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Original Article

Troxerutin Exerts Anti-Pulmonary Fibrosis Activity in Nicotine-Induced Lung Fibrosis on Zebra Fish Model

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ABSTRACT

Background: Cigarette smoking is the major obstacles to lung cell proteostatic imbalance due to the exposure of the respiratory epithelial cell to toxic oxidants. The most active ingredient presents in the cigarette smokers, nicotine causes number of health risk by synthesis of free radicals which alters the individual cell DNA stability. Troxerutin, a flavonoid derived from the Rutin, possess antioxidant potential which has been investigated for its anti-pulmonary fibrosis activity against nicotine associated inflammation in the gills of the Zebra fish. **Objectives:** The current study was carried out to analyse toxicity of nicotine in zebrafish model and the restoration of gill architecture by the action of troxerutin. **Materials and Methods:** The activity of troxerutin was investigated by evaluation of antioxidant levels, gene expression, and tissue architecture via histopathological studies of the nicotine-induced group. **Results:** Superoxide Dismutase (SOD) and Catalase (CAT) levels significantly decreased in the nicotine-induced group in our study than in the treated groups after the administration of Troxerutin. Myeloperoxidase (MPO), Nitrous Acid (NO), and Lipid Peroxidation (LPO) levels elevated in induced group which reduced significantly in Troxerutin-treated groups. It exerted protective effect by reducing the histopathological alterations linked to nicotine-induced group, enhancing antioxidant defence, modifying the oxidative status by scavenging free radicals and supressing inflammatory gene expression of IL-10, IL-1 β pro-inflammatory cytokine in Zebra fish model. **Conclusion:** The current study confirms the potential function of troxerutin in repairing nicotine-induced pulmonary fibrosis in the Zebrafish model.

Keywords: Nicotine, Zebra fish, Antioxidant, Troxerutin, Lipid peroxidation, Gene expression.

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INTRODUCTION

Nicotine, a principal alkaloid isolated from the species Nicotiana tabacum (*leaves*) and Nicotiana rustica (*stem*). It is a pharmacological agent in causing lung disorders by generating free radical species. The major consumption of nicotine is by cigarette smoking, it is easily absorbed by the blood stream into the circulatory system thereby reaches the brain. Nicotine deposit antioxidant defence system by imposing more oxidative stress to the cells[1] resulting in release of free radicals causing damage to DNA, Lipids and Proteins at the cellular levels**.** Nicotine affects expression of genes, hormone secretion, modulates the action of enzymes associated with the activity.[2] This, adverse cellular effects caused by free radicals can be altered by the action of pharmacologically active compound (flavonoid). Here we employed, Troxerutin trihydroxy ethylated, a bioflavonoid derived from rutin, present in tea, coffee, vegetables, fruits and cereal grains.[3] Troxerutin has been linked to a number of biological effects, including anti-erythrocytic, anti-fibrinolytic, anti-inflammatory, anti-neoplastic, anti-oxidant, and anti-thrombotic action.[4] Nicotine intake in the form electronic cigarettes or snuffing, have been studied using the zebrafish embryo models.[5] The major goal of this investigation was to study the therapeutic potential of troxerutin on nicotine induced zebrafish model by estimating the level enzymatic antioxidants, such as SOD and CAT, gill architecture and levels of inflammatory cytokine gene expression, IL-10 and IL-1β.

MATERIALS AND METHODS

Zebrafish Collection and maintenance

The Zebra fish were maintained in tap water that had been dechlorinated in order to accustomed to lab environment with continuous aeration. The pH was maintained between 7.3 and 7.5 and the water temperature was regulated between 26 and 27°C. The Zebra fish were fed with commercial food pellets twice a day.

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Determination of LC_{50} value for Nicotine and Pulmonary inflammation induction

LC_{50} was carried out by Alvarez *et al.*, 2018 [6] the following concentration ranging from 1.0, 2.0, 3.0, 4.0 and 5.0 mg/L respectively for 7 days. Each concentration involves ten fishes.

LC_{50} Value Troxerutin

Zebrafish were treated according to body weight for a week to determine the LC_{50} concentration of troxerutin. During the first stage, oral gavage was used to administer 2 to 10 mg/kg of troxerutin to the fishes. Up to 10 mg/kg, no lethal effect was seen. Further, high concentration of 10 to 50 mg/kg was tried over a week.

