Opuntia monocantha attenuates $MnCl_2$ induced hepatotoxicity, oxidative stress and hepatic inflammation in wistar rats

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Abstract: The inevitable significance of medicinal plants has been accepted worldwide for curing and treatment purposes. Likewise, *Opuntia monocantha* (OM) was implicated traditionally for numerous pathologies due to diversified pharmacological activities. The current study was focused on elucidating the hepato-protective and anti-inflammatory potential of *OM* in $MnCl_2$ induced hepatotoxicity and liver inflammation. The $MnCl_2$ was administered at a dose of 6.0 mg/kg for 30 days (p.o) to induce hepatotoxicity and liver inflammation. An *in vivo* model was employed for investigating hepato-protective effect of orally given ethanolic extract of OM. Outcomes revealed that administration of ethanolic extract of OM at different doses (250, 500, and 750 mg/kg) remarkably reduced oxidative stress and hepatic inflammation. Moreover, histopathological observations were compared with diseased rats which supported protective effect of plant extract. Therefore, protective effect of ethanolic extract of OM was exhibited in wrister rats by attenuating liver inflammation an oxidative stress.

Keywords: Inflammation, Celecoxib, oxidative stress, Opuntia monocantha, $MnCl_2$

Introduction

Multiple etiologies such as viral infections, alcohol, drugs and non-alcoholic steatohepatitis (NASH) are involved in generating inflammation and liver injury caused fibrosis which refers to a normal healing mechanism (Tsuchida and Friedman, 2017). Chronic hepatic injury and inflammation are commonly known as resident hepatic astrocytes being stimulated into fibroblast-like cells in course of hepatic fibrinogenesis (Seki and Schwabe, 2015). Mainly, Liver immune cells trigger macrophage activation (Li et al., 2017) which in turn directly activate myo-fibroblasts by liberating numbers of chemokines and cytokines (Pellicoro et al., 2014). Furthermore, these cells generate pro-inflammatory cytokines which includes tumor necrosis factor- (TNF-), interleukin (IL-1) and (IL-6) thereby, stimulate nonstop inflammation.

Almost all tissues comprise the trace element known as manganese (Mn) which is considered vital for the regular *in vivo* metabolism of carbohydrates, lipids, proteins and amino acids (Aschner and Aschner, 2005). Humans seldom report Mn deficit, while the toxicity related to its excess exposure is more frequent (Erikson et al., 2007). Mn poisoning is prevalent in the individuals who work in mining and welding industries where workers remain at constant risk of its exposure through aerosols containing this element (Santamaria et al., 2007). Moreover, Mn toxicity is evident in people consuming contaminated well water or recieving parenteral nutrition in case of liver disorders (Williams et al., 2012). Researchers have efficiently indicated the relationship between Mn exposure and its accumulation in the brain and liver (O'Neal and Zheng, 2015). Besides, amounts of Mn have been seen elevated in a dose dependent manner in different organs and tissues over the period of time. Therefore, building up of Mn in liver cause hepatic damage which in turn decline its elimination rate (Kwakye et al., 2015) whereas, in brain the excessive amount of Mn may results in accuracy comparable to Parkinson`s disease (Zalups and Koropatnick, 2010).

Multiple investigations have revealed that hepatic inflammation is predominantly attributed by mitochondrial dysfunction (Caldwell et al., 1999). However, hepatic inflammation augments oxidative stress and alleviate mitochondrial activity in the liver. Oxidative stress is generated by the excessive production of reactive oxygen species (ROS) or by the depletion of anti-oxidants at cellular level (Deng et al., 2009). Thus, ATP generation and free beta-oxidation of fatty acids are major mitochondrial processes which regulate hepatocyte energy homeostasis (Grattagliano et al., 2012). Owing to injury, several immune responses are crucial in hepatic inflammation. Literature is evident that the interaction between medicines and the immune system has a well-defined mechanism with ground assumptions in both adaptive as well as innate immune responses (Hoque et al., 2014).

Modified pro- and antioxidant pathways by mitochondrial dysfunction results in the emergence of high un-metabolized fatty acids in the cytosol which resultantly produce ROS (Begriche et al., 2013). High generation of ROS along with the byproducts of catecholamine oxidation declares Mn more toxic (Parenti et al., 1988). Different antioxidant mechanisms are primarily tissue and intracellular ROS compartments and involve antioxidants such as glutathione (GSH) and antioxidants (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Wilhelm, 1996). These defense mechanisms prevent cells from lipid peroxidation, protein oxidation and potentially harmful DNA oxidation (LPO).

Natural compounds having antioxidant potential are a new potential source to reduce ROS (Ahmad et al., 2023, Hadi et al., 2022). OM belongs to Cactaceae family and commonly known as "Dropping prickly pear" or "Chnutarthar" holding a characteristic succulent stem (Bari et al., 2012). It is widely distributed in Uruguay, Argentina and Brazil while the plants are also cultivated in different subtropical and tropical regions such as China, Cuba, South Africa, Australia and India (Valente et al., 2010). It is previously reported with numerous biological activities due to several phytochemicals. Multiple aliments like bronchial asthma, diabetes, burns, and acid reflux have traditionally been treated with OM effectively (Kim et al., 2016, Yang et al., 2008). Other

applications includes as a laxative, carminative, antipyretic, remedy for urinary tract tumors, piles, mild inflammations, anemia, ulcers and spleen enlargement (Ballabeni et al. 2010).

