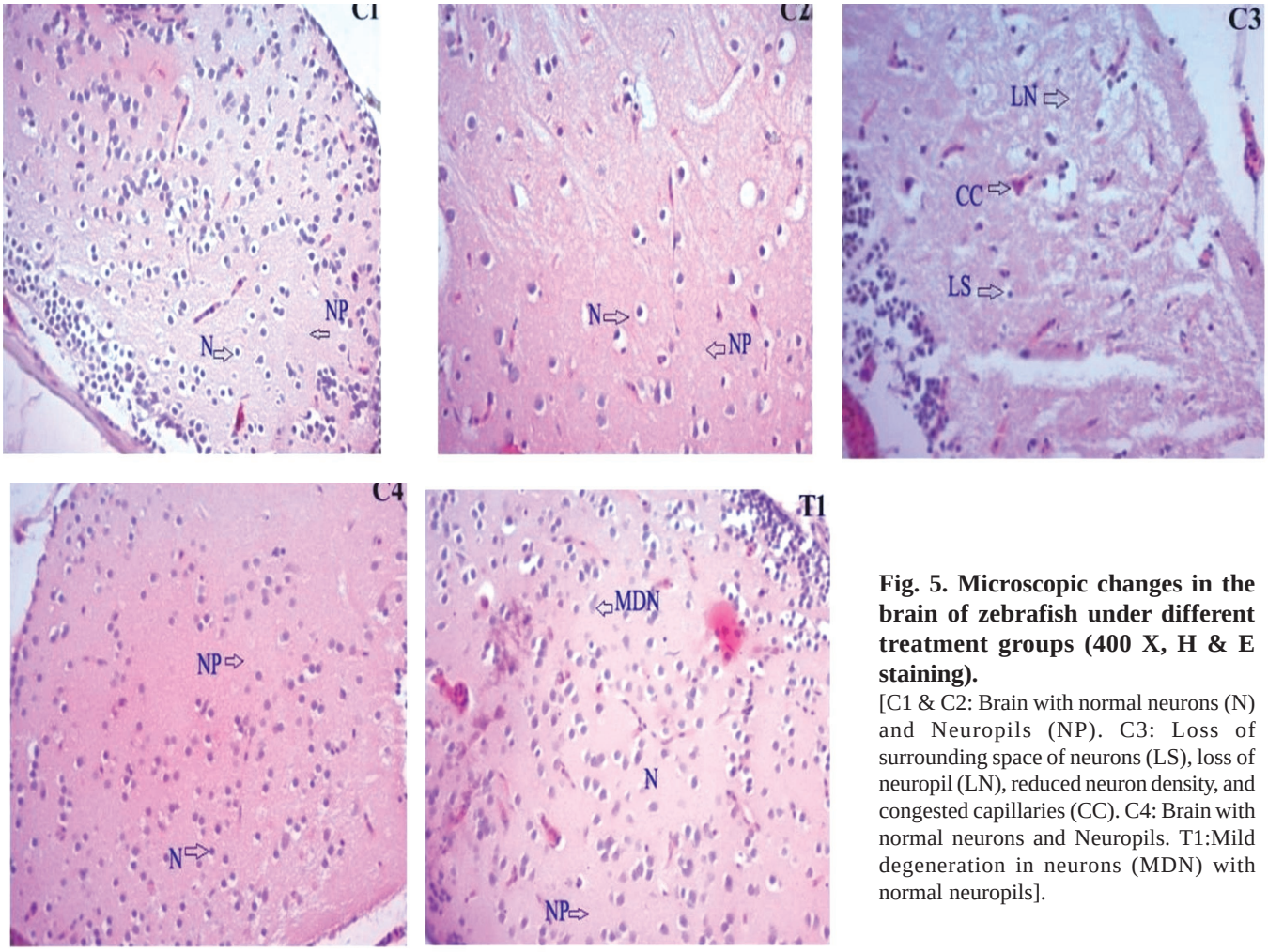

Fig. 5. Microscopic changes in the brain of zebrafish under different treatment groups (400 X, H & E staining).

[C1 & C2: Brain with normal neurons (N) and Neuropils (NP). C3: Loss of surrounding space of neurons (LS), loss of neuropil (LN), reduced neuron density, and congested capillaries (CC). C4: Brain with normal neurons and Neuropils. T1:Mild degeneration in neurons (MDN) with normal neuropils].

mild degenerative changes in neurons with normal neuropils. The microscopic changes in the brain of fish of the CA treatment group were mild compared to those observed in the toxicity group. Similar to CA treatment, *Petroselinum crispum* has been reported to possess the neuroprotective effect in mice exposed to Cd (Maodaa *et al.* 2016). The use of plant material or phytochemicals to combat the toxicity of xenobiotics is an important aspect of biomedicine.

It has been documented that co-treatment with *Spirulina platensis* markedly improved the oxidative stress and histological alterations in organs of rats exposed to arsenic (Korany *et al.* 2019). The protective effect of CA seed-powder against the cadmium-induced oxidative stress-mediated changes might be due to the potency of flavonoids present in the CA seed which could counteract the free radicals generated.

CONCLUSION

Feeding of 10 mg powder of CA seed/fish/day partially ameliorated the pathological changes in liver, kidney, gills and brain of zebrafish exposed to 1 ppm cadmium chloride for 21 days. The antioxidant effect of flavonoids present in *Cassia absus* might be responsible for protection against oxidative stress-mediated changes caused by cadmium in zebrafish. Further study may be performed to explore the pharmacological potentials of the phytochemicals present in the CA seed.

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