Experimental design

The Zebra fishes were divided into five groups: the control group, the nicotine-induced group, the troxerutin-treated group (5, 10 and 15 mg/kg) with nicotine. Ten fishes are in each group. A nicotine dosage of 2.5 mg/kg was used for a week to cause lung inflammation. Troxerutin was administered orally as 5, 10, and 15 mg/kg daily for 8 days following the induction period for the treatment groups, respectively. In order to conduct experiments, fish were euthanized on the eighth day and gill tissue was retrieved.

Estimation of Myeloperoxidase (MPO) activity

O-dianisidine dihydrochloride (o-dianisidine) solution 16.7 mg in 90 mL DH_2O and 10 mL of potassium phosphate buffer was prepared (50 µL of diluted H_2O_2 (4 µL of 30% H_2O_2 diluted in 96 µL of dH_2O) was added to the O-dianisidine mixture 20 mg of gill tissue was grinded using 1 mL buffer. 14 µL of tissue homogenate was added with O-dianisidine mixture with 400 µL H_2O_2. The absorbance was measured between the interval 30 sec - 120 sec at 450 nm using a spectrophotometer. The MPO activity was represented as units per milligrams of tissue.[7]

Determination of Nitrous Oxide (NO) Assay

The estimation of NO was carried out by Giustarini *et al.* method[8] using Griess reagent. The nitric oxide concentration was expressed in µM/mL.

Estimation of Lipid Peroxidation assay (LPO)

The homogenate of Zebra fish gill tissue was subjected to determine lipid peroxidation by Thiobarbituric Acid method (TBA) De Leon *et al.*[9] A secondary product of LPO, Malonaldehyde (MDA), was measured using TBA reagent. Pink colour development is read at absorbance of 532 nm and expressed in nanomoles/milligrams of protein.

Analysis of Superoxide Dismutase (SOD) activity

The activity of SOD is performed as per the method reported by Saggu, *et al.*[10] Zebrafish tissue homogenate is assessed by the ability of the superoxide ions in tissue to react with the chemical compound Nitro Blue Tetrazolium (NBT), reducing it to generate a colour (blue) that is read at 560 nm. SOD is expressed in units per milligrams of protein.

Estimation of Catalase activity

The activity of catalase was measured by Goth.[11] To the 0.2 mL of gill tissue homogenate, 1 mL of H_2O_2 (65 µM) and 1mL of 32.4 mM of ammonium molybdate were allowed to react and observed for yellow colour development and observed at 405 nm.

$$\text{Catalase activity (KU/L)} = x271$$

Gene expression studies

DNA was isolated from the gill tissue and homogenated with digestion buffer, incubation overnight at 55°C, and then dried. The air-dried pellet was dissolved in 50 µL of TE buffer and Thermo cycler gradient PCR was performed for primers designed using NCBI Primer-BLAST tool, as mentioned below.

Gene	Forward Primer	Reverse primer
IL-10	5′ ACCAGCAGTT ATTCGCACTCA 3′	5′ TCAATCCAAA ACACTTACGCATT 3′
IL 1 β	5′ TGTTTTCCTC CACAGAGCGT 3	5′GGAGGAAGTG AAAACAGGGGA 3′
β-Actin	5′ TCAAGGTGG GTGTCTTTCCTG 3′	5′ATTTGCGGTG GACGATGGAG 3′

Histopathology Studies

Histopathological analysis was performed to determine the presence of monocyte infiltration and alterations in the gill tissue. Gill tissues were preserved using the Bouin solution. The picric acid is then removed from the tissues by rinsing them four times in 70% ethanol for 15 min. Embedding, fixing, and dehydrating samples as described by Scheil *et al.*[12]

Statistical analysis

Statistical difference and significance between treated and induced group were determined by one-way Analysis of Variance (ANOVA) followed by Tukey's multiple comparison by GraphPad Prism version 8.0.2.

RESULTS

Determination of Nicotine and Troxerutin dosage for Pulmonary inflammation and treatment

The zebra fishes were incubated for a period of 7 days with different concentrations of nicotine ranging from 1.0 to 5.0 mg/L.

No mortality was observed up to 3.0mg/L of troxerutin for 7 days. From 0.4 to 1.0 mg/L dose-related toxicity was observed. There was a noted mortality at the rate of 5.0 mg/L (Figure 1). Based on these acute toxicity studies, we decided to select the concentration at 2.5 mg/L as a fixed LC_{50} dosage to induce gill inflammation throughout the studies. Similarly, the Zebra fish was fed with the maximum dose of Troxerutin (10 to 50 mg/kg). No adverse effects or mortality were observed at the chosen concentrations. Therefore, 5, 10 and 15 mg/Kg was considered oral dosage range concentration for the treatment.