Materials and methods

Materials

All chemicals used in the study and Manganese chloride (MnCl2, MW 125.84) were of good quality and procured from Sigma Chemical (St. Louis, MO) unless otherwise stated. Ultrapure water was obtained from Millipore Academic Systems (Bedford, MA, USA). A solution was prepared by combining 6.0 mg/mL MnCl2 solution with sterile saline. Solution was periodically prepared and kept at room temperature.

Plant collection

OM plant wall was collected from the botanical garden of the Govt. College University, Lahore where it was accurately identified and allotted an ID number (Gc.herb.bot: 3655).

Preparation of extract

Prior to surface sterilization, plant was collected, thoroughly washed with distilled water to remove dust and contaminants. The OM was pulverized with an electric mixer, dried in the shade, and stored in an airtight container. The OM was ground to a fine powder and weighed on a digital scale before being immersed in a 70/30 aqueous ethanol solution. After thoroughly mixing the powder using an electric mixer, it was placed in a closed container and refrigerated at 4 °C for 2 days (Odey et al., 2012, Chouhan and Meena, 2015). The resulting filtrate was centrifuged at 2,000 rpm for 15–20 minutes to separate the concentrated phase, which accounted for approximately 10% of the initial volume or placed in a rotary evaporator at 37–40 °C to remove excess solvent to obtain concentrated extract. The crude mixture was then filtered through cheesecloth and Whattman filter paper. Concentrated extracts were kept at 4 °C while diluting stock solutions were used to prepare working aliquots for specific molecular assays.

Experimental protocols

Study design flow sheet

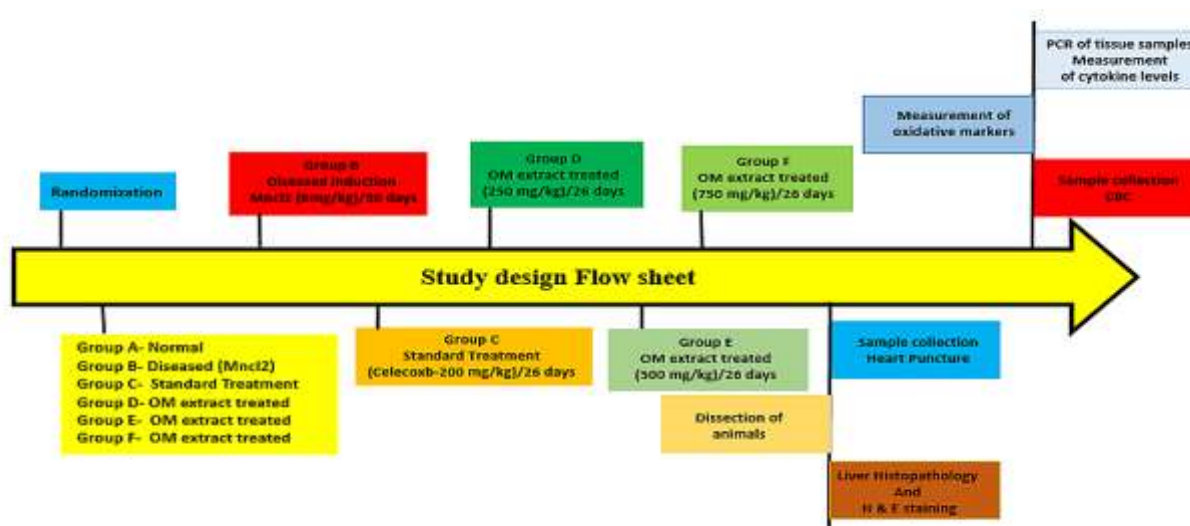

Fig. 1: Representation of study design

Animals

The present study used Swiss albino Wistar rats (180-225 g) which were taken from the central animal house and housed in standard conditions which included single polypropylene cage, specific temperature (25 ± 2 °C), relative humidity (60-80%) and kept in dark/light cycle for 12 h. The animals were fed standard pellet diets and were given unlimited access to water. Experimentation and protocols were approved by "The University of Lahore" which were accompanied by International Ethical Care procedures (IREC No: 33215). Animals were segregated in four groups (six rats each).

1. Group A normal.
2. Group B diseased $MnCl_2$ treated.
3. Group C $MnCl_2$ treated with (celecoxib 200mg/kg).
4. Group D $MnCl_2$ treated with (250mg/kg OM extract).
5. Group E $MnCl_2$ treated with (500mg/kg OM extract).
6. Group F $MnCl_2$ treated with (750 mg/kg OM extract).

$MnCl_2$-Induced Hepatotoxicity and Drug Treatments

Groups of animals (B, C, D, E, and F) were orally administered MnCl2 (6.0 mg/kg/body weight) once daily for 30 days. Animals were dosed with 250, 500, and 750 mg/kg of OM plant extract daily for 26 days. Selection of dose was entirely based on previous research (Wang et al., 2013, Abid et al., 2021). Animals received the same volume of sterile saline as a typical population. Twenty-four hours after the last dose of A was administered, this study was approved by the Government College University, Faisalabad in compliance with animal welfare standards. Animals were executed by decapitation after which their livers were rapidly removed and weighed.

Hematology and clinical chemistry assay

Serum aspartate aminotransferase (AST) and alkaline phosphatase (PLT) levels were measured using chemical analyzers in order to detect white blood cells (WBC), red blood cells (RBC), hemoglobin (Hb) and platelets (PLT) levels.

Samples preparation

Blood samples were taken via heart puncture at the end of the study. Blood was tested for oxidative stress markers like SOD, GPx, and Catalase, as well as pro-inflammatory markers (IL-1, IL-6, TNF-α) and the anti-inflammatory marker (IL-10).

Determination of catalase activity

We followed the Aebi's methodology (Ruiz-Larrea et al., 1994), serum catalase was determined using a spectrophotometric UV meter. The measurement was made at 240 nm. The serum from rats was homogenized in a 150 milli molar phosphate buffer with a pH of 7 and centrifuged at 6000 rpm at 4 °C. A UV spectrophotometer was used to measure the absorbance. Results were compared to a standard curve generated by the known enzyme catalase.

Determination of superoxide dismutase (SOD) activity

The quantity measure is dependent on the enzyme's intensity to obstruct the scavenging effect on superoxide anion radicals was investigated using a modified NBT reduction method. In

comparison to a blank, resulting mixture was checked for absorbance at 560 nm. The procedure was carried out using the previously described method (Tang et al., 2007).

Determination of glutathione peroxidase (GPx) activity

The amount calculated is determined by the strength of the enzyme's metabolic network that produces anti-oxidant effects pieces. Over-regulation of its activities has been proposed as a promising strategy for controlling inflammation (Li et al., 2018).

Evaluation of mRNA expression levels of TNF-α, IL-1β, IL-6 and IL-10

On the 56th day of the investigation, blood was collected, and RNA was extracted using the TRIzol system, following standard manufacturer's bearings (Thermo Fisher Scientific, America).

TNF-α: 3'-GTCTACTCCTCAGAGCCC-5'Forward
5-TGAGATCCATGCCATTGGCC-3′ Reverse
IL-1β: 5'-GTCCTCTGCCAAGTCAGGTC-3' Forward
5'-CAGGGAGGGAAACACAGGTT-3' Reverse
IL-6: 5'-CCCACCAGGAACGAAAGTCA-3' Forward
5' ACTGGCTGGAAGTCTCTTGC-3' Reverse
IL-10: 5'-TTGAACCACCCGCATCTAC-3' Forward
5'-CCAAGGAGTTGCTCCCGTTA-3' Reverse

The item was enhanced with the help of thermal cycler for 45 cycles of denaturation (95 °C for 10 seconds), annealing (60 °C for 20 seconds) and extension (72 °C for 30 seconds). Results were evaluated using (RT-PCR) via the Bio-Rad framework. The cDNA was created using RNA. The appropriate primer was used to create duplicates of the pro-inflammatory arbiters via RT-PCR (Jin et al., 2008).

GAPDH 5'-AGTGCCAGCCTCGTCTCATA-3'
Forward 5'-ACCAGCTTCCCATTCTCAGC-3' Reverse was used as a house-keeping gene.

Assessment of Hematological and biochemical markers

On day 56, hematological samples were obtained via cardiac puncture. Afterwards, hemoglobin levels as well as inflammatory cells including WBC count, neutrophils, eosinophils, lymphocytes and platelets were determined with hematology analyzer. While creatinine and urea levels were also assessed using a chemistry analyzer.

Histopathology

Liver sample preparations were formalin fixed. After gradually immersing in ethanol with incremental concentrations, it was treated with xylene and embedded in paraffin wax. Microtome slices cut into 5.0 mm pieces were stained using H&E stain. The histological sections were examined and viewed under a microscope at different resolutions.

Statistical analysis

The study's findings were statistically analyzed using analysis of variances (ANOVA) one way using Tukey test with level of significance, 0.05 using GraphPad Prism (version 7.0).