Effect of Troxerutin on MPO and NO levels

Nicotine-induced group showed significant increase (*p< 0.05) in MPO and NO levels (Figure 2). MPO and NO levels were significantly reduced in the troxerutin treatment groups in dose-dependent manner (Figure 3).

Effect of Troxerutin on Lipid Peroxidation (LPO) Levels

Nicotine treated group was increased significantly with the levels of LPO in the gill tissues ($^{\neq}p$< 0.05)**.** Troxerutin treatment of the nicotine group significantly decreased LPO levels (*p< 0.05). LPO levels were gradually reduced dose-related manner (Figure 4).

Estimation of troxerutin on Superoxide Dismutase (SOD) and Catalase (CAT)

The levels of SOD were reduced in nicotine induced group indicating the toxicity caused by the nicotine on the gills (Figure 5). Treatment with troxerutin in all concentrations was effective in increasing scavenging activity of SOD and CAT levels (Figure 6) in a dose- related manner (*p< 0.05).

Gene expression studies

The possible effects of troxerutin administration on IL-10 (inflammatory gene) and IL-1β (pro-inflammatory cytokine) in adult zebrafish after exposure of nicotine were evaluated. The level of IL-1β is increased and IL-10 decreased on the action of nicotine in induced zebrafish. We observed a decrease in IL-1β and increase in IL-10 level on treatment with troxerutin at varying concentrations from 5 to 15 mg/kg between when compared to control gene (β-actin) Figure 7. However, on treating the fish with troxerutin, IL-10 expression was upregulated and IL-1β expression was downregulated in a dose-dependent manner.

Histopathology

Histopathological studies showed the presence of gill inflammation and damaged structure in nicotine induced group as well as in the troxerutin treated group (Figure 8B). However, no differences were observed in fishes treated with 15mg troxerutin compared with control (Figure 8A). Repaired gill architecture with healthy epithelial cell and absence of lamellar fusion was observed on treatment with troxerutin at 15 mg concentration (Figure 8E).

DISCUSSION

The toxic compound nicotine cause inflammation and releases free radical which generates the production of ROS and RNS resulting in deleterious effect on the antioxidant defence system[2] This causes destruction of the gill tissue by the action of nicotine which is indicated by the elevating the levels of MPO, NO, LPO. Our study reveals that levels of MPO were markedly reduced after treatment with Troxerutin, suggesting that it has a strong antioxidant activity. Hence, Troxerutin protects the structural damage to the gill and by improving its inflammation. This

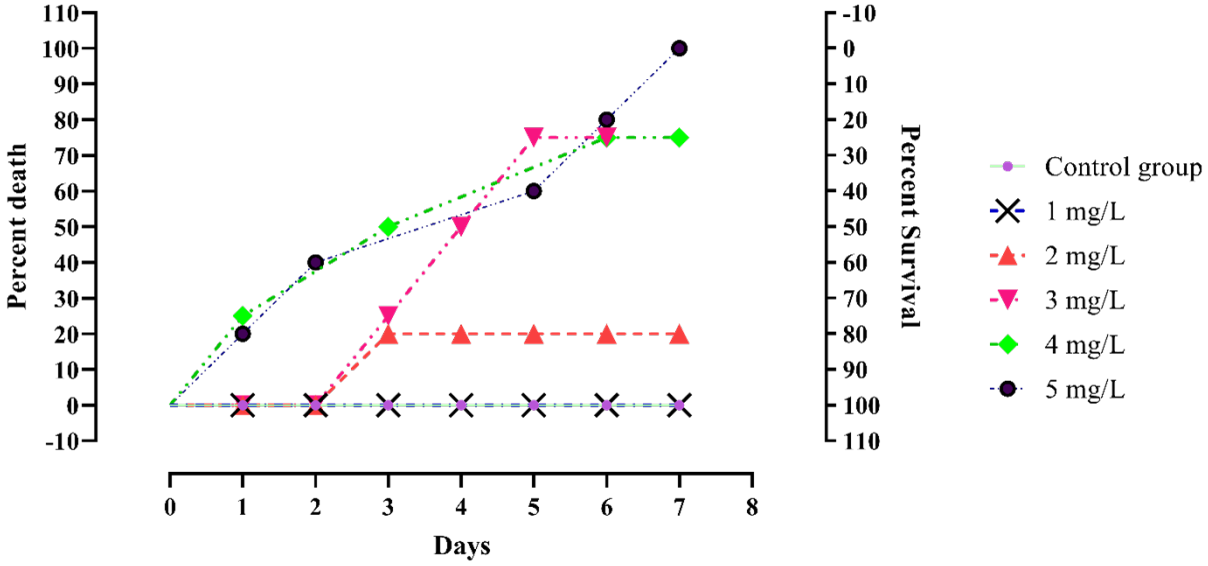

Figure 1: Toxic effect of nicotine was studied. The LC_{50} value of nicotine 2.5 mg/L was fixed based on the acute toxicity.