Results

OM regulates liver enzymes

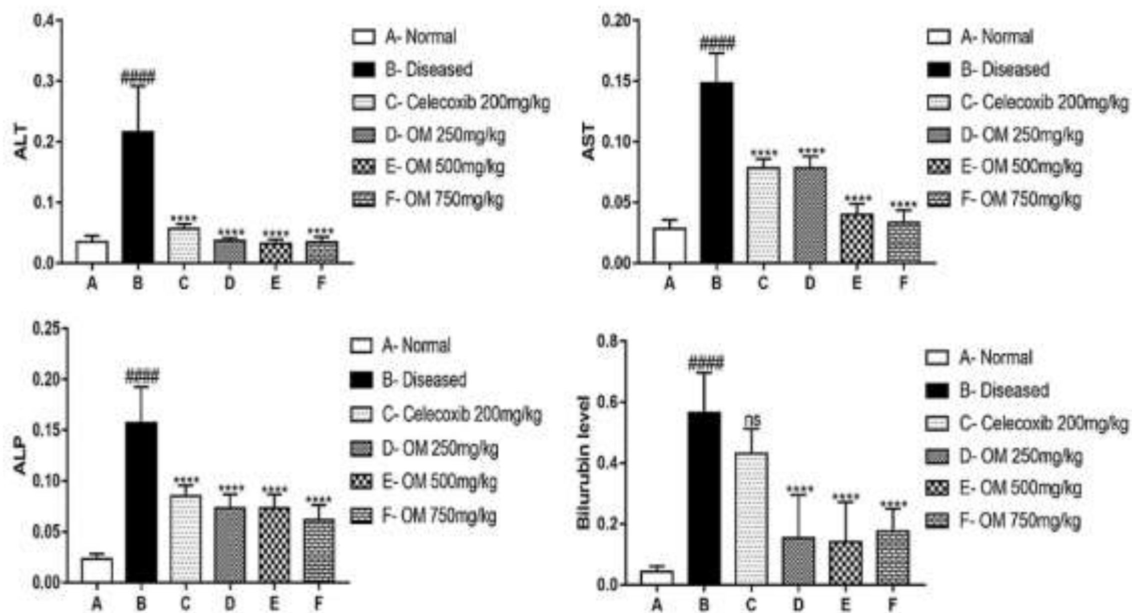

Fig. 2: ALP, F (5, 30) = 33.75, (P0.0001), ALT, F (5, 30) = 32.07, (P0.0001), AST, F (5, 30) = 72.79, (P0.0001), and bilirubin levels in group D compared group B after multiple administrations of OM (250, 500, and 750 mg/kg) (disease group). Tukey's test for important variations: reveals a significant difference from group B that is remarkable and compares to the conventional treatment.

ALP levels in group B were significantly increased, F (3, 20) = 60.62, (p0.0001), whereas ALP levels in group D dramatically decreased with OM treatment. Chronic MnCl2 injection significantly elevated ALP, ALT, AST, and bilirubin levels (p0.0001). F (5, 20) = 347.1, ($p<0.0001$). It was F (5 20) = 33.98 on the ALT scale. The AST data showed F (3, 20) = 49.25 having reduced AST levels in treated group rats compared to group B rats, and the comparison of the illness and OM treated groups revealed (p 0.0001), respectively. The animals treated with OM were found to have elevated levels of bilirubin. F (5 20) = 60.11, ($p<0.0001$). Prolonged treatment with OM extract (250, 500, and 750 mg/kg/day) improved the levels of bilirubin and liver enzymes in the treated group rats, whereas chronic treatment with $MnCl_2$ increased both inflammation and liver enzyme levels in diseased group rats.

OM improves the SOD, Catalase, and GPx levels

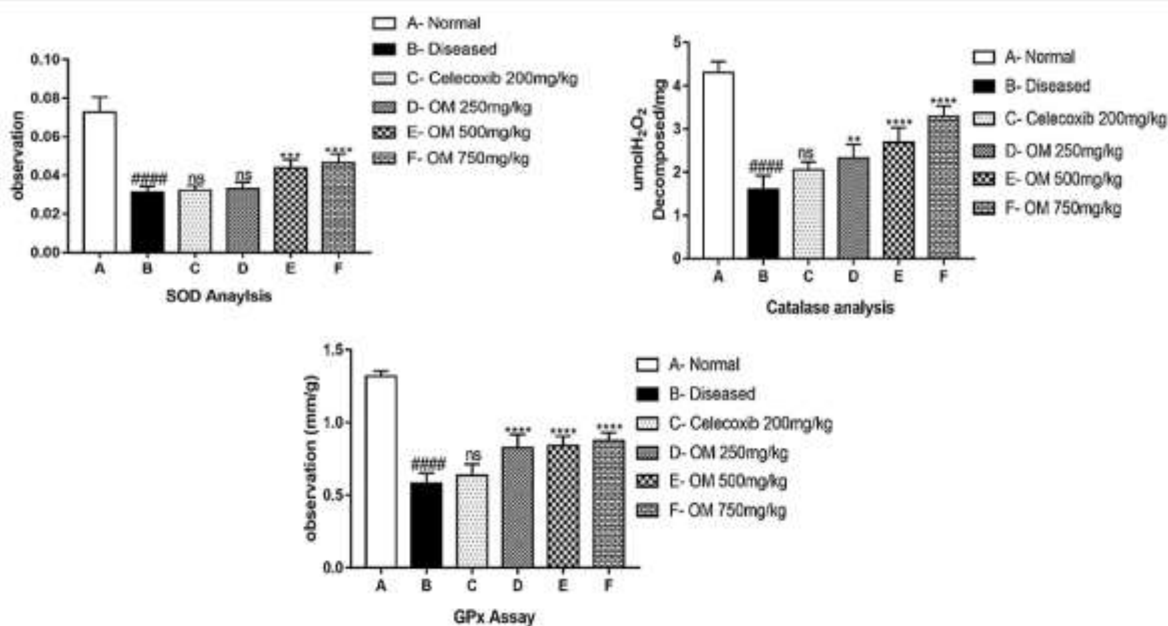

Fig. 3: The effects of repeated administration of OM (250, 500, and 750 mg/kg) on lipid peroxidation demonstrate improved SOD, Catalase, and GPx levels in the D, E, and F groups when compared to group B (F (5, 30) = 68.81, P0.0001, and 67.39, P0.0001, respectively) (disease group). The values were means and standard deviations (n = 6). Tukey's test revealed significant differences: A notable (***) shift from group B (####) denoted significance in comparison to the usual course of treatment.

The catalase level in group B reduced significantly with chronic $MnCl_2$ treatment (p0.0001), whereas the SOD level in groups D, E, and F were also declined significantly with chronic OM treatment. (p0.0001). F (3, 20) = 347.1, ($p < 0.0001$). Compared with catalase levels in group B (p 0.0001), these results were accompanied by a significant reduction in SOD levels in groups B (p 0.0001) and C (p = 0.9826), with repeated measurement again F (5, 25) = 94.71, respectively (p0.0001). The results were not significant compared with group B with repeated measures F (3.20) = 102.1 However, there was observed a significant decrease in group B compared with group F treated with OM (p 0.0016). (p0.0001). Chronic injection of $MnCl_2$ into diseased mice resulted in inflammation as well as decreased GPx levels.

OM suppress mRNA expression levels of TNF-α, IL-1β, IL-6 and IL-10 expression

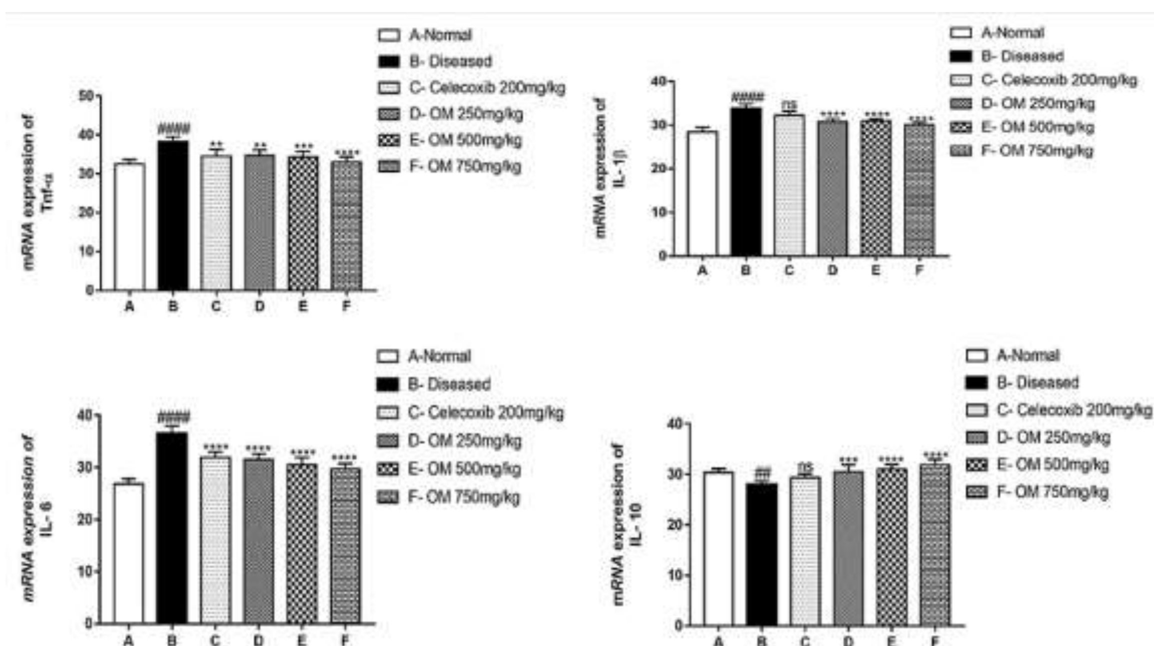

Fig. 4: In groups D, E, and F compared to the control group, the effects of repeated administration of OM (250, 500, and 750 mg/kg) suppressed pro-inflammatory cytokine levels (TNF-) F (5, 30) = 11.93, (P0.0001), (IL-1) F (5, 30) = 27.2, (P0.0001), (IL-6) F (5, 30) = 41.98, (P0.0001), and (IL-10) F (5, 30) = 11.47 The values (n = 6) represent means and standard deviations. Tukey's test for significant differences revealed a substantial difference when compared to group B and to the usual course of treatment.

TNF-α levels were substantially higher in group B (p 0.0001) compared to groups receiving standard treatment (p = 0.7332) and those receiving OM treatment (p = 0.0043). IL-1 mRNA expression was noticeably lower in the OM-treated group. F (5, 20) = 13.49, (P<0.0001). Moreover, IL-6 mRNA expression was likewise decreased in treatment group D when compared to group B. (1.8239 0.2466). (3.4883 0.4961). Similarly, it was found that in group B, IL-4 levels were lower (p0.0001) than those of group A. whereas, IL-4 levels in treatment group D (P = 0.0014) were considerably increased than in control group C (P = 0.05).