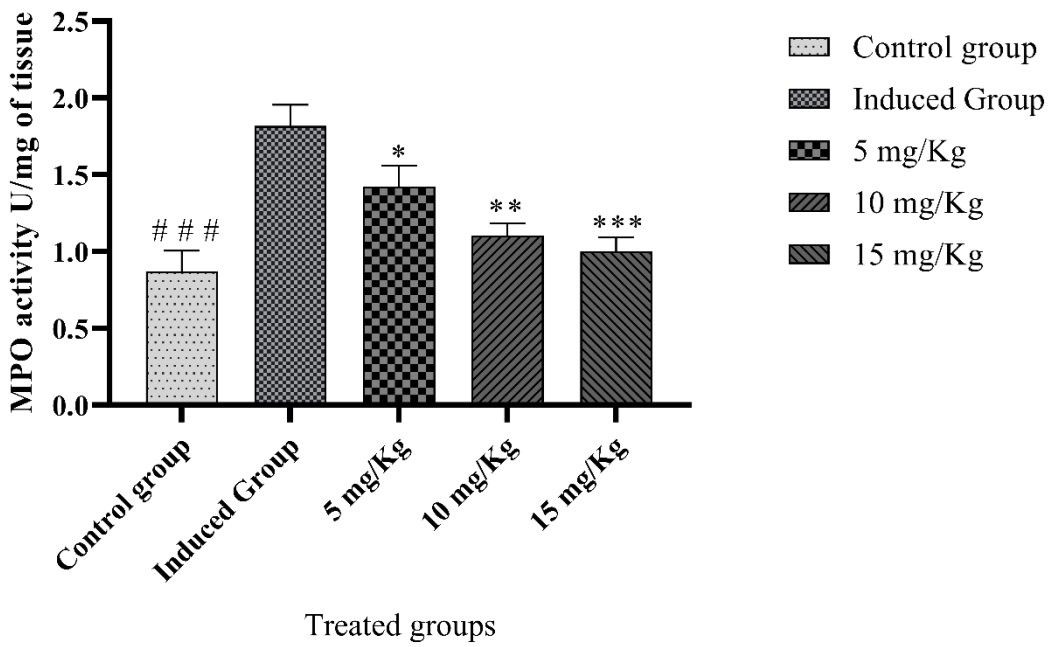

Figure 2: Effect of nicotine induced troxerutin on MPO activity in zebra fish.

MPO estimation between control, induced and treated groups were studied. MPO level of treated groups was significantly decreased when compared to induced group (*p<0.05).

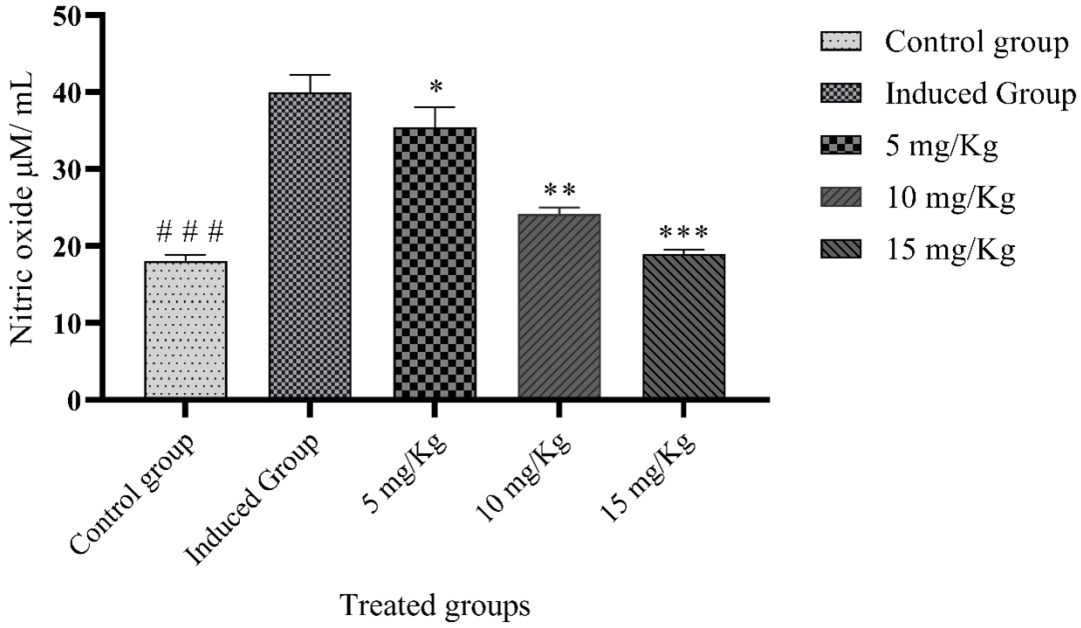

Figure 3: Effect of nicotine induced troxerutin on nitric oxide assay in zebra fish. NO estimation between control, induced and treated groups were studied. NO level of treated groups was significantly decreased when compared to induced group (*p< 0.05).

inflammation caused by nicotine was indicated by the rise in the gill MPO activity. From our present research, it seems likely that Troxerutin underlie its protective effects to the lesions of the gills by proving its antioxidant activity. During respiratory burst, MPO utilises H_2O_2 to make Hypochlorous Acid (HCl), which combines with protein molecules, unsaturated fatty acids, and other oxidizable group to form protein adducts and genetic alterations, triggering the signalling system.[13] This effect is counteracted by the action of Troxerutin by increasing the MPO level.

On treatment with troxerutin, NO levels reduced significantly in dose-dependent manner**.** The rise in the level of NO which was

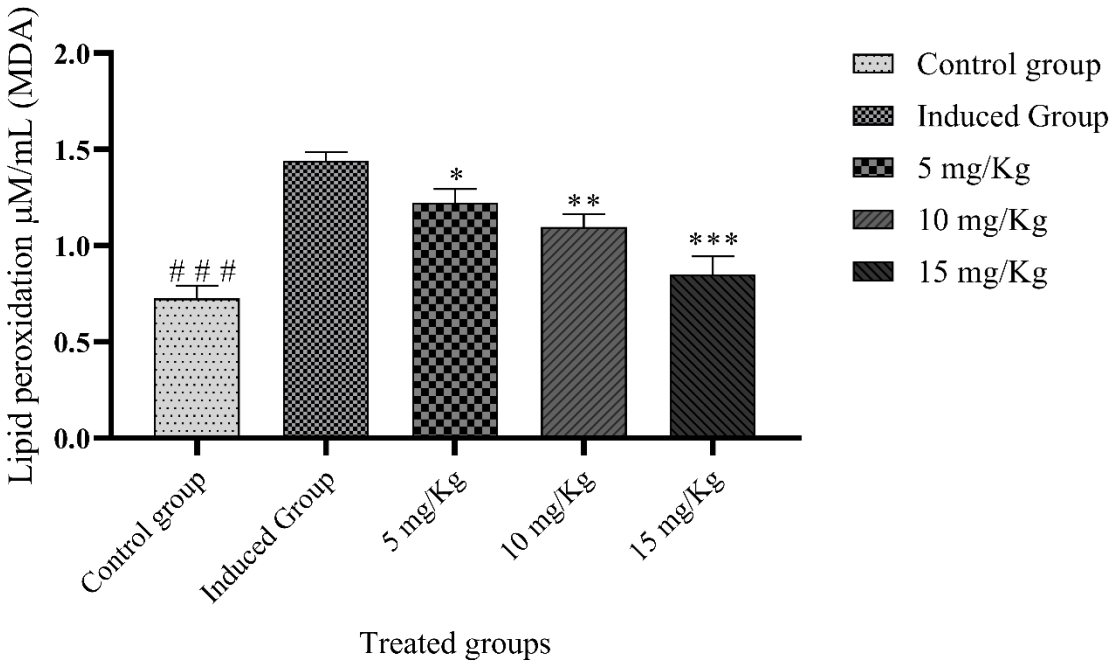

Figure 4: Effect of nicotine induced troxerutin on lipid peroxidation assay in zebra fish. LPO estimation between control, induced and treated groups were studied. LPO level of treated groups was significantly decreased when compared to induced group (∗p<0.05).