OM Normalized Biochemical and Hematological Markers

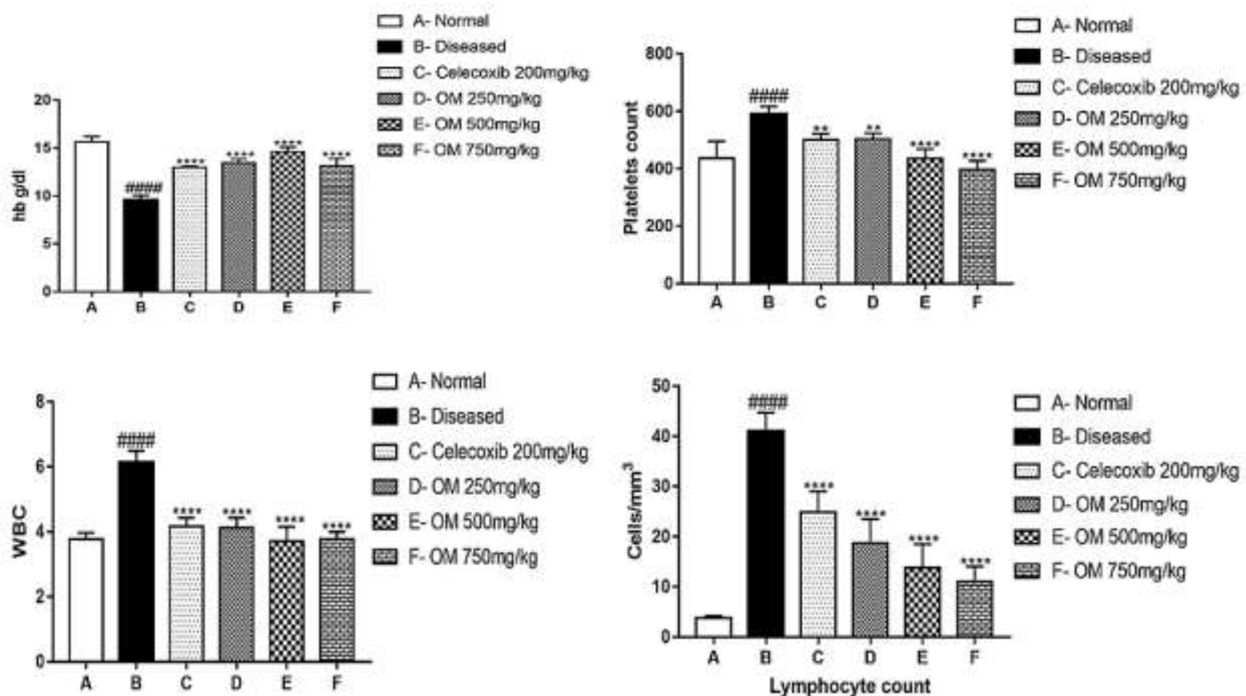

Fig. 5: When compared to the diseased group B and the control group C, the medication (250, 500, and 750 mg/kg) in group D significantly declined WBC, Platelet, lymphocyte count, neutrophils and biochemical parameters including AST and ALT.

Repeated administration of OM to rats in groups D, E, and F resulted in a lymphocyte count F (5 20) = 195.2 (P 0.0001), whereas repeated administration of OM to rats in group B resulted in an increase in WBC counts. Decreased (p0. 001). F (5, 20) = 177 (p<0.0001). Moreover, OM increases his Hb levels in group D rats compared to group B animals and standard-treated group C animals (p0.0001). F (5, 20) = 273 (p<0.0001) A statistically significant difference was found using Tukey's test. Comparing the platelet counts of group B and group A rats, group B rats had significantly higher platelet counts (p<0.0001).

Quantification by HPLC

HPLC was used to determine the quantity of plant extracts. Quercetin (2.14g/g), benzoic acid (1.24g/g), gallic acid (0.87g/g), vanillic acid (4.87g/g), sinapic acid (12.1g/g), M-coumaric acid

(2.23g/g), syringic acid (0.11g/g), and P-coumaric acid (0.38g/g) were all quantified in the plant's ethanolic extract.

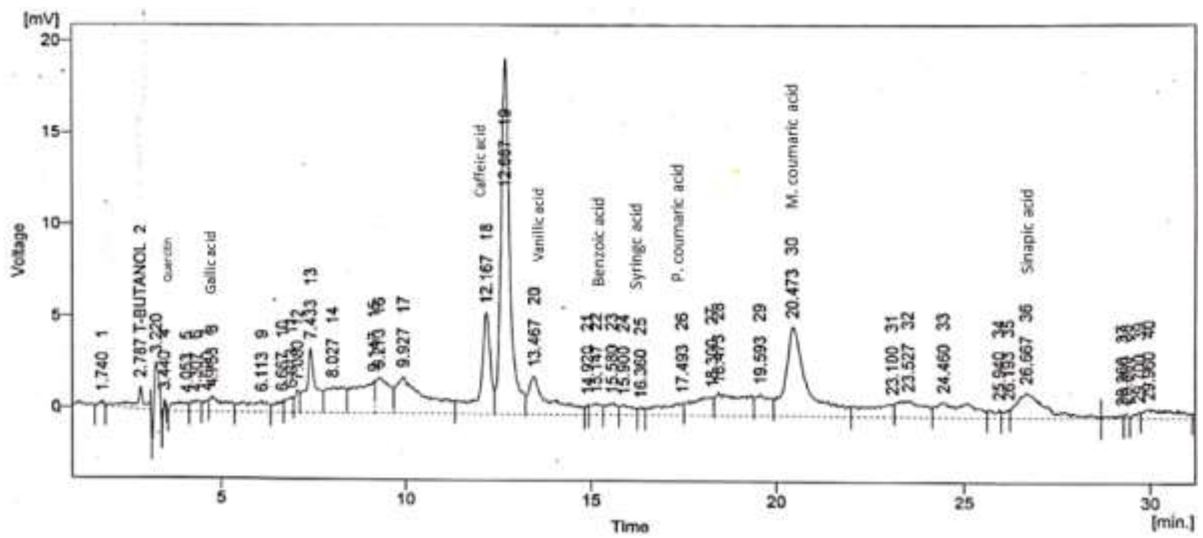

Fig. 6: HPLC chromatogram of Phenolic, and Flavonoids compound detected in ethanolic extract of OM.