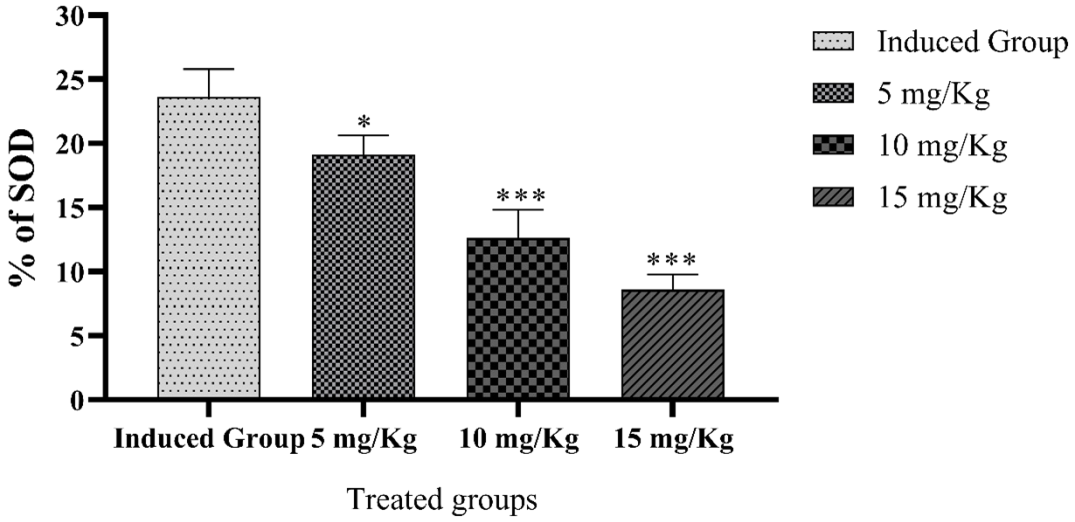

Figure 5: Effect of nicotine induced troxerutin on SOD activity in zebra fish. SOD estimation between induced and treated groups were studied. SOD level of treated groups was significantly decreased when compared to induced group (∗p<0.05).

generated by the action of NO synthase (iNOS) in the process of inflammation, and preventing the iNOS expression might be an indicator in regulating anti-inflammatory mechanism.[14] According to our results, troxerutin at varying concentration inhibits the iNOS production and also reduce the generation of NO levels. The inhibition of iNOS expression is mediated by the troxerutin's anti-inflammatory properties.

Troxerutin has an antioxidant effect against nicotine exposure in gills due to its scavenger action against lipid radicals. LPO initiation is carried by superoxide, hydroxyl radicals and hydrogen peroxide which impair the cells by inactivating the enzymes that associate with membranes and receptors, and also by depolymerization of polysaccharide as well as proteins.[15] LPO is characterised by a free radical chain action mediated by the abstraction of atom of hydrogen from polyunsaturated fatty acid

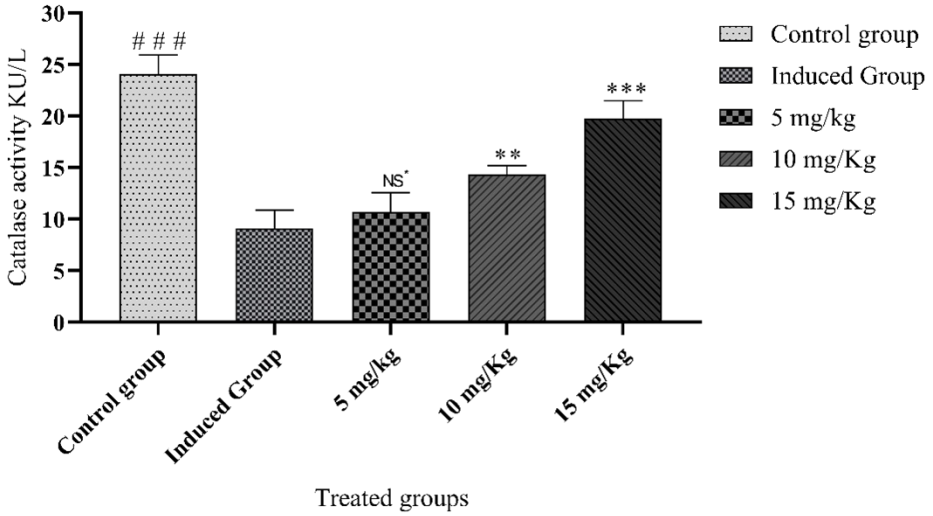

Figure 6: Effect of nicotine induced troxerutin on catalase activity in zebra fish. Catalase activity between control, induced and treated groups were studied. Catalase level of treated groups was significantly increased when compared to induced group (*p<0.05).

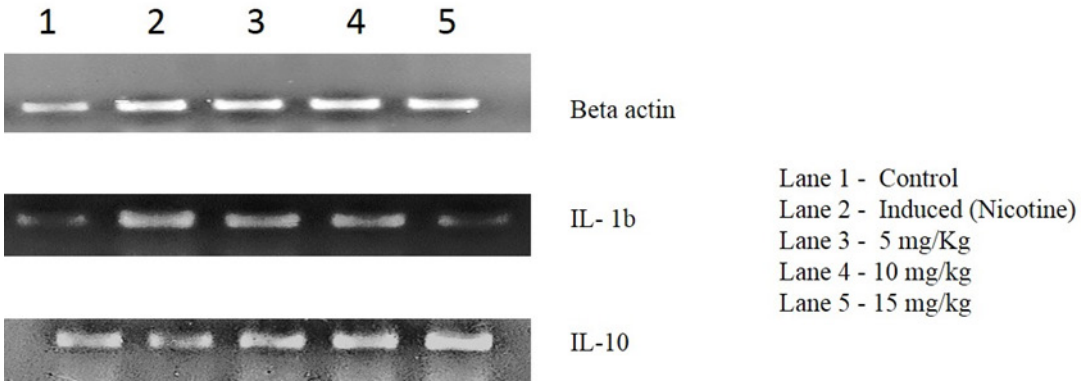

Figure 7: Gene Expression studies.

Pro-inflammatory and anti-inflammatory cytokines. IL-1β and IL-10 were investigated. Down-regulated gene expression levels were observed for IL-1β and an upregulated expression was observed for IL- 10 gene, in a dose-dependent manner.

side chain. By scavenging free radicals and restoring normal cell activity, certain dose of troxerutin considerably reduced the damage caused by nicotine.[16]

Troxerutin was shown to have a high superoxide radical scavenging activity when the activity of SOD and CAT were assessed. The decrease in SOD activity in nicotine-treated fish's gills may be caused by the increased use of antioxidants to block lipid peroxidation. SOD is a crucial enzyme for defending the cells against the nicotine induced ROS that are produced for energy production during aerobic respiration. Additionally, it changes more toxic superoxide anion radicals into harmless hydrogen peroxide.[17] Troxerutin protects the cells against oxidative stress. The activity of SOD is decreased in the nicotine induced group, treatment of troxerutin increased SOD and catalase activities. Catalase, an enzymatic antioxidant enhanced in the hepatocytes is a determinant of cellular resistance to the toxicity of hydrogen peroxide was lowered which encompasses a diminished capacity of cells to detoxify hydrogen peroxide.[18] Troxerutin treatment groups significantly increased CAT levels in a dose-related manner. Low levels of CAT were observed in the nicotine induced group which is due to the increased oxidative stress and may lead to gill damage this action was reverted by the action of Troxerutin. Similar results were reported[19] by the action of resveratrol on antioxidant enzymes. The pathophysiologic imbalance within ROS and the antioxidant defence system, favours to progression and pathogenesis of several diseases. In our investigation, the nicotine-treated group showed evidence of inflammation of gill tissue and damaged gill filaments, while the effects were less pronounced on treatment with troxerutin. As inflammation rises, neutrophils and lymphocytes will aggregate more often at the gill lamellae, damaging the filaments of the gills, according to a prior investigation.[20] The expression of IL-10 is reduced and IL-1β expression is increased by the action of nicotine treated groups.