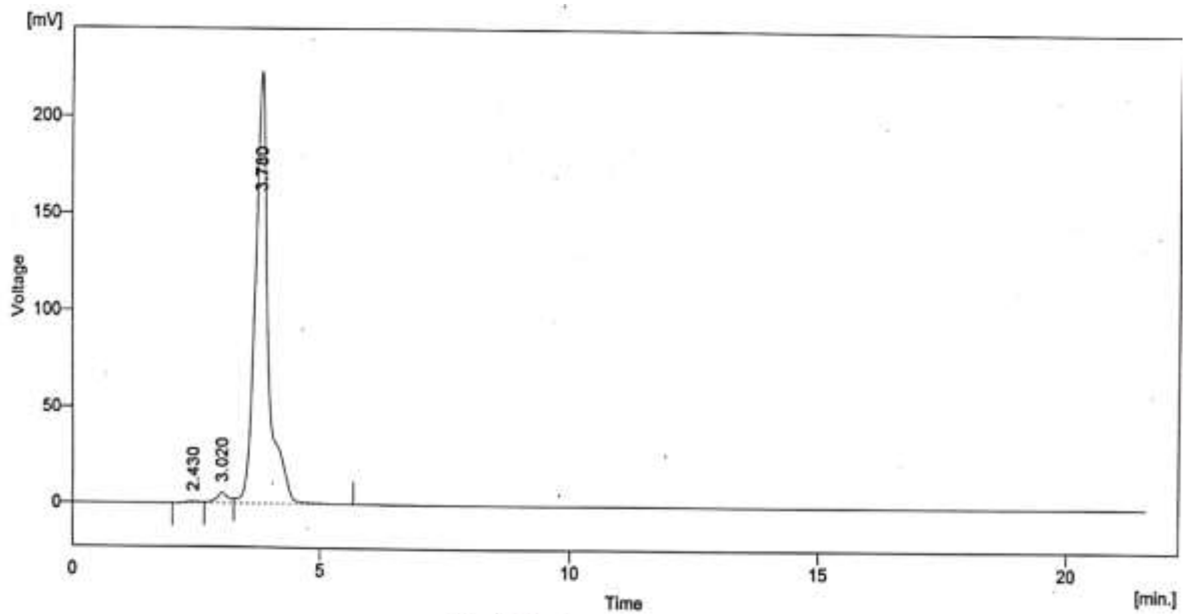

Fig. 7: HPLC chromatogram of Vitamin C compound detected in ethanolic extract of OM.

OM causing protective effects on liver.

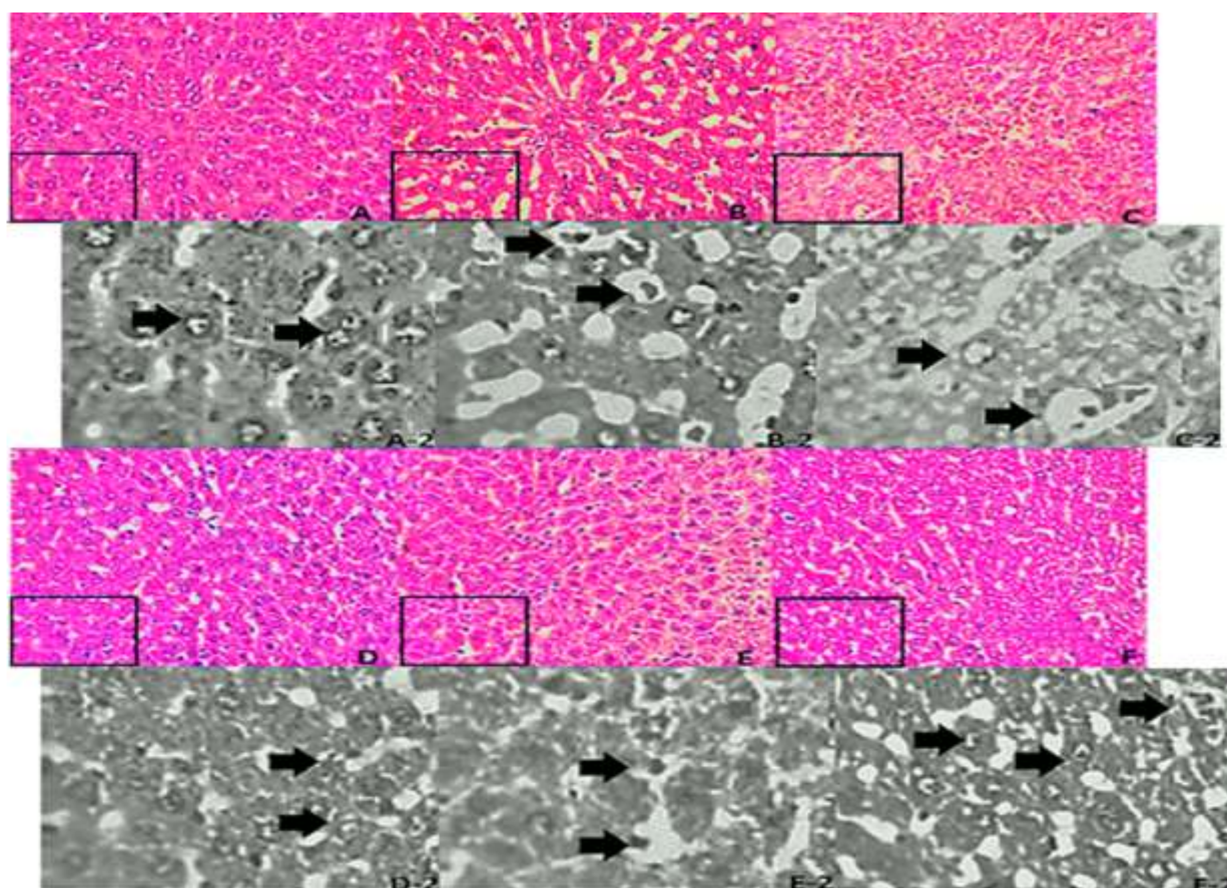

Fig. 8: A collection of liver tissue, magnified and stained in H&E, exhibited normal histological liver appearance control rats (A). (B) In contrast to ill rats, which displayed hepatocyte degeneration together with intra-lobular mononuclear inflammatory infiltrations and Mallory bodies, celecoxib-treated rodents exhibited diffuse small-scale vesicular hepatocyte degeneration. (250 mg/kg, 500 mg/kg, and 750 mg/kg D, E, and F) In groups of rats that underwent OM H&E staining at a magnification of 40x, a focused network with a mildly provocative mononuclear accumulation was visible.