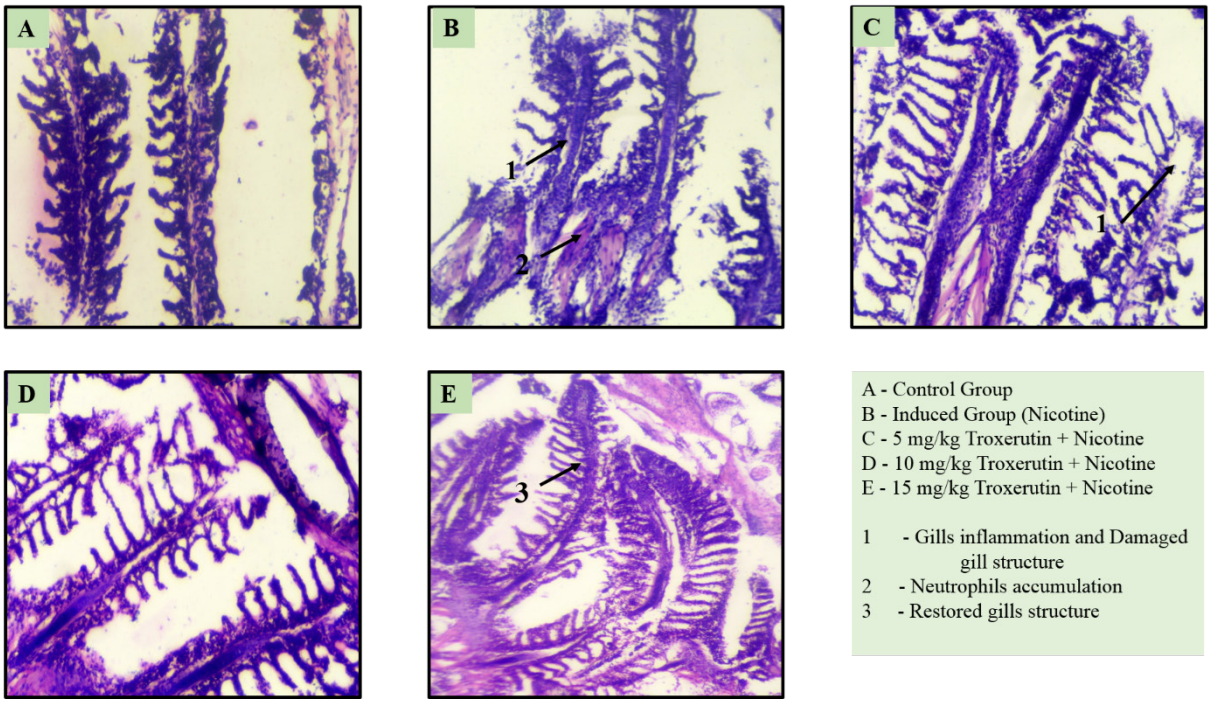

Figure 8: Histopathological studies.

Histopathological studies showed the presence of gill inflammation and damaged structure in nicotine induced group. Repaired gill architecture with healthy epithelial cell and absence of lamellar fusion was observed on treatment with troxerutin at 15 mg concentration.

However, on treating the zebrafish with troxerutin, the levels of IL-10 expression were upregulated and IL-1β expression was downregulated in a dose-dependent manner. The present finding reveals that troxerutin treated group in combination with nicotine decreased the levels of IL-10 and increased the IL-1β expression. Tong Xu Guan[21] reported that, troxerutin not only decreased the levels of TNF-α, NF-κB, IL-1β and IL-6 gene expression and but also increased IL-10 level. According to our study, troxerutin significantly increased LPO, MPO in the gill tissue homogenate and decreased enzymatic antioxidant levels (SOD, CAT) and also increased the NO generation. Troxerutin had a capacity in inhibiting the inflammatory response, by suppressing the both local and systemic inflammatory response, which has a potency in releasing higher levels of NO.[22]

CONCLUSION

Nicotine at the dose of 0.3 mg/L for 7 days caused inflammation to the gill tissue of the Zebra fish. According to our study, Zebra fish induced with nicotine increased the levels of LPO, MPO, NO and antioxidant enzymes (SOD, CAT) were decreased. On treatment with troxerutin the above antioxidant levels were increased and LPO, MPO, NO levels were decreased. The expression of IL-1β is increased in the group induced with nicotine and treatment with troxerutin resulted in a decrease level of IL-1β. Based on the observation, it was confirmed that troxerutin restored the gill damage which was affected by nicotine action. The results also demonstrate that troxerutin has therapeutic potential for the treatment of nicotine-induced gill disease -based on its antioxidant and anti-inflammatory properties.

CONFLICT OF INTEREST

There is no conflict of interest.

ABBREVIATIONS

COPD: Chronic obstructive pulmonary disease; **CSE:** Cigarette smoke extracts; **IL-1β:** Interleukin 1 beta; **IL-10:** Interleukin 10; **ROS:** Reactive oxygen species; **MPO:** Myeloperoxidase; **NO:** Nitric oxide; **LPO:** Lipid peroxidation; **DMSO:** Dimethyl sulfoxide; **KOH:** Potassium hydroxide; **Cig:** Cigarette; **PCR:** Polymerase chain reaction.

SUMMARY

This work reported the activity of troxerutin in nicotine induced Zebra fish model. In our study, the activity of troxerutin in reducing MPO, LPO and NO levels and increasing the activity of enzymatic antioxidants like CAT, SOD levels were demonstrated. Troxerutin also decreased IL-1β gene expression and restored the gill architecture in nicotine exposed group. More research is needed to understand the underlying mechanism of troxerutin in suppressing nicotine.

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