Discussion

We investigated the mechanism by which OM alleviated $MnCl_2$ induced liver inflammation in rats. Since $Mncl_2$ is an iron antagonist which can replace Mg^{2+} in some enzymes. Thus, owing to its specific ionic radius, it may interfere with calcium (Ca^{2+}) metabolism and cause hepatic inflammation. However, Mn mainly accumulates in mitochondria and affects mitochondrial functioning both in vivo and in vitro. As a matter of fact, the mechanism of $MnCl_2$ accumulation is unknown (Zhang et al., 2003, Abid et al., 2020)). It causes varying degrees of inflammation and liver toxicity due to nodular cirrhosis, hepatocyte proliferation, and parenchymal necrosis (Apte et al., 2002). It is believed that, $MnCl_2$ being a natural high-dose hepato-toxicant (Sepúlveda et al., 2012) is not solely dependent on oxidative stress induction to CCl_4 toxicity. The $MnCl_2$ induced hepatic inflammation model was used to assess various pathogens, factors and signaling pathways that highlight the onset of liver disease and oxidative stress. Different ethanolic extract doses of OM (250, 500, and 750 mg/kg/day) produced $MnCl_2$ therapy in addition to preventing hepatocyte oxidative stress and guarded against oxidative stress-induced cell death. It also suppressed the inflammatory response brought on by $MnCl_2$ dosing. Therefore, by imitating the clinical settings which includes variety of elements aimed at enhancing liver damage and numerous systems which further support the development, the inflammation and hepatotoxic models of $MnCl_2$ is aided (Greenwel et al., 2000). Due to limited application of $MnCl_2$ as a toxicant model in comparison to

other test animal models, particularly in chronic model studies, its precise contribution to $MnCl_2$ induced necrosis is also unclear. It ought to be applied in the next data driven research initiatives. When compared to lower levels in the OM-treated groups D, E, and F, the MnCl2-treated group had elevated serum levels of bilirubin, ALT, ALP and AST. A drop in GPx-affected rat livers, an increase in Catalase content, and a decrease in SOD activity have all been connected to oxidative stress in the current MnCl2-treated population. These findings come from controlled group rat trials using histology. Previous studies have demonstrated that the body's resistance to oxidative stress is what causes the impacts of its protective and anti-oxidant processes (Pallottini et al., 2006). It damaged the ALT and AST enzymes in the hepatic circulation and hepatocyte membranes causing inflammation and elevated liver enzyme levels (Nissar et al., 2013).

In the current study, OM administration of the extract at doses of 250, 500, and 750 mg/kg significantly reduced liver inflammation, increased liver capacity, declined oxidative stress and improved histological results when compared to diseased rats. Similar as well as the beneficial effects of OM extract on liver inflammation have been considered. CCl_4 has recently been employed in similar research involving hepatic swell and ethanol-induced fibrosis in animal models (Saito et al., 2014). According to the underlying initial pro-inflammatory cytokines released by macrophages (Simpson et al., 1997), the liver is considered to have a significant role in regulating cytokine production. Hepatotoxic $MnCl_2$ increased pro-inflammatory molecules such TNF-, IL-1, and IL-6, which are crucial in inflammation (Wu et al., 2010).

The results of our investigation comply with earlier studies. Anti-inflammatory cytokines like IL-10 play a crucial function. However, it was found that the diseased group exhibited lower levels of IL-10 while the healthy group showed higher levels of IL-4. This suggests that OM extract is helpful in delivering positive outcomes and may even prevent the progression of disease (Prasad, 2017). In contrast to animals in the $MnCl_2$ induced illness group, OM extract completely diminished the levels of pro-inflammatory cytokines (TNF-α, IL-1, and IL-6) and increased the levels of anti-inflammatory cytokine (IL-10). Thus, findings revealed that one of the potential mechanisms behind OM anti-inflammatory action might be compared with mitigating activity, which elevated the levels of anti-inflammatory cytokine by suppressing levels of pro-inflammatory cytokines.

Similar to this, enhanced liver inflammation was identified as a measure of hepatic inflammation in rats with liver illness treated with $MnCl_2$, leading to changes in biochemical and hematological levels. Higher levels of TNF-α and pro-inflammatory cytokines in $MnCl_2$ induced rats supported the theory that in the latter stages, MnCl2 induced liver cell inflammation contribute to hepatocyte necrosis. Levels of TNF-α, as a measure of OM effectiveness against inflammatory activity, were dramatically reduced by the ethanolic extract of OM.

Conclusion

According to the study's findings, ethanolic extract of OM and its constituents lowered down the levels of inflammatory cytokines in addition to oxidative stress. Thus, by enhancing liver functions

and acting as an anti-oxidant as well as an anti-inflammatory, extracts of varying doses also have generated hepatoprotective effects.